Manuscript title:

Epigenetic regulation of *Dlg1,* via *Kaiso*, alters mitotic spindle polarity and promotes intestinal tumourigenesis.

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Abstract

Both alterations to the epigenome and loss of polarity have been linked to cancer initiation, progression and metastasis. It has previously been demonstrated that loss of the epigenetic reader protein Kaiso suppresses intestinal tumourigenesis in the *Apc*$^{+/min}$ mouse model, in which altered polarity plays a key role. Thus, we investigated the link between *Kaiso* deficiency, polarity and suppression of intestinal tumourigenesis. We used *Kaiso* deficient mice to conditionally delete *Apc* within the intestinal epithelia and demonstrated up-regulation of the spindle polarity genes *Dlg1* and *Dlgap1*. To understand the role of *Dlg1* we generated *Villin-creApc*$^{+/min}$*Dlg1*$^{flx/flx}$ *Kaiso*$^{-/y}$ mice to analyse gene expression, survival, tumour burden and spindle orientation. *In vivo* analysis of the *Dlg1* deficient intestine revealed improper orientation of mitotic spindles and a decreased rate of cellular migration. Loss of *Dlg1* decreased survival in *Apc*$^{+/min}$ mice, validating its role as a tumour suppressor in the intestine. Significantly the increased survival of *Apc*$^{+/min}$*Kaiso*$^{y/-}$ mice was shown to be dependent on *Dlg1* expression. Taken together this data indicates that maintenance of spindle polarity in the intestinal crypt requires appropriate regulation of *Dlg1* expression. As *Dlg1* loss leads to incorrect spindle orientation and a delay in cells transiting the intestinal crypt. We propose that the delayed exit from the crypt increase the window in which spontaneous mutations can become fixed, producing a "tumour-permissive" environment, without an increase in mutation rate.

Implications:

Loss of mitotic spindle polarity delays the exit of cells from the intestinal crypt and promotes a tumourigenic environment.

Introduction

Recent work recognising the importance of the link between polarity and epigenetic pathways in cancer onset and progression (1) has highlighted a need to increase our understanding of these relationships. DNA methylation of CpG dinucleotides is a fundamental epigenetic modification. Within gene promoters' methylation leads to transcriptional silencing and is exploited by a range of different cancer types, including colorectal cancer, to silence tumour suppressor genes (2,3). There is now a growing body of evidence that aberrant epigenetic silencing of polarity genes influences tumourigenesis in a variety of settings (4-6). Mechanistically, methylated CpG dinucleotides are recognised and bound to by proteins from the methyl binding family, which then recruit transcriptional repressor complexes (7). These proteins play a key role in disease by identifying tumour suppressor genes that have been aberrantly methylated (2,3,8). The methyl binding protein Kaiso, a POZ/BTB (Broad Complex Tramtrak, Bric a brac/pox virus and zinc finger) family zinc finger transcription factor protein, has been shown to play a key role in intestinal tumorigenesis. POZ/BTB proteins are a well conserved protein family (9) which have been indicated to play a role in both development and cancer (10,11). Despite the requirement of Kaiso for amphibian development (11), *Kaiso* null mice are viable and do not show any notable abnormalities in either development or reproduction (12). In cancer cells *Kaiso* appears to have a pro-tumourigenic function. Its deficiency relieved repression of tumour suppressor and DNA repair genes (13,14) in colon cancer. While its nuclear localisation is indicative of higher grade and metastatic breast and prostate cancers (15). In addition to its roles as a transcriptional repressor it can also bind to δ-Catenin and behave as a transcriptional activator(16,17). Further, *in vivo* studies have shown that *Kaiso* overexpression

promotes intestinal inflammation and intestinal tumourigenesis (18,19), while its absence increased survival of *Apc*$^{+/min}$ mice by delaying the onset of intestinal tumourigenesis (12). The *Apc*$^{+/min}$ mouse is a model of the human disease familial adenomatous polyposis (FAP). In both *Apc*$^{+/min}$ mice and FAP patients, mutation of the wild-type Apc allele (or 'second hit') is required for adenoma formation (20). *APC* mutation and subsequent activation of the canonical Wnt pathway is an early, if not the first step, in the development of more than 80% of all sporadic colorectal cancers (21,22). Due to its role in mediating epigenetic silencing of tumour suppressor gene in human CRC (13) and the low frequency of mutations observed in the disease (0% observed in 72 cases (23,24)), Kaiso represents a potentially druggable target. However, the mechanism of intestinal tumour suppression due to *Kaiso* deficiency *in vivo* has yet to be explored. As the dividing cells in the pre-cancerous and cancerous intestinal tissue of *Apc*$^{+/min}$ mice display alterations to the spindle polarity (25) we have investigated whether alterations to spindle polarity play a role in the intestinal tumour suppression observed in the *Apc*$^{+/min}$*Kaiso*$^{y/-}$ mouse model. We have demonstrated that the polarity and tumour suppressor gene *Dlg1* is mis-regulated in the absence of *Kaiso*. Dlg1 forms part of the Scribble/Lgl/Dlg polarity complex, which has been shown to play an important role in the maintenance of epithelial integrity (26). Mutations in this complex associated with the progression of a range of different epithelial cancer types (27-29) and mutations within the Dlg family have been observed in 12 of 72 (16.7%) of colorectal cancer patients (23,24). We further demonstrate that the absence of *Dlg1* alters spindle polarity, delays migration and promotes tumourigenesis and progression in the *Apc*$^{+/min}$ and *Apc*$^{+/min}$*Kaiso*$^{y/-}$ setting. Taken together this increases the evidence demonstrating

the importance of epigenetic regulation in maintenance of spindle polarity in intestinal homeostasis and disease

Materials and Methods

Mice

All animal procedures were conducted in accordance with institutional animal care guidelines and UK Home Office regulations. Mice were maintained in a SPF barrier facility in conventional open top cages on Eco-Pure Chips 6 Premium bedding (Datesand, UK) under a 12h light cycle, with IPS 5008 diet (Labdiet-IPS Ltd, UK) provided for nutritional support. To enrich environment, we provided irradiated sunflower seeds (at weaning only), Techniplast mouse houses (Techniplast, UK) and small chewsticks (Labdiet-IPS Ltd, UK). All mice were from a mixed background and were homozygous with respect to the C57Bl/6 Pla2g2a (also called Mom-1) allele. Experimental animals were between 10-15 weeks old with siblings used as controls. The alleles for the *Ah-cre(30)*, Apc^{flx} (31), $Apc^{+/min}$ (32), *Villin-Cre* (33), $Dlg1^{flx}$ (34), $Kaiso^{y/-}$ (12), alleles have been described previously. Genotyping conditions available upon request. Induction of the *Ah-cre* transgene was performed by administering 3 intra-peritoneal (I.P.) injections of β-naphthoflavone (BNF; Sigma, UK) at 80 mg/kg in a 24-hour period. For BrdU labelling, mice were injected I.P. with 0.15 mL of BrdU (Amersham, UK). Following *cre* induction loxP flanked alleles are referred to as deleted in intestinal epithelial cells (ΔIEC).

Microarray Expression Analysis

Ten-week-old male mice were used for the array analysis. Three mice for each of the desired genotypes were used. Three-centimetre portions of the SI located 5cm from the stomach were placed in RNAlater (after removing any mesentery and ensuring that no Peyer's patches were present). The tissue was then homogenized in Trizol reagent and RNA extracted using standard phenol-chloroform methodologies.

Samples were sent to the Molecular Biology Core facility at the Cancer Research UK Molecular Biology Core Facility at The University of Manchester. Where biotinylated target cRNA was generated and hybridised to Affymetrix Mouse430A_2 gene expression chips. The raw data from these microarrays is available at http://www.ebi.ac.uk/arrayexpress/. The data was normalised and analysed to generate differential expression tables using the AffyImGUI package for linear modelling of microarray data (35).

Tissue isolation, reporter visualization, immunohistochemistry (IHC)

Mice were euthanised at specified time points and the small intestine (S.I.) removed and flushed with water. Intestines were dissected as follows: The proximal 7 cm was mounted, fixed overnight in 10% formalin, and paraffin embedded. The following 3 cm was opened and placed into RNA later (Sigma), ensuring that all mesentery and Peyers patches were removed. The following 5 cm was divided into 1-cm lengths, bundled using surgical tape, and then fixed in 4% formaldehyde at 4°C for no more than 24 h before processing into wax blocks by conventional means. Section were cut at 5 µm thickness, dewaxed, and rehydrated into PBS. Staining was performed using the Envision+ mouse or rabbit kit (Dako, Agilent Ltd, Cheshire, UK) according to manufacturer's instructions. To identify cells which had lost *Dlg1* we stained using a validated rabbit polyclonal anti-Dlg1 antibody (Cat No# STJ111281, St John's Antibodies, London, UK) at 1:200. Cells which had incorporated BRDU were identified using an anti-BrdU antibody conjugate (Roche, UK) at 1:50, cells positive for DNA damage marker γH2AX were identified using anti-γH2AX (Upstate 05636)

Cellular analysis was performed on >25 whole crypts from at least three mice of each genotype.

Cellular analysis

Cellular analysis was performed on >25 whole crypts from at least three mice of each genotype. Apoptotic and mitotic index were scored from haematoxylin-and-eosin-stained sections as previously described (36). The cells between the base of the crypt and the junction with the villus was designated as the proliferative zone. For migration analysis mice of 60-80 days of age were given an IP injection of BrDU 2hours or 24hours prior to culling and dissection. Immunohistochemical analysis for BrDU incorporation was performed on formalin fixed small intestinal rolls, and the number BrDU positive cells and their location (with 0 being the bottom of the crypt) was measured on 50 half-crypts per mouse, minimum of 4 mice. Statistical analysis of the cumulative frequency of positive cells was performed using a two-tailed Kolmogorov-Smirnov test, on graphs P values are indicated as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$. Measurement of mitotic angle was performed on H&E slides stained sections. The angles of 200 mitosis in which the two spindle poles were easily observed were measured per mouse, with the angle of a direct line drawn between the two spindle poles and the line of the basement membrane measured. Measurements of 0˚-45˚ and 135˚-180˚ were classified as “planar”, being parallel to the basement membrane, whereas measurements of 45˚-135˚ were classified as being “Apico-basal”, being perpendicular to the basement membrane and dividing in the orientation of apico-basal polarity. The Chi-squared test was performed to assess statistical differences between the two genotypes, on graphs P values are indicated as follows: $*P < 0.05$; $**P < 0.01$; $***P < 0.001$.

Chromatin Immunoprecipitation (ChIP) Assay

ChIP assays were performed to assess Kaiso binding to the promoters of *Dlgap1* and *Dlg1* in murine samples from aged $Apc^{+/min}$ mice, using polyps and adjacent normal tissue from the small intestine and the large intestine. Anti-Kaiso antibody (Santa Cruz, CA, USA, sc-23871) was used to identify DNA binding partners of Kaiso, and primers for *Dlgap1* and *Dlg1* were used to confirm presence of these genes (primers detailed in Supplementary Fig 2).

siRNA against *Apc* mRNA was purchased from Bioneer (Bioneer, Daejeon, South Korea). The *Apc* siRNA (sense 5'- GCAGGCGUAGAGUAUUCAU -3', antisense 5'-AUGAAUACUCUACGCCUGC -3') was transfected into CT-26 cells using Lipofectamine RNAiMax (Invitrogen, Carlsbad, CA, USA), and knockdown levels of were confirmed using qRT-PCR (available upon request). After transfection, the cells were harvested, and ChIP analysis performed.

qRT-PCR Analysis

The following methods were all performed according to manufacturer's instructions unless otherwise stated. For analysis of gene expression in the intestine epithelia, three to five mice from each control and experimental group were harvested. RNA was extracted from a 0.5 cm portion of small intestine taken ~5 cm distally from the stomach and stored at -80 in RNAlater (Sigma, UK). Total RNA was extracted using the RNeasy kit (Qiagen, UK) and DNase treated using the Turbo DNase kit (Ambion, UK). Complimentary DNA (cDNA) was transcribed from 1 ug of RNA using random hexamers (Promega, UK) and Superscript III (Invitrogen, UK) kits. For relative quantitation, all samples were run in duplicate on the StepOnePlus PCR machine

using Fast Sybr Green master mix (Applied Biosystems, UK). The threshold cycle (Ct) values of each gene analysed were normalised to a reference gene. For expression analysis (qRT-PCR) Ct values were normalised against the ActB gene. Differences between groups were assessed using the 2-ΔΔCT method[47]. Two-tailed Mann-Whitney U (M.W.) tests were performed on the ΔCt values to determine significance (P=<0.05) differences between groups. Oligonucleotide sequences are available upon request.

Survival

A minimum of 14 mice per cohort were used to assess survival. Mice were aged until they showed symptoms of intestinal tumour burden (pale feet, bloating, prolapse or piloerection). Survival data was analyzed using the Kaplan-Meier test. If not indicated otherwise, the statistical mean is presented, and error bars represent standard deviation, on graphs P values are indicated as follows: ∗P < 0.05; ∗∗P < 0.01; ∗∗∗P <0 .001.

Tumour burden and grading

Tumour burden was measured at point of death with the number of macroscopic lesions and their size from fresh tissue. Grading was performed microscopically using H&E slides of small intestine taken from aged mice, tumours were microscopically identified as; single crypt lesion (grade 1), microadenoma (grade 2), adenoma (grade 3), adenocarcinoma with stromal invasion (grade 4) and adenocarcinoma with smooth muscle invasion (grade 5).

Intestinal organoid culture

Intestinal organoid culture was performed using the method described by Sato *et al* (37). Organoids were grown in Nunc LabTek Chamber Well slides for whole mount immunofluorescence. Organoids were fixed overnight in 4% PFA, then permeabilised using 1% Triton-X100 for 1hr at 37˚C. Alexa fluor® Phalloidin at 1:150 and Neomarkers Anti-lysozyme (Rb-327-A1) at 1:300 were used as primary antibodies.

Results

Apc deficient small intestine crypts are resistant to Kaiso deficiency

The delayed onset of adenomas in the *Apc*$^{+/min}$*Kaiso*$^{y/-}$ mouse is potentially due to a role for Kaiso in the Wnt signalling pathway. To investigate the direct effects of *Kaiso* status upon gene expression and Wnt signalling we analysed the phenotype of a conditional deletion of *Apc* in the context of *Kaiso* deficiency. *Kaiso*$^{y/-}$ deficient mice were crossed with mice carrying an *Ah-cre* transgene and a *loxP*-flanked *Apc* allele, following induction with BNF *Apc* is deleted in the small intestine (S.I.) crypts. We generated and compared cohorts of *Ah-cre*$^{+}$ *Kaiso*$^{+/+}$ (WT), *Ah-cre*$^{+}$ *Kaiso*$^{-/y}$ (*Kaiso*$^{-/y}$), *Ah-cre*$^{+}$ *Apc*$^{flx/flx}$ *Kaiso*$^{+/+}$(*Apc*$^{\Delta IEC/\Delta IEC}$) and *Ah-cre*$^{+}$ *Apc*$^{flx/flx}$ *Kaiso*$^{y/-}$ (*Apc*$^{\Delta IEC/\Delta IEC}$*Kaiso*$^{-/y}$) mice. At 4 days post induction (d.p.i.), comparing WT and *Kaiso*$^{-/y}$ indicated the absence of *Kaiso* does not grossly alter the crypts (Figure 1A and B). Similarly the *Apc*$^{\Delta IEC/\Delta IEC}$ mice, which are characterized by enlarged, aberrant crypts (Figure 1C) (as previously reported (38)), appeared unaltered in *Apc*$^{\Delta IEC/\Delta IEC}$*Kaiso*$^{y/-}$ setting (Figure 1D). To examine cell division and migration, mice were killed 2hrs or 24hrs after injection with BrdU. BrdU is incorporated into the DNA of cells at S phase and is bioavailable for less than 2hrs. Therefore, by killing mice at 2hrs and 24hrs after exposure, we could track the movement of BrdU-positive cells from the proliferation zone in the crypt on to the villus by immunohistochemistry. The position of BrdU-positive cells was plotted as the cumulative frequency of cells 2hrs and 24hrs after labelling (Figure 1E-H). Migration patterns of the S.I. were not significantly altered by the absence of *Kaiso*, however the data indicated a trend towards an increase in the speed of migration of *Kaiso* deficient cells from the proliferative zone onto the villi. In *Apc*$^{\Delta IEC/\Delta IEC}$ mice cell proliferation occurs independently of position and migration of cells along the crypt-villus axis is

perturbed (Figure 1F), as previously reported (27), the additional loss of *Kaiso* failed to alter this phenotype (Figure 1F).

Further, it has been previously shown that Paneth cell localisation is altered in the induced $Apc^{\Delta IEC/\Delta IEC}$ S.I. (27), the additional loss of *Kaiso* had no effect on this phenotype (Figure 1I). Finally, analysis of Wnt target genes demonstrated that the increase in Wnt target gene expression synonymous with *Apc* deletion was not significantly altered in the absence of *Kaiso* (Figure 1J and Supplementary Figure 1). In conclusion, the lack of any gross phenotype in either the $Kaiso^{y/-}$ or the $Apc^{\Delta IEC/\Delta IEC}Kaiso^{y/-}$ mice suggested that repression of intestinal tumourigenesis in the $Apc^{+/min}Kaiso^{y/-}$ mice is Wnt independent.

The polarity genes Dlg1 and Dlgap1 are upregulated within the Kaiso deficient small intestine

As *Kaiso* deficiency is well tolerated by the intestine we aimed to identify novel roles for *Kaiso* in the normal and *Apc* deficient intestine which may influence tumourigenesis. To identify genes mis-regulated in the absence of *Kaiso*, we generated microarray gene expression profiles on small intestine from WT and $Kaiso^{-/y}$ mice (raw data is available from www.ebi.ac.uk/array express under accession number E-MTAB-6009). After normalisation genes differentially expressed were identified and ranked according to their statistical significance (Supplementary Table 1). As expected the genes *Kaiso* and *Hprt* which were deleted in the generation of the model were highly down regulated and the most significantly altered. As *Kaiso* is a mediator of epigenetic transcriptional repression we identified the genes that were upregulated by its loss and candidates for a role in

tumourigenesis. The analysis identified the gene Discs Large Homologue Associated Protein 1 (*Dlgap1*) (Supplementary Table 1) which was significantly 4.9-fold up-regulated in the *Kaiso*$^{-/y}$ intestine. Dlgap1 is a highly conserved protein which interacts with the guanylate kinase-like domain of the important cancer regulatory protein Discs Large Homolog 1 (*Dlg1*), a binding partner of the key Wnt regulator *Apc*. To support the evidence that these genes are regulated by *Kaiso* we analysed whether the sequence of the promoter regions of these genes contained the *Kaiso* binding sequences TCCTGCNA (minimum binding sequence of CTGCNA) and CGCG (39). *In silico* analysis of the 5kb promoter regions upstream of the *Dlg1* and *Dlgap1* ATG start codons confirmed the presence of 5 minimum Kaiso binding motifs and 1 complete Kaiso binding motif in *Dlg1* and 1 minimum Kaiso binding motif in the *Dlgap1* promoter region, indicating its potential to regulate expression of these genes (Supplementary Figure 2). To confirm the upregulation of gene expression we used QRT-PCR analysis to demonstrate a significant 1.5-fold and 3.3-fold upregulation of *Dlgap1* and *Dlg1* expression, respectively (Figure 2A), in the *Kaiso* deficient intestine. This was further confirmed by immunohistochemistry which showed a dramatic increase in Dlg1 protein levels within the intestinal epithelium of *Kaiso* deficient mice (Figure 1B and C). *Dlg1* is important in maintenance of spindle polarity and is a binding partner of *Apc*. As spindle polarity is altered in the *Apc*$^{+/min}$ intestine (25), the upregulation of *Dlg1* suggested that it may play a functional role in suppression of intestinal tumourigenesis and the increased survival of *Apc*^{min} *Kaiso*$^{y/-}$ mice. To confirm a direct role for Kaiso to regulate *Dlg1* and *Dlgap1* expression Chromatin immunoprecipitation (ChIP) was utilised to pull down DNA associated with the Kaiso protein in both normal and polyp tissue from the small and large intestine of *Apc*^{min} mice. Kaiso binding to either *Dlgap1* or *Dlg1* was not observed in 10 normal

small intestinal samples or 5 normal large intestinal samples (Fig 2D). In contrast within *Apc*^{min} polyps *Dlgap1* binding was observed in 3/10 small intestinal tumours and 3/5 colonic polyps, with *Dlg1* binding observed in 1/10 small intestinal tumours and 3/5 colonic polyps (Fig 2D). These data indicate that Kaiso has the ability to bind to *Dlgap1* and *Dlg1*, but does so only an *Apc* deficient environment. To confirm this finding, ChIP was also used to assess Kaiso binding to *Dlgap* and *Dlg1* in the mouse colon carcinoma cell line CT-26 in which *Apc* is intact. Using siRNA methods to generate a greater than 8 fold knock-down *Apc* (Figure 2E), demonstrated that binding of *Kaiso* to the promoters of *Dlgap1* and *Dlg1* was significantly increased in the in an *Apc* deficient setting. (Figure 2F). Taken together this data indicates that regulation of expression of *Dlgap1* and *Dlg1* in the normal intestine is most likely indirect, however in a tumour permissive environment, Kaiso can directly bind to the promoters and regulate expression.

Dlg1 loss disrupts cell division polarity and migration rates in intestine

Dlg1 is known to regulate cell polarity and is a key part of the scribble/dlg/lgl polarity complex (40) and the *Apc* destruction complex. To investigate the role of *Dlg1* in the intestine we analysed *villin-creDlg1*$^{flx/flx}$ mice (34), in which exon 4 of the *Dlg1* gene is flanked by LoxP and driven by endogenous expression of Cre from the *Villin* promoter to initiate loss of *Dlg1* at embryonic E9 within the intestinal epithelium (41). Survival analysis was performed by aging cohorts of *villin-cre Dlg1*$^{+/+}$ (WT) and *villin-cre Dlg1*$^{flx/flx}$ (*Dlg1*$^{\Delta IEC/\Delta IEC}$) mice. Overall there was no significant difference in survival between WT and *Dlg1*$^{\Delta IEC/\Delta IEC}$ and at death the intestinal epithelium of *Dlg1*$^{\Delta IEC/\Delta IEC}$ mice appeared functionally normal (Figure 3A). Histological

assessment of *Dlg1*$^{\Delta IEC/\Delta IEC}$ intestinal tissue revealed that *Dlg1* deficiency does not alter crypt length (number of cells), apoptosis (number of apoptotic bodies) or proliferation (Supplementary Figure 3 A, B and C). In summary, it appears that *Dlg1* loss is well tolerated by the intestinal epithelium and alone is not sufficient to induce colorectal tumourigenesis. As *Dlg1* is known to form part of the important Scribble/Lgl/Dlg polarity complex, we next explored the role of these gene in the *Dlg1* deficient intestine. Expression qRT-PCR analysis of the polarity genes associated with *Dlg1* indicated a significant reduction in expression of the Scribble (*Scrib1*) gene which forms part of the polarity complex) (Figure 3B). The lack of any gross phenotype as a result of loss of *Dlg1* and significant reduction in *Scrib* expression suggested that other cues from the extracellular matrix (ECM) or redundancy amongst *Dlg* family members may compensate for the absence of *Dlg1* in order to maintain the correct architecture of the intestinal epithelia *in vivo* (42). However, further investigation revealed no expressional compensation by other *Dlg* family member genes (supplementary Figure 4), indicating that extremely low levels of Dlg1 expression as observed in the *Dlg1*$^{\Delta IEC/\Delta IEC}$ model are sufficient to maintain intestinal crypt morphology.

To investigate the role of *Dlg1* loss on polarity, specifically within the intestinal epithelium in the absence of ECM cues, small intestinal organoid cultures were made from both WT and *Dlg1*$^{\Delta IEC/\Delta IEC}$ tissue. Whole mount immunofluorescence for the marker of the apical tip/ brush border of intestinal epithelial cells revealed no obvious cell polarity defects as a result of *Dlg1* deficiency within the intestinal epithelium (Figure 3C & D). As well as playing a key role in maintaining normal apical basal polarity *Dlg1* has a role in spindle orientation during mitosis. Within the WT crypt 86% of cell divisions occurred in a “planar” orientation, with mitotic spindles

running parallel to the basement membrane while the remaining 14% of mitotic spindles occurred in a “apico-basal” direction (Figure 3E). Analysis of the *Dlg1*$^{\Delta IEC/\Delta IEC}$ intestine demonstrated a significant shift in spindle orientation to 79% planar to 21% apico-basal(Figure 3F;Chi-squared test P=0.0437). As planar cell divisions are in line with normal migration along the crypt villus axis any alterations may lead to delayed exit of cells from the crypt. Further BrDU analysis at a 2h time point indicated that *Dlg1* deficiency did not alter the position of cells undergoing mitosis (Figure 3G). However, at 24h the *Dlg1* deficient BrDU^{+} cells were significantly lower down the crypt-villus axis compared to wild type controls (Figure 3G; (Kolmogorov-Smirnov p<0.0001). Potentially the delay in *Dlg1* deficient cells exiting the crypt may impact on tumourigenesis as it could increase the time for oncogenic mutations to become fixed, creating a tumour-permissive environment. Levels of cell DNA mutation within the intestine were assessed by qRT-PCR for a range of DNA repair pathway genes, and the protein localisation of the DNA damage marker γH2AX was assessed by immunohistochemistry and counted, showing no difference (supplementary Fig 5).This was expected as it is not thought that the rate of mutation is controlled by *Dlg1*, simply the rate at which mutated cells migrate.

Dlg1 loss promotes intestinal tumourigenesis in Apc$^{+/min}$ *mice*

To explore the role of *Dlg1* in intestinal tumourigenesis *VillinCre*$^{+}$ *Dlg1*$^{flx/flx}$ mutant mice were crossed with *Apc*$^{+/min}$ mice to generate *Apc*$^{+/min}$ and *Apc*$^{+/min}$ *VillinCre*$^{+}$ *Dlg1*$^{fl/fl}$ (*Apc*^{min}*Dlg1* $^{\Delta IEC/\Delta IEC}$) cohorts. To confirm our previous finding for the role of *Dlg1* in spindle orientation we repeated the analysis on the normal crypts in these mice. As predicted we observed a significant increase in apico-basal mitotic spindles from 14% in WT (Figure 3E) to 19% in *Apc*$^{+/min}$ (Figure 4A; Chi-squared test

P<0.001) to 32% in *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ mice (Figure 4B; Chi-squared test P>0.001). The mice were aged and harvested until they reached a humane endpoint indicating an intestinal tumour burden. Survival analysis indicated a significant decrease in survival of *Apc*^{min}*Dlg1*$^{\Delta IEC/\Delta IEC}$ in comparison to *Apc*$^{+/min}$ mice (Figure 4C, Log-Rank(Mantel-Cox) P=0.0309). At point of harvest there was no significant difference to the total median tumour burden between the cohorts, indicating mice were analysed at equivalent stages (Figure 4D). However, it was noted that even though it did not achieve statistical significance the median number of tumours in the small intestine increased from 17 in the *Apc*$^{+/min}$ mice to 30 in the *Apc*^{min}*Dlg1*$^{\Delta IEC/\Delta IEC}$ cohort (Figure 4E), with the *Apc*^{min}*Dlg1*$^{\Delta IEC/\Delta IEC}$ mice showing a significant increase in inter-mouse variability. To understand why *Apc*^{min}*Dlg1*$^{\Delta IEC/\Delta IEC}$ reached the endpoint sooner than *Apc*$^{+/min}$ mice, we examined the grade of tumours in the mice, as loss of spindle polarity is associated with EMT and progression. Microscopic grading of the observed lesions identified a significant increase in the transition of single crypt lesions to more advance tumour types, including adenocarcinomas with stromal and smooth muscle invasion rarely observed in the *Apc*^{min} mice (Figure 4G). In conclusion, the data indicates that that *Dlg1* acts as a tumour suppressor in the murine intestine by preventing loss of spindle polarity, supporting cell migration rates and preventing progression of lesions.

Tumour suppression due to Kaiso deficiency is Dlg1 dependent

Previously we reported that loss of *Kaiso* suppressed tumourigenesis in the *Apc*$^{+/min}$ model. Here we have identified that *Dlg1* is upregulated in the *Kaiso* null intestinal epithelium and acts as a tumour suppressor in the *Apc*$^{+/min}$ model. To assess if the increased survival of *Apc*^{min} *Kaiso*$^{y/-}$ mice is dependent on the presence of a

functional *Dlg1* gene and/or related to spindle polarity, we generated *Apc^{min} Kaiso$^{y/-}$ Dlg1$^{\Delta IEC/\Delta IEC}$* mice. Analysis of spindle polarity indicated that the proportion of aberrantly orientated spindles was significantly reduced from 19% in the *Apc$^{+/min}$* mice to 12% in the *Apc^{min} Kaiso$^{-/-}$* (Figure 5Aand B; Chi-squared test P<0.001). A similar level to the WT setting (Figure 3E) and a potential reason for the subtle increase in cell migration from villus to crypt (Figure 1E). We next examined the *Apc$^{+/min}$ Kaiso$^{-/-}$Dlg1$^{flx/flx}$* mice and demonstrated that the improvement in percentage of mis-orientated divisions due to *Kaiso* loss was lost with the additional absence of *Dlg1 (*Figure 5A and B; Chi-squared test P<0.001). Significantly there was no difference in the percentage of aberrant mitosis in the apico-basal orientation between the *Apc^{min}Dlg1$^{\Delta IEC/\Delta IEC}$* and *Apc^{min} Kaiso$^{y/-}$Dlg1$^{\Delta IEC/\Delta IEC}$* intestines (Figure 5A), and no change in total levels of cell proliferation between the cohorts (Figure 5C). To investigate whether the improvement in the proportion of mitotic spindles in the planar orientated in *Apc$^{+/min}$ Kaiso$^{y/-}$* intestine was important in the tumour suppression we generated cohorts of *Apc^{min} Kaiso$^{y/-}$Dlg1$^{\Delta IEC/\Delta IEC}$* mice and aged until humane endpoint was reached. As previously observed the loss of *Kaiso* increased survival (Figure 5D; *Apc$^{+/min}$ Kaiso$^{y/-}$* mice were only aged to 200 days to confirm increased survival and then sacrificed). As reported earlier (Figure 4C) there is a significant decrease in survival of of *Apc^{min} Dlg1$^{\Delta IEC/\Delta IEC}$* which was not rescued by the additional loss of *Kaiso* (Figure 5D). Further the increased aggression of lesions observed in *Apc^{min}Dlg1$^{\Delta IEC/\Delta IEC}$* again presented in the *Apc^{min} Kaiso$^{y/-}$Dlg1$^{\Delta IEC/\Delta IEC}$* (Figure 5E). In conclusion, this data suggests that the tumour resistance observed in the intestine *Apc^{min}Kaiso$^{y/-}$* mice requires expression of the *Dlg1* gene. We propose that the appropriate expression of *Dlg1* prevents mitosis occurring at an aberrant

apico-basal orientation, maintains the rates of cell migration onto the villus and therefore may decrease the window of opportunity for tumourigenesis (Figure 6).

Discussion

In summary, here we have demonstrated that in the absence of the epigenetic regulator *Kaiso* there is an increase in expression of the polarity proteins *Dlg1* and *Dlgap1.* The absence of *Dlg1* results in an increase in the number of baso-lateral cell divisions within the intestinal crypt and a decrease in cell migration rates. On the $Apc^{+/min}$ background the absence of *Dlg1* decrease survival due to an increase in the number and aggressiveness of tumours. Further the loss of *Dlg1* overcomes the suppression of intestinal tumorigenesis observed in the $Apc^{+/min}Kaiso^{-/y}$ mouse model. Suggesting that the mechanisms that maintain planal mitotic orientation and migration rates have important anti-tumourigenic roles. Although there are many potential mechanisms for the control of migration rates along the crypt-villus axis, including cell:cell signalling and negative draw of cells through the sloughing of cells at the villus tip, there is much evidence to support the concept that mitosis supports normal cellular migration along the crypt villus axis through mitotic pressure (43,44). This leads to the assumption that the angle of mitosis within the crypt could play a part in normal migration patterns. The rapid cell migration along the crypt-villus axis is one of the driving factors which controls the quick turn over of cells within the intestinal epithelium and is essential to minimise the risk of tumourigenesis. It achieves this by flushing undetected mutant cells from the epithelium before they can disrupt intestinal homeostasis. Through delayed migration it can be hypothesised that $Apc^{+/min}$ cells which have lost the wild type copy of *Apc* (through random mutations, loss of heterozygosity or epigenetic silencing) may remain within the intestinal epithelium for a longer period of time, increasing the opportunity for lesion formation. An alternative hypothesis is that the increased crypt residence time of a cell increases the likelihood that it will accumulate additional oncogenic

mutations e.g. *K-ras, Smad4* or *p53*. This may account for the increased aggressiveness of the tumours observed in the *Apc*$^{+/min}$ *Dlg1*$^{-/-}$ model. The role of cell migration speed within the intestinal epithelium in creating a tumour permissive environment has been hypothesised elsewhere (45,46), and in fact many previously established chemo-preventative agents play a role in increasing cellular migration (47), which may contribute to their efficacy in delaying tumour onset.

Normal planar cell divisions within the crypt can support mitotic pressure for migration to occur in a straight line, the quickest route from crypt base to villus tip, whereas apico-basal divisions could logically result in “side-to-side” cell movement as well as “upward”, which could be responsible for the increased migration time observed in *Dlg1*$^{\Delta IEC / \Delta IEC}$ mice (Figure 6). This has been previously shown in astrocyte cells in which deficiency of the Dlg1:Dlgap1 complex, also identified in this study, leads to inefficient migration(40). Further, while severely abnormal angles of mitosis may lead to delamination of cells they would normally undergo apoptosis. Any further suppression of apoptosis leads to formation of disorganised masses of aberrant cells with characteristics of tumorigenesis and EMT, potentially accounting for the increase in more advanced lesion observed here. However, it must be noted that during mitosis within the intestinal crypt, cells step out of the epithelial sheet but maintain contact with surrounding cells and divide more luminally before the daughter cells return to the intestinal epithelium proper. It is possible that the orientation of cell division makes less difference to the final position of the daughter cells within the intestinal epithelium than the process by which cells return to the epithelial sheet.

Previous reports have linked epigenetic modification to altered expression of genes associated with cell polarity (48), thereby enabling transcriptional repressors such as *Kaiso* to regulate cell polarity. However further work using chromatin immuno-precipitation techniques is required to confirm that these genes are direct targets of *Kaiso*. The work we present here supports the idea that transcriptional repression of polarity associated genes plays a role in tumourigenesis and tumour invasion (49), and that inhibition of such epigenetic regulators may be of therapeutic value. However, it should be noted that the phenotype associated with the loss of *Kaiso* may also reflect yet unidentified changes in genes associated with its transcriptional activation functions. At the very least, identification and exploration of the targets of such epigenetic transcriptional regulators which may influence cell polarity could prove to be of value to the field of cancer research. As *Kaiso* inhibition has been suggested as a potential therapeutic strategy for the treatment of colorectal cancer, and inhibitors for this transcriptional repressor are currently being investigated (50). The work presented here indicates that the tumour-suppressive effect of *Kaiso* loss is limited to systems in which functional Dlg1 is present at the initiation stage of a tumour. It remains to be determined whether targeting Kaiso in a higher-grade tumour will have any beneficial effect and whether it would be dependent on *Dlg1* remaining unmutated within the patient's tumour. As such, the efficacy of such a treatment would be limited to lower grade tumours where *Dlg1* remains functional, and tumour resistance to treatment could develop through additional mutations in *Dlg1*.

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Figure legends

Fig. 1. Loss of *Kaiso* does not alter the gross phenotype of *WT* and *Apc* deficient SI crypts. Haematoxylin and eosin staining showing no difference between WT (**A**) and *Kaiso*$^{-/y}$ (**B**) or *Ah-cre Apc*$^{\Delta IEC/\Delta IEC}$ (**C**) and *Ah-creApc*$^{\Delta IEC/\Delta IEC}$ *Kaiso*$^{-/y}$ (**D**) S.I., note the enlarged crypts in the *Apc*$^{\Delta IEC/\Delta IEC}$ mice are retained in *Apc*$^{\Delta IEC/\Delta IEC}$*Kaiso*$^{-/y}$ ([indicates height of crypts), Scale bars represent 50μm. (**E & F**) Mice were injected with BrdU to mark cells in S phase at 2h and track migration onto villi 24h later. (**E**) WT 2h (—), *Kaiso*$^{-/y}$ 2h (---), WT 24h (···), *Kaiso*$^{-/y}$ 24h (-·--) and **(F**) *Apc*□*IEC/*□*EC* 2h (—), *Apc*$^{\Delta IEC/\Delta IEC}$ *Kaiso*$^{-/y}$ 2h (---), *Apc*$^{\Delta IEC/\Delta IEC}$ 24h (···), *Apc*$^{\Delta IEC/\Delta IEC}$ *Kaiso*$^{-/y}$ 24h (-·--) BrdU-positive cells indicated no alteration to the position and size of the proliferative zones or rate of migration (absent in the *Apc*$^{\Delta IEC/\Delta IEC}$ S.I.), n≥4, with representative images of 24hr BrDU in *WT* and *Kaiso*$^{-/y}$ small intestine (**G & H**), scale bar represents 50μm. (**I**) The absence of *Kaiso* does not alter the normal position of Paneth cells or rescue their mis-localisation in the *Apc*$^{\Delta IEC/\Delta IEC}$ S.I; WT (—), *Kaiso*$^{-/y}$ (---), *Apc*$^{\Delta IEC/\Delta IEC}$ (···) and *Apc*$^{\Delta IEC/\Delta IEC}$ *Kaiso*$^{-/y}$ (-·--), n≥4. (**J**) Relative expression analysis indicated no significant alteration to Wnt target genes in the absence of *Kaiso* (WT-black, *Kaiso*$^{-/y}$-white, *Apc*$^{\Delta IEC/\Delta IEC}$ -dark grey and *Apc*$^{\Delta IEC/\Delta IEC}$ *Kaiso*$^{-/y}$-light grey), error bars indicate standard deviation, n≥4

Fig. 2. Upregulation of *Dlg1* and *Dlgap1* in the *Kaiso* deficient intestine. (**A**) qRT-PCR analysis indicating down regulation of *Kaiso* and up regulation of *Dlg1* and *Dlgap1* in the *Kaiso*$^{-/y}$ intestine (WT-black bars; *Kaiso*$^{-/y}$ - grey bars), error bars indicate standard deviation, n≥4. Immunohistochemistry comparing WT (**B**) to *Kaiso*$^{-/y}$ small intestine**(C)** demonstrating an increase in *Dlg1* staining in the intestinal epithelial of the *Kaiso* deficient intestine, scale bar represents 100μm. ChiP analysis

was used to assess the ability of Kaiso to bind *Dlgap1* and *Dlg1* in murine small intestine, large intestine, small intestinal polyps and large intestinal polyps (**D**), Apc SiRNA enabled a more than 8 fold knock down of Apc within the murine cell line CT-26 (**E**), and ChIP analysis was used to assess the ability of Kaiso to bind to *Dlgap1* and *Dlg1* when CT-26 cells were treated with either control scrambled siRNA (light grey) or *Apc* siRNA (dark grey) (**F**), Error bars represent standard deviation.

Fig. 3. Absence of *Dlg1* is tolerated by the S.I. but disrupts mitotic spindle orientation and migration. (**A**) Kaplan-Meier survival analysis of aged WT (—) and *vilCre-Dlg1*$^{\Delta IEC/\Delta IEC}$ (…) cohorts indicates no difference in overall survival. (**B**) *Scrib* is significantly (Mann-Whitney; P=0.002) downregulated in the *Dlg1*$^{\Delta IEC/\Delta IEC}$ intestine, error bars indicate standard deviation, n≥4. (**C & D**) Whole mount immunofluorescence of WT (**C**) and *Dlg1*$^{\Delta IEC/\Delta IEC}$ (**D**) organoids demonstrating the maintenance of cellular polarity (green-phalloidin, red-lysozyme expression and blue-DAPI). Analysis of mitotic angles in WT (**E**) and *Dlg1*$^{\Delta IEC/\Delta IEC}$ (**F**) indicates a significant decrease in divisions occurring in a planar orientation (white segments; **E** *right panel* red circle indicating planal mitosis) and an increase in apico-basal divisions (grey segments; **F** *right panel* red circle indicating apico-basal mitosis) as a result of *Dlg1* deficiency, n≥6. (**G**) Comparison of the position of BrdU+ cells at 2h (—) and 24h (…)in WT (black) and *Dlg1*$^{\Delta IEC/\Delta IEC}$ (grey) indicating a delay in migration in the *Dlg1*$^{\Delta IEC/\Delta IEC}$ crypt (Kolmogorov-Smirnov test $p<0.0001$), with representative images of 24hr BrDU, n≥4 (**H&I**).

Fig. 4. *Dlg1* is a tumour suppressor in the murine intestine. Analysis of mitotic spindle angles in crypts of *Apc*$^{+/min}$ (**A**) and *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ (**B**) crypts indicates loss of *Dlg1* leads to a significant decrease in the proportion of cells dividing in a

normal planar orientation (Chi-squared test P>0.001; grey). (**C)** Kaplan-Meier analysis demonstrating a significant reduction in overall survival of *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ (black line) mice compared to *Apc*$^{+/min}$ (Grey line)(Log-rank (Mantel-Cox) P=0.0309). The total number of tumours (**D**) and their burden (**E**) were not significantly altered in *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ mice, although they both displayed an increased inter mouse variability (Levene's test for equality of variances p=0.018). (**F**) Lesions observed within *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ (black bars) small intestine at end point were significantly more severe than those observed in *Apc*$^{+/min}$ (grey bars, error bars indicate standard deviation)(Chi-squared test). SI= Stromal Invasion, SMI= smooth muscle invasion, n≥28 lesions counted from 4mice. (**G**) Lesion from an *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ mouse indicating progression of tumour to adenocarcinoma with submucosal (↑) and smooth muscle invasion (🡅), scale bar represents 500µm.

Fig. 5. The *Kaiso* deficient intestine requires an intact *Dlg1* to manifest its tumour resistance. (**A**) The proportion of aberrantly orientated mitotic spindles is significantly reduced in the *Apc*$^{+/min}$*Kaiso*$^{y/-}$ but increased in the *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$, and *Apc*$^{+/min}$*Kaiso*$^{y/-}$*Dlg1*$^{\Delta IEC/\Delta IEC}$, when compared to *Apc*$^{+/min}$ intestine. Statistics were performed using Mann Whitney, * indicates p≤0.05, n≥6. (**B**) Representative images of *Apc*$^{+/min}$, *Apc*$^{+/min}$*Dlg1*$^{\Delta IEC/\Delta IEC}$, *Apc*$^{+/min}$*Kaiso*$^{y/-}$ and *Apc*$^{+/min}$*Kaiso*$^{y/-}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ intestine, with indicated aberrant apico-basally orientated cell divisions(←) and planar cell divisions (↑) Scale bars represent 100µm. BrDU analysis (**C**) indicated there were no differences in total levels of proliferation within the intestine, n≥4. (**D**) Kaplan-Meier survival analysis demonstrating a decreased survival of *Apc*$^{+/min\Delta IEC/\Delta IEC}$ (grey dotted line) and *Apc*$^{+/min}$*Kaiso*$^{y/-}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ (grey solid line; Log-rank (Mantel-Cox) P=0.0441) compared to *Apc*$^{+/min}$ (black solid) and *Apc*$^{+/min}$*Kaiso*$^{y/-}$(black dashed)

mice, vertical mark indicate mouse disease free survival n≥9. (**E**) Adenocarcinoma with sub-mucosal invasion (←)(from an *Apc*$^{+/min}$*Kaiso*$^{y/-}$*Dlg1*$^{\Delta IEC/\Delta IEC}$ mouse, scale bar represents 500µm.

Fig. 6. Model of tumour suppression by *Dlg1*. (**A**) In the normal intestine Dlg1 plays an important role in maintaining the planal orientation of mitosis to ensure that division occurs in the same direction as migration. (**B**) In the *Dlg1* deficient intestine alteration to the angles of mitosis conflict with the direction of cell migration leading to longer transit times from the crypt-base to the villus. Potentially increasing the window of opportunity for fixation of a mutant cell, which may then migrate in multiple directions and increasing the chance of invasion into the sub-mucosa.

Supplementary Information Legends

Supplementary Fig. 1. Relative expression analysis indicated no significant alteration to Wnt target genes expression in the intestine of the *Kaiso* deficient mouse. Graph of the dCt values for the fold expression change data in Fig. 1.

Supplementary Fig. 2. (A&B) *Dlg1* and *Dlgap1* promoter regions contain *Kaiso* binding sequences. The region 5kb upstream of the *Dlg1* exon 1 (NR_045516) and Dlgap1 (NM_177639), *Kaiso* binding domains ((full -TTCCTGCNA (red) & minimum-CTGCNA (green)) and CGCG (blue). Primers locations used for ChIP analysis are indicated in yellow.

Supplementary Fig. 3. The loss of *Dlg1* is tolerated by the small intestinal epithelium. Comparing WT and *Dlg1*$^{\Delta IEC/\Delta IEC}$ at death indicated there were no significant changes in (**A**) cellular height of crypt, number of apoptotic (**B**) and mitotic (**C**) bodies per 50 half crypts.

Supplementary Fig. 4. Intestinal samples from *Dlg1*$^{\Delta IEC/\Delta IEC}$ (black bars) show significantly reduced *Dlg1* expression compared to Wildtype (grey bars), but there is no significant changes in expression levels of other *Dlg* family members.

Supplementary Fig. 5 qRT PCR analysis indicated that *Dlg1* deficiency within the intestinal epithelium resulted in no significant change in expression levels of a range of DNA repair genes which are associate with the 5 main DNA repair pathways, as indicated (**A**), with immunohistochemical analysis for the DNA damage marker γH2AX showing no significant difference between *WT* and *Dlg1*$^{\Delta IEC/\Delta IEC}$ crypts (**B**).

Supplementary Table 1. Top 50 differential expressed genes in the *Kaiso*$^{-/y}$ intestine versus *WT*.