Physiological expression of the PI3K activating mutation *Pik3ca*^{H1047R} combines with *Apc* loss to promote development of invasive intestinal adenocarcinomas in mice

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Abstract

PIK3CA, the gene encoding the p110 α catalytic subunit of phosphoinositide 3-kinase (PI3K), is mutated in around 20% of sporadic colorectal cancers (CRCs) but the role of these mutations in the pathogenesis of CRC remains unclear. We used a novel mouse model to investigate the role of *Pik3ca*^{H1047R} mutation, the most common *PIK3CA* mutation in CRC, during the development and progression of intestinal cancer. Our results demonstrate that *Pik3ca*^{H1047R}, when expressed at physiological levels, is insufficient to initiate intestinal tumourigenesis. However, in the context of *Apc* loss, which is observed in 80% of CRCs, and by itself results in benign intestinal adenomas, *Pik3ca*^{H1047R} mutation promote the development of highly aggressive and invasive adenocarcinomas in both the small and large intestines. Our findings show that an activating *Pik3ca* mutation can cooperate with *Apc* loss to drive the progression of gastrointestinal cancer and thus this disease may be susceptible to therapeutic targeting using PI3K pathway inhibitors.

Keywords: Phosphatidylinositol 3 kinase (PI3K), colorectal cancer, *Pik3ca*, Adenomatous polyposis coli (*Apc*), mouse model.

Summary statement: We used a novel mouse model to investigate the role of a common *PI3K* pathway mutation observed in human cancers and identified that when combined with loss of the *Apc* gene, intestinal tumourigenesis is enhanced compared to *Apc* loss alone.

Abbreviations:

APC, adenomatous polyposis coli; Cdx2, caudal type homeobox 2; CRC, colorectal cancer; Cre, cre recombinase; FAP, familial adenomatous polyposis; Gpa33, glycoprotein A33; H&E, haematoxylin and eosin; HCC, hepatocellular carcinoma; IHC, immunohistochemistry; PI3K, phosphatidylinositol 3 kinase; pRps6, phosphorylated S6; TCGA, The Cancer Genome Atlas network; WT, wild type.

Introduction

Colorectal cancer (CRC) is the third most common cancer worldwide with poor prognosis and survival accounting for more than 600,000 deaths globally in 2008 [1]. Most CRCs undergo a well described multistage pathogenesis known as the adenoma-carcinoma sequence involving the progression of benign adenomas to carcinoma over 10-17 years [2]. Investigations into the underlying molecular mechanisms and genetic events occurring at each stage of pathogenesis have revealed this process to be multifactorial, involving chromosomal abnormalities, loss of tumour suppressor genes and activation of oncogenes [3].

Familial adenomatous polyposis (FAP) accounts for ~1% of all CRC cases and is the result of inherited mutations in the adenomatous polyposis coli (*APC*) tumour suppressor gene [4]. *APC* mutation leads to formation of many benign dysplastic polyps and adenomas in the small and large intestines and a 90% lifetime chance of progression to CRC [3]. *APC* loss is also detected in ~80% of sporadic CRCs making it one of the most common initiating genetic alterations in CRC [5]. The molecular mechanisms driving progression of *APC*-initiated adenomas to adenocarcinomas are poorly understood.

The phosphoinositide 3-kinase (PI3K)/AKT pathway plays a critical role in regulating numerous cellular processes critical in cancer development, including growth, proliferation, migration, apoptosis and survival [6]. Mutations in *PIK3CA*, the gene encoding the p110 α catalytic subunit of PI3K, have been identified in many human cancers including 20-30% of sporadic CRCs [7, 8]. Around 80% of these mutations cluster in three hot-spots; two in the helical domain (E542 and E545) and one in the kinase domain (H1047) [7, 8]. *PIK3CA* mutations have been detected in CRCs but not adenomas [8] suggesting their involvement in the later stages of CRC progression.

We have generated mice harbouring the most commonly detected *PIK3CA* mutation in human cancer (H1047R) conditionally knocked into the endogenous *Pik3ca* locus to explore the consequences of *Pik3ca* mutation in the gastrointestinal tract. In a system ideal for mimicking sporadic disease, we demonstrate that the *Pik3ca*^{H1047R} mutation, by itself, is not tumourigenic. However when expressed in combination with the loss of *Apc* gene function, we observe invasive intestinal adenocarcinomas. This demonstrates a direct link between mutations in the PI3K/Akt and Wnt/ β -catenin pathways and the development of these adenocarcinomas.

Experimental

Mice

Mice harbouring a single latent, Cre recombinase (Cre)-inducible knock-in of the *Pik3ca*^{H1047R} mutation (*Pik3ca*^{Lat-H1047R}) [9] and/or two Cre-inducible *Apc* loss-of-function alleles (*Apc*^{580S/580S}) [10] were crossed with mice heterozygously expressing RU486-activatable Cre-progesterone receptor ligand-binding domain fusion knocked into the intestinal epithelial cell-specific Gpa33 locus (*Gpa33Cre*^{PR2}) [11]. Mice generated (equal numbers of male and females) expressed heterozygous mutant *Pik3ca*^{H1047R} and/or homozygous *Apc* loss of function (now *Apc*^{580D/580D} when alleles deleted) in the gastrointestinal tract. Transgenic B6;129S4-*Gt*(ROSA)26Sor^{tm1Sor/J} (*Rosa*^{lacZ}) reporter mice were used for verification of Cre-mediated recombination [12]. All mice strains were maintained on C57BL/6 backgrounds.

All animal experiments were approved by the Peter MacCallum Cancer Centre Animal Experimental Ethics Committee and conducted in accordance with the *NHMRC Australian Code of Practice for the Care and Use of Animals for Scientific Purposes*. Mice were closely monitored and humanely euthanized when they exhibited >10% weight loss, rectal bleeding or other signs of distress.

Induction of conditional Cre recombinase expression

To induce recombination, all mice were fed chow containing RU486 at a concentration of 20mg/kg (dissolved in ethanol, mixed with peanut oil, and added to mouse food softened with sucrose water) for four weeks from 6 weeks of age.

β-Galactosidase staining

Intestinal tissues from *Gpa33Cre*^{PR2}:*Rosa*^{lacZ} and *Rosa*^{lacZ} control mice were harvested, rolled, fixed, embedded in optimal cutting temperature media for cryosectioning and stained for expression of β-galactosidase as previously described [13].

Histology and immunohistochemistry

Small and large intestines of mice were removed and flushed with phosphate-buffered saline. A small piece of the proximal end of each, and any tumours if present, were snap frozen for RNA extraction. Remaining intestinal tissues were fixed as a “Swiss roll” on surgical tape in 10% neutral buffered formalin for 24 hours before paraffin embedding.

Sections were stained with haematoxylin/eosin (H&E) for histological analysis or used for immunohistochemistry (IHC). Primary antibodies for IHC included β-catenin (BD Biosciences, Franklin Lakes NJ, USA), p-Akt^{S473} (CS3787, Cell Signalling Technology, Danvers MA, USA), pAkt^{T308} (CS4056, Cell Signalling Technology) and pRps6 (CS2211, Cell Signalling Technology).

Sequencing

To confirm knock-in of the *Pik3ca*^{H1047R} mutation, RNA was extracted from tissues and tumours and reverse transcribed to cDNA which was sequenced with *Pik3ca*-specific primers (forward, 5' –CAAGAGTACACCAAGACCAGAGAGTT – 3' and reverse, 5' – TCCAATCCATTTTGTCTGCC – 3') using PCR cycle conditions of 95°C for 30 seconds, 55°C for 30 seconds and 72°C for 45 seconds for 35 cycles.

Results

Confirmation of GpaA33Cre-mediated recombination in the intestinal tract by β-galactosidase expression

The efficiency of *Gpa33*-driven Cre-mediated recombination, and thus conditional induction of *Pik3ca*^{H1047R} expression in the gastrointestinal tract, was assessed using the Cre-inducible *Rosa26*^{lacZ} reporter mouse strain (Figure 1A). Cre-recombination was observed in 1-5% of crypts in the small and large intestines while no staining was detected in other tissues such as the liver (Figure 1B). As the mouse intestinal epithelium renews every 4-5 days, only recombination that occurs in the stem cell at the base of the crypt is reflected in positive β-galactosidase staining beyond a few days.

Expression of the $Pik3ca^{H1047R}$ mutation alone in the intestines does not initiate tumourigenesis

To explore the role of $Pik3ca^{H1047R}$ mutation in intestinal cancer *in vivo*, we crossed $Pik3ca^{Lat-H1047R}$ mice (Figure 2A, [9]) with $Gpa33^{CrePR2}$ mice [11]. The resulting $Gpa33^{CrePR2/+};Pik3ca^{Lat-H1047R/+}$ mice express wild-type $Pik3ca$ until fed the progesterone agonist RU486 which induces Cre expression leading to the heterozygous expression of $Pik3ca^{H1047R}$ in the gastrointestinal tract. Following induction, mice expressing heterozygous $Pik3ca^{H1047R}$ were monitored for up to 2 years at which time they were sacrificed and the gastrointestinal tract analysed. No evidence of tumours or adenomas were detected (Figure 2C, Table 1).

Expression of the $Pik3ca^{H1047R}$ mutation and Apc loss in the intestine synergize to cause tumourigenesis

To explore if $Pik3ca^{H1047R}$ mutation could promote intestinal tumourigenesis in the context of homozygous Apc loss, we crossed the $Gpa33^{CrePR2/+};Pik3ca^{Lat-H1047R/WT}$ with the conditional Apc^{580S} mouse in which exon 14 of the Apc gene is flanked by loxP sites [10]. RU486 exposure to both the control $Gpa33^{CrePR2/+};Apc^{580S/580S}$ mice and the double mutant $Gpa33^{CrePR2/+};Pik3ca^{Lat-H1047R/WT};Apc^{580S/580S}$ mice induces Cre resulting in exon 14 deletion and a frameshift at codon 580 in both alleles of the Apc gene leading to the generation of a truncated, non-functional Apc protein (Figure 2B) either alone or alongside concurrent knock-in of the $Pik3ca^{H1047R}$ mutation. After Cre induction, floxed exons in Apc and $Pik3ca$ are deleted and control and double mutant mice are now referred to as $Gpa33^{CrePR2/+};Apc^{580D/580D}$ and $Gpa33^{CrePR2/+};Pik3ca^{H1047R/WT};Apc^{580D/580D}$ respectively.

Upon sacrifice, intestinal tumours were identified macroscopically in 59% of $Pik3ca^{H1047R};Apc^{580D/580D}$ ($Gpa33^{CrePR2/+};Pik3ca^{H1047R/WT};Apc^{580D/580D}$) mice which was dramatically increased compared to control ($Gpa33^{CrePR2/+};Apc^{580D/580D}$) ($Gpa33^{CrePR2/+};Apc^{580D/580D}$) and $Pik3ca^{H1047R}$ ($Gpa33^{CrePR2/+};Pik3ca^{H1047R/WT}$) mice ($p<0.0001$, Figure 2C). The double mutant $Pik3ca^{H1047R};Apc^{580D/580D}$ mice presented with up to 3 tumour masses greater than 4x4mm in size in both the small intestine and colon (Table 1).

Histological analysis identified tumours in $Pik3ca^{H1047R};Apc^{580D/580D}$ mice as aggressive adenocarcinomas with extensive invasion into the submucosa (Figure 3C). Areas of dysplasia were also observed adjacent to the adenocarcinomas. Adenocarcinomas were distributed throughout the intestine (9 in the duodenum, 6 in the jejunum, 7 in the ileum and 5 in the distal colon, Figure 2E). Three $Pik3ca^{H1047R};Apc^{580D/580D}$ mice (7%) presented with 1-4 small (<4mm) polyps observed macroscopically in addition to adenocarcinoma. These were pathologically confirmed as adenomas, although some exhibited early signs of invasion (Figure 3C). No single mutant mice developed adenocarcinomas and only one $Apc^{580D/580D}$ mouse (4%) developed adenomas.

Sanger sequencing confirmed presence of the $Pik3ca^{H1047R}$ mutation, and the silent base changes engineered into the mutant $Pik3ca$ exon 20, in the adenocarcinomas (Figure 4). IHC staining for phosphorylated S6 (pRps6), a downstream target of PI3K/Akt activity, demonstrated consistent staining across tumours (Figure 5Ai) which was more intense than in adjacent normal intestine (Figure 5Aii) confirming increased PI3K pathway activity in the tumours.

The *Pik3ca*^{H1047R} mouse was also crossed with the *Apc*^{Min} mouse, which possesses a heterozygous germline *Apc* mutation predisposing mice to development of multiple adenomas [14]. As previously demonstrated, the latency for intestinal tumour-free survival in *Pik3ca*^{H1047R};*Apc*^{580D/580D} mice is ~200 days (Figure 2B). *Apc*^{Min} mice have a reduced lifespan (<150 days) as a result of anaemia associated with polyp burden in the small intestine (Supplementary figure 1A), yet despite this narrow window in which tumourigenesis can occur, one of the twelve (8%) *Pik3ca*^{H1047R};*Apc*^{Min} mice developed a large invasive adenocarcinoma with confirmed presence of *Pik3ca*^{H1047R} mutation and *Apc* loss (Supplementary figure 1B). The single *Pik3ca*^{H1047R};*Apc*^{Min} mouse which possessed an intestinal adenocarcinoma also developed a colonic adenoma which did not express the *Pik3ca*^{H1047R} mutation (Supplementary figure 1C) but expressed higher β -catenin levels characteristic of *Apc* loss of function (Supplementary figure 1D).

Loss of Cdx2 expression observed in adenocarcinomas in areas of enhanced nuclear β -catenin expression

β -catenin localization was used to evaluate loss of *Apc* function. High levels of nuclear β -catenin staining, corresponding to regions of *Apc* depletion, are clearly visible in tumours compared to predominately cytoplasmic staining observed in the surrounding normal tissue (Figures 5B-C). Interestingly, in *Pik3ca*^{H1047R};*Apc*^{580D/580D} tumour tissue with high nuclear β -catenin staining, we observed a complete loss of *Cdx2* expression (Figure 5B-C).

Development of hepatocellular carcinomas in all mice as a result of hypomorphic unrecombined *Apc* alleles

All *Apc*^{580D/580D} mice, regardless of their *Pik3ca* status, developed hepatocellular carcinomas (HCCs) requiring mice to be sacrificed (Supplementary figure 2A-C) in spite of the fact that RU486 induction of *Gpa33Cre*^{PR2} does not express Cre in the liver (Figure 1B). The lack of expression of the *Pik3ca*^{H1047R} mutation in the HCCs and normal liver in these mice was confirmed by sequencing RNA extracted from these tissues (Supplementary Figure 2D). Previous research has demonstrated the presence of *loxP* cassettes in this strain to be hypomorphic and sufficient to reduce activity of the floxed *Apc* alleles prior to recombination (*Apc*^{580S/580S}) [15]. The liver is particularly sensitive to this resulting in HCC development even with unrecombined alleles. Here, HCC growth resulted in mice being sacrificed before intestinal tumour development accounting for the incomplete (59%) penetrance of tumour formation in *Pik3ca*^{H1047R};*Apc*^{580D/580D} mice.

Discussion

Even though mutations in the *PIK3CA* gene are commonly found in human CRC, the underlying molecular role they play in the mechanisms of intestinal carcinogenesis have not been fully elucidated. Here, we describe a model in which the physiological expression of the *Pik3ca*^{H1047R} mutation synergizes with the targeted loss of *Apc* to induce intestinal tumourigenesis. We demonstrate that *Pik3ca*^{H1047R} expression alone is insufficient to initiate intestinal tumourigenesis yet is able to drive progression of adenomas, initiated by inactivation of *Apc*, to aggressive and invasive intestinal adenocarcinomas. This result is consistent with our previous findings that *Pik3ca*^{H1047R} expression alone does not induce tumourigenesis in the ovary [9] or mammary gland [13].

Mutations in the *APC* gene are observed in over 80% of CRCs [5] and ~30% of sporadic CRCs possess *PIK3CA* mutations [7, 8]. Analysis of The Cancer Genome Atlas (TCGA) Network database of 212 CRCs found 14% harboured mutations in both *PIK3CA* and *APC* (Supplementary figure 3, [16]). This cooperative interaction between the WNT/ β -catenin and PI3K pathways in cancer is supported by previous studies showing loss of *Apc* together with *Pten*, a negative regulator of the PI3K enzyme, synergize in the progression of intestinal [17], ovarian [18] and bladder [19] tumourigenesis. Furthermore, *PIK3CA* mutations are commonly co-expressed with APC loss/ β -catenin mutation in 14% of CRCs [16]. When extended to include other genes in the PI3K/Akt and Wnt/ β -catenin pathways (*PIK3R1* and *PTEN*, and *CTNBL1*, respectively), this increases to ~22% of cases which possess a mutation or homozygous deletion in one or more genes in both of these pathways [16], further highlighting the importance of these two signalling cascades in CRC tumourigenesis.

Cdx2 is required for the regulation of intestinal epithelium homeostasis, development and differentiation [20]. Loss of *CDX2* mRNA and CDX2 protein expression has previously been associated with increased invasiveness in human CRC cell lines and poor prognosis in ~80% of poorly differentiated, advance stage adenocarcinoma samples from patients [20, 21]. Thus, loss of Cdx2 expression in *Pik3ca*^{H1047R}.*Apc*^{580D/580D} tumours is consistent with them being advanced stage aggressive carcinomas as observed in late stage CRCs in humans.

Cdx2 is also considered to be a tumour suppressor in the adult colon. This is considered to be the result of down-regulation, not genetic alterations, due to the low number of *CDX2* mutations observed in CRCs [20-23]. *In vitro* studies demonstrated PTEN stimulated expression of Cdx2 in colon cancer cell lines while inhibition of PI3K by wortmannin also produced a similar effect suggesting that Cdx2 is a target of the PTEN/PI3K signalling pathway [24]. As such, we postulate that constitutive activation of the PI3K pathway by the *Pik3ca*^{H1047R} mutation in our system may be down regulating Cdx2 and its action as a tumour suppressor further contributing to the intestinal tumourigenesis observed.

Our data contrasts with a recent study where the transgenic expression of a constitutively active PI3K protein was sufficient to initiate aggressive tumour formation in the mouse colon within 60 days [25] which became slightly more aggressive upon spontaneous loss of *Apc* [26]. The tumourigenesis observed with PI3K activation alone in their model may be due to the different mutant protein used to dominantly activate the PI3K enzyme (a fusion of p110 α and the iSH2 domain of p85 compared to our single amino acid substitution). Also, they used a transgenic approach to express their mutant PI3K which is likely to result in the over-expression of PI3K and potentially within cells that may not normally express PI3K [25]. In our model, both expression of the *Pik3ca*^{H1047R} mutation and loss of both *Apc* alleles are targeted to the same cell in the intestine and, importantly, the *Pik3ca*^{H1047R} mutation is knocked-in to the endogenous gene and thus expression is under the control of the endogenous promoter. As a result, the mutant protein is expressed at physiological levels and only in cells that normally express *Pik3ca*. Thus our model more accurately reflects the scenario of a heterozygous somatic mutation, as occurs in human CRC.

Evidence is growing that *Pik3ca*^{H1047R} mutation is a weak oncogene, and that multiple hits within the PI3K pathway itself, or in other oncogenic pathways, are necessary for it to contribute to tumourigenesis [9, 13]. Our current data further supports these findings, indicating that *Pik3ca* mutations alone, when expressed at physiological levels, are unable to initiate tumourigenesis in the intestines but have a role in driving the progression of adenomas to invasive adenocarcinomas.

The ability of *Pik3ca*^{H1047R} to cooperate with *Apc* loss to drive progression to adenocarcinoma implies that therapeutic targeting of the PI3K pathway could be beneficial in limiting disease progression in CRC patients. Furthermore, this novel model of intestinal carcinoma will provide a valuable tool for further examining the role of the PI3K signalling cascade in CRC.

Authors' contribution

Wayne Phillips conceived the idea. Lauren Hare, Toby Phesse, Karen Montgomery and Kevin Mills performed the experiments. Robert Ramsay, Mattias Ernst and Joan Heath made contributions to the conception and design of *in vivo* experiments and provided reagents. Paul Waring provided pathological analyses of tissues. All authors participated in analysis, discussion and interpretation of results. Lauren Hare wrote the manuscript. All authors read and approved the manuscript.

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Table 1. Incidence of intestinal tumours in control, *Pik3ca*^{H1047R}, *Apc*^{580D/580D} and *Pik3ca*^{H1047R};*Apc*^{580D/580D} mice.

Genotype	Number of mice	Number of mice with adenomas	Number of mice with adenocarcinomas	Total number of adenomas	Total number of adenocarcinomas
Control	19	0	0	0	0
<i>Pik3ca</i> ^{H1047R}	33	0	0	0	0
<i>Apc</i> ^{580D/580D}	28	1 (4%)	0	4	0
<i>Pik3ca</i> ^{H1047R} ; <i>Apc</i> ^{580D/580D}	46	3 (7%)	27* (59%)	7	28

*p<0.0001, compared to wild-type and single mutant controls, Fishers exact test.

Figure legends

Figure 1. Confirmation of Cre recombinase excision using the Rosa26 *lacZ* reporter mouse.

(A) Schematic of the genetic strategy used to assess *lacZ* expression using the *lox-stop-lox Rosa26^{lacZ}* mouse strain crossed with the RU486 inducible *Gpa33Cre* mouse strain. Upon exposure to RU486, Cre excises the floxed stop codon only in intestinal cells which express *Gpa33* resulting in *lacZ* expression (blue) in these cells which is detectable by β -galactosidase staining. (B) *lacZ* expression in tissues 90 days post induction of Cre recombinase expression by RU486 from a reporter positive *Rosa^{lacZ}* (*Gpa33Cre^{+/-}:Pik3ca^{H1047R/WT}:Rosa^{lacZ/WT}*) mouse and from a control no *Gpa33Cre* (*Gpa33Cre^{-/-}:Pik3ca^{H1047R/WT}:Rosa^{lacZ/WT}*) mouse. Colon and small intestinal tissues showed positive staining in our test mouse (higher magnification inset) and no staining in the control mouse. Liver was stained as a negative tissue control and showed no positive staining in either the *Rosa^{lacZ}* reporter or the *Gpa33Cre* control mice. Scale bars, 50 μ m.

Figure 2. Mutant *Pik3ca^{H1047R}* and *Apc* loss leads to intestinal adenocarcinoma.

(A) Schematic of the exon-switch strategy used to engineer a conditional knock-in of mutant *Pik3ca^{H1047R}*. *LoxP* sites were placed on either side of exon 20 of the *Pik3ca* gene and a duplicate copy of exon 20 containing the mutant sequence (red) placed downstream of the wild type exon (lat-H1047R). Before Cre exposure the wild-type exon is expressed as normal and only after Cre expression is the floxed wild-type exon excised and replaced by the duplicate exon resulting in expression of the *Pik3ca^{H1047R}* mutation [9]. (B) The *Apc* conditional knock-out mouse strategy used involves the flanking of exon 14 with *loxP* sites (purple) which is illustrated before (580S) and after (580D) Cre expression [10]. Ex = exon. (C) Kaplan-Meier analysis reveals a median intestinal tumour free survival of 506 days in *Pik3ca^{H1047R}:Apc^{580D/580D}* mice compared to *Apc^{580D/580D}*, *Pik3ca^{H1047R}* and control mice, all of which remained tumour free (*n* in parentheses). (D) Macroscopic images of large tumours (black arrows) observed in both the small and large intestines of *Pik3ca^{H1047R}:Apc^{580D/580D}*. Scale bars, 1cm. (E) Graphed are the location proportions of adenocarcinomas observed across the small intestine and colon in *Pik3ca^{H1047R}:Apc^{580D/580D}* mice.

Figure 3. Pathological analysis of intestinal tumours in the *Pik3ca^{H1047R}:Apc^{580D/580D}* mice determined they are invasive adenocarcinomas.

(A) Haematoxylin and eosin (H&E) staining demonstrated tumours observed in *Pik3ca^{H1047R}:Apc^{580D/580D}* mice were invasive adenocarcinomas (higher magnification of boxed area in B). (C) Additional lesions observed included adenomas with early invasion (higher magnification of boxed area in D). (E) *Apc^{580D/580D}* mice only presented with adenomas (higher magnification of boxed area in F). Scale bars, 100 μ m.

Figure 4. Sanger sequencing of intestinal tumours from *Pik3ca^{H1047R}:Apc^{580D/580D}* mice confirmed the presence of the *Pik3ca^{H1047R}* mutation.

RNA was extracted from the tumours, reverse transcribed into cDNA and Sanger sequenced. Sequencing from exon 19 into exon 20 of the *Pik3ca* transcript confirmed the introduction of the mutant exon by the presence of all silent base changes

engineered into the mutant exon 20 of the mouse (indicated by orange boxes, [9]) as well as the base change responsible for the *Pik3ca*^{H1047R} mutation (indicated by the blue box). The portion of sequence spanning the mutation site (labelled H1047R) indicates the A>G base change resulting in the histidine (H) to arginine (R) amino acid change.

Figure 5. Intestinal adenocarcinomas from *Pik3ca*^{H1047R};*Apc*^{580D/580D} mice show high PI3K and β -catenin signalling.

Representative histological images and IHC staining of adenocarcinomas observed in the small intestine and colon of *Pik3ca*^{H1047R};*Apc*^{580D/580D} mice are shown. (A) Staining for phosphorylated S6 (pRps6) in an invasive adenocarcinoma (i) and adjacent normal small intestine (ii) (higher resolution images inset). (B) Colon and (C) small intestine adenocarcinomas were stained for β -catenin, to evaluate loss of Apc activity, and Cdx2, an intestinal cell marker, was used to demonstrate the intestinal origin of the tumours. Boxes indicated areas presented in higher resolution below each image. Areas which stained differentially for β -catenin and Cdx2 are separated by the dashed line. Scale bars, 100 μ m.

Supplementary figure legends

Supplementary figure 1. An intestinal adenocarcinoma was observed in a single *Pik3ca*^{H1047R};*Apc*^{Min} mouse.

(A) Kaplan-Meier curve of overall survival in a cohort of control *Apc*^{Min} alone (*Gpa33Cre*^{+/-};*Apc*^{Min/WT}) mice compared to a cohort of mutant *Pik3ca*^{H1047R};*Apc*^{Min} (*Gpa33Cre*^{+/-};*Pik3ca*^{H1047R/WT};*Apc*^{Min/WT}) mice (*n* in parentheses). (B) Upon dissection of the GI tract, a large polyp was observed in the jejunum of the SI (black arrow). A smaller polyp was observed in the colon (black arrow head). Scale bar, 1cm. (C) RNA was extracted from polyps as well as normal colon and SI tissue. The RNA was reverse transcribed to cDNA and Sanger sequenced for the *Pik3ca*^{H1047R} mutation. The wild-type *Pik3ca* gene sequence is shown (labelled WT *Pik3ca*) as well as the silent base changes engineered into the mutant *Pik3ca*^{H1047R} which are identified by the orange boxes. (D) Pathological analysis of H&E sections from these polyps revealed the jejunum growth was an invasive adenocarcinoma while the colon polyp was an adenoma. IHC staining for β -catenin of FFPE sections of these tumours was conducted to confirm loss of Apc activity (higher magnification inset). Dashed line separates area of normal colon (Co) from the adenoma (Ad). Scale bars: bracketed = 25 μ m, unbracketed = 100 μ m.

Supplementary figure 2. Hypomorphic *Apc*^{580S/580S} alleles cause the development of hepatocellular carcinoma (HCC) regardless of *Pik3ca* mutation status.

(A) Kaplan-Meier analysis of overall survival of *Pik3ca*^{H1047R};*Apc*^{580D/580D} mutant (*n* = 46) and *Apc*^{580D/580D} alone (*n* = 28) control mice revealed no significant difference. (B) Gross morphology of HCCs observed in three *Pik3ca*^{H1047R};*Apc*^{580D/580D} mutant and *Apc*^{580S/580S} alone control mice livers. Dashed white lines outline the tumours observed. Scale bars, 1cm (C) Tumours were dissected, fixed, embedded in paraffin and sectioned. Sections were H&E stained for histopathological assessment (higher magnifications are inset.) Scale bars, 1000 μ m (Li: liver, Tu: tumour). (D) Sanger sequencing of HCCs and adjacent normal liver tissue from both *Pik3ca*;*Apc* (*Gpa33Cre*^{+/-};*Pik3ca*^{H1047R/WT};*Apc*^{580D/580D}) mice and *Apc* alone (*Gpa33Cre*^{+/-};*Apc*^{580D/580D}) control mice revealed wild-type *Pik3ca* sequence in all samples

examined. The wild-type *Pik3ca* gene sequence is shown (labelled WT *Pik3ca*) as well as the mutant *Pik3ca* sequence containing silent base changes engineered into the mutant *Pik3ca*^{H1047R} which are identified by the orange boxes.

Supplementary figure 3. Examination of the TCGA database reveals co-occurring PI3K/Akt and Wnt/β-catenin pathway mutations are common in CRC. Analysis of mutations and/or homozygous loss of the *PIK3CA*, *PTEN* and *PIK3R1* genes and the *APC* and *CTNNB1* genes encoding members of the PI3K/Akt and Wnt/β-catenin pathways respectively, in 212 CRC tumours, reveals co-occurrence of mutation or loss in 46 of human CRCs (data accessed from <http://www.cbiportal.org>).

Figure 1

Figure 2

Figure 3

Figure 4

Figure 5

Supplementary Figure 1

Supplementary Figure 2

Supplementary Figure 3

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