Stage dependent expression and tumour suppressive function of *FAM134B (JK1)* in colon cancer

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Abstract

The aims of the present study are to investigate sub-cellular location, differential expression in different cancer stages and functional role of *FAM134B* in colon cancer development.

Immunocytochemistry was used to study the location of expression of *FAM134B* in the colon cancer cells. In addition, *FAM134B* expression was quantified at protein and mRNA levels in cell lines (SW480, SW48, HCT116, FHC) using Western blot and real-time PCR. *In vitro* functional assays (cell proliferation, wound healing, clonogenic, cell cycle, apoptotic assays) and an *in vivo* xenotransplantation mouse models were used to investigate the molecular role of *FAM134B* in cancer cell biology in response to FAM134B silencing with shRNA lentiviral particles. The examination showed that FAM134B protein was noted in both cytoplasm and nuclei of cancer cells. In cancer cells derived from stage IV colon cancer (HCT116), the level of *FAM134B* expression was remarkably reduced when compared to non-cancer colon cells (FHC) and cancer cells derived from stage II colon cancer (SW480). *FAM134B* knockdown significantly ($p < 0.05$) increased the proliferation of colon cancer cells following lentiviral transfection when compared to scrambled control or untreated counterparts. Furthermore, *FAM134B* suppression significantly increased (34-52%; $p < 0.05$) the clonogenic capacity and wound healing potential of cancer cells. Cell cycle analysis revealed that *FAM134B* down-regulation significantly increases the proportion of cells performing DNA synthesis ($p < 0.01$). The *in vivo* xenotransplantation model showed that higher-grade tumours developed in mice receiving *FAM134B* knockdown cells compared to scrambled control cell receiving mice. Furthermore, weights and sizes of the tumours were higher in mice receiving *FAM134B* knockdown cells when compared to the control group. To conclude, reduced expression in colon cancer of advanced cancer stages and the effects of its down regulation both *in vitro* and *in vivo* indicated that *FAM134B* acts as a cancer suppressor gene in colon cancer. **Keywords:** FAM134B; JK1; tumour suppressor; colon cancer.

Introduction

FAM 134B (Family with sequence similarity 134, member B), also called *JK1*, is located at chromosome 5p15.1, downstream of δ -catenin. Our group first reported the importance of this gene in human disease.¹ *FAM 134B* was noted to play important roles in the pathogenesis of oesophageal squamous cell carcinoma.¹ This gene encodes a cis-golgi transmembrane protein, which is essential for the long-term survival of nociceptive and autonomic ganglion neurons.² Hereditary sensory and autonomic neuropathy type IIB is associated with the mutations of *FAM134B*.^{3,4} In addition, this gene may have a role in susceptibility to vascular dementia.⁵ Also, over expression of *FAM134B* mRNA was associated in patients with increased risk of allergic rhinitis.⁶ Scheubert and co-workers noted that *FAM134B* acts as a biomarker for pluripotency of stem cells in mice. The finding was based on the genetic algorithm and bioinformatics on multiple Gene Expression Omnibus (GEO) data sets.⁷ A recent study noted that *FAM134B* regulated endoplasmic reticulum turnover by selective autophagy.⁸

In colorectal cancer, we have found that *FAM134B* is a growth-related gene, which has a significant role in cancer pathogenesis by exhibiting the properties of a tumour suppressor gene.⁹⁻¹¹ In a large cohort of patients with colorectal cancer tissues samples, *FAM134B* mRNA expression and DNA copy number were noted to be significantly lower in colorectal cancer when compared to non-cancer colon tissues or pre-invasive colorectal lesions (colorectal adenomas).⁹⁻¹¹ In addition, a functional study by our team demonstrated that *FAM134B* inhibits the invasion and migration of colon cancer *in vitro*.¹⁰ The results of these studies imply that *FAM134B* is involved with different biological networks in colorectal cancer pathogenesis. Despite its clinical and pathological significance, *FAM134B*'s tumour suppressor properties and its impact in cancer cell biology has not been studied in depth. In addition, the stage dependant expression and localisation of *FAM134B* in cancer cells are still

unclear. In this study, we aim to examine the cellular distribution, expression profiling and downstream effects of *FAM134B* suppression on cell lines derived from different pathological stages of colon cancer both *in vitro* and *in vivo*.

Results

Localization of FAM134B in colon cancer

Both cytoplasmic and nuclear localization of *FAM134B* protein was observed with a predominant cytoplasmic expression in colon cancer cells (Figure 1A and supplementary data 1). *FAM134B* protein co-localized with cis-golgi marker protein giantin in studied colon cancer cells (Figure 1A). Immunocytochemistry analyses also revealed cytoplasmic and nuclear expression of *FAM134B* protein in all cell lines (Figure 1B). The expression level of *FAM134B* protein was noted to be reduced in colon cancer cell line derived from stage III colon cancer (SW-48) when compared to a cancer cell line derived from stage II colon cancer (SW-480) and normal colon cell line (FHC) (Figure 2A). The relative protein band intensity of *FAM134B* expressions in FHC and SW-480 were 0.93 ± 0.1 and 0.63 ± 0.3 respectively. On the other hand, the intensity of *FAM134B* protein expressions were 0.2 ± 0.4 and 0.11 ± 0.02 in SW-48 and HCT116 cells respectively (Figure 2E).

FAM134B mRNA and protein expression is stage dependent in colon cancers

Expression levels of *FAM134B* at both mRNA and protein levels showed significant changes in the three colon cancer cell lines derived from cancers of different pathological stages when compared with normal colon epithelial cells after normalizing with housekeeping gene *GAPDH*. With the advancement of cancer stages (Stage III & Stage IV) *FAM134B* mRNA expression was found to be markedly reduced in cancer cells from the cell lines derived from stage 3 or stage 4 colon cancers (SW48 and HCT116) in comparison to those from stage 2 colon cancer (SW480) and non-neoplastic colon epithelial cells (Figure 2B-C). The inverse ratio of *FAM134B* mRNA expression in FHC and SW-480 cell were 2.125 ± 0.24 and 1.952 ± 0.53 respectively whereas the inverse ratio in HCT116 was 0.524 ± 0.07 (2B). The fold change of *FAM134B* mRNA expression also showed similar

fashion (2C). In concordance to the mRNA expression, *FAM134B* protein (~54 kDa) expression also showed significant reduction in more advanced colon cancer cell lines (HCT116, SW-48 versus SW480/ FHC) (Figure 2D-E).

Knockdown of *FAM134B* gene

Cells treated with *FAM134B* shRNA showed significant reduction of *FAM134B* protein levels when compared to the control and non-transfected wild type cells (Figure 3C-D). *FAM134B* mRNA levels in shRNA treated cells showed ~ a 4-fold suppression of *FAM134B* in both SW-480 and SW-48 cell lines when compared to non-transfected and control shRNA treated cells (Figure 3A & B). The comparison of *FAM134B* expression in shRNA treated, scrambled control and wild type cells were presented in supplementary data 2.

Tumour suppressor properties of *FAM134B* *in vitro*

Colon cancer cells treated with *FAM134B* shRNA showed increased cell proliferation when compared to control and non-transfected cells (wild type counterparts). The increased proliferation of cells was observed in all five days of the experiment with a similar trend in each cell line (Figure 4). In addition, *FAM134B* shRNA treated cells had a higher cell migration potential than the control and wild type cells as they healed the created wound faster when compared to their counterpart (Figure 5). *FAM134B* suppressed cancer cells healed the wound completely on 4th day of initial scratch whereas non-treated and control cells took more time to heal the wounds. Similarly, *FAM134B* shRNA treated colon cancer cells showed remarkably increased colony formation properties in comparison to the control and non-transfected wild type colon cancer cells (Figure 6A-D).

Apoptotic changes

There was no statistically significant difference of apoptotic cell populations noted among the different cells groups (*FAM134B* shRNA treated, scrambled control and wild type non-transfected cells). Flow cytometric analysis showed a slight decrease (2-4%) of apoptotic cells in *FAM134B* shRNA treated colon cancer cells (7A-D). Also, there was no notable change observed with nuclear staining among the *FAM134B* shRNA and control cells groups (Supplementary data 3 and 4).

Alteration of cell cycle kinetics

Cell cycle analysis revealed an accumulation of cells in the S phase of the cycle in *FAM134B* suppressed cells when compared to control and non-transfected wild type cells. In case of control SW-480 cells, the percentage of S phase cells was $30.9 \pm 4.5\%$, while in *FAM134B* knockdown SW-480 cells, the S phase population increased remarkably to $43.0 \pm 4.13\%$ ($p < 0.01$) (8A-B). Similarly, in *FAM134B* shRNA treated SW-48 cells, the proportion of S phase cell was significantly ($p = 0.0083$) increased (32.1% versus $43.1 \pm 3.1\%$) when compared to that of control cells (Figure 8C-D). These increments were parallel with a decreased in cell population in the G2/M phase. These results indicated that regulation of the synthesis phase of the cell cycle could be altered by the suppression of *FAM134B*.

***In-vivo* confirmation of *FAM134B* tumour suppressor properties**

The functional role of *FAM134B* in colon cancer pathogenesis was investigated *in vivo* by subcutaneously injecting *FAM134B* knockdown and control cells into severe combined immunodeficiency (SCID) mice. Local tumours were formed in all experimental animals (12/12) at the site of injection. The tumours were detectable by palpation after one week of cancer cell implantation. Mice treated with *FAM134B* knockdown cells (SW-480

and SW-48) produced significantly larger tumours compared to the control cell ($196 \pm 1.4 \text{ mm}^3$ versus $48 \pm 1.5 \text{ mm}^3$ and $171.5 \pm 2.6 \text{ mm}^3$ versus $71.87 \pm 1.8 \text{ mm}^3$, respectively) treated mice (Figure 9A). Pathological examination of the tumours confirmed that the tumours produced in mice were adenocarcinomas, the tumour type that was expected (9C-F). In addition, mice treated with *FAM134B* knockdown SW-48 colon cancer cells produced higher grade and more poorly differentiated cancer when compared to control cells (9E-F). The weights of tumours from *FAM134B* downregulation cells treated mice were higher when compared to that of control groups (Figure 9B). In mice receiving *FAM134B* suppressive cells (SW-48 and SW-480) the tumours weight were 0.2 ± 0.08 and 0.315 ± 0.07 whereas in control groups the weight were 0.113 ± 0.05 and 0.124 ± 0.04 , respectively (Figure 9B).

Discussion

The results of this study indicate that *FAM134B* plays a direct role in cancer pathogenesis through regulation of cellular proliferation and mobility, operating as a tumour suppressor.

As the first of its sub-studies, this research investigated the physical localization of *FAM134B* protein in cell lines of different colon cancer stages. Immuno-staining of colon cancer cells revealed a cytoplasmic and nuclear distribution of the protein with predominantly cytoplasmic expression (Figure 1). A previous study by our team also noted nuclear localization of *FAM134B* protein in colorectal carcinoma, adenoma and non-tumour mucosa.¹¹ *FAM134B* was noted to co-localize with the cis-golgi matrix protein giantin in the cell lines tested in our research. Kurth and co-workers previously demonstrated that *FAM134B* is a cytosolic (cis-golgi matrix) protein in N2a cells, a tumour cell line that is associated with autonomic ganglion neurons.² Another study by Khaminets *et al* reported that *FAM134B* protein co-localized with endoplasmic reticulum marker proteins (KDEL, CLIMP and SEC61B) in A549, HeLa and U20S cells.⁸ These results, along with our own observation of nuclear staining for *FAM134B* support the potential for the protein to have multiple localizations in the cell.

The translocation of *FAM134B* protein to the nucleus in cancer tissues observed in our previous study and this research indicates the possibility of nucleus-cytoplasmic translocation of *FAM134B* protein in a certain subset of colorectal cancers, for reasons yet unknown. The tumour microenvironment, especially hypoxia may induce the translocation of this novel protein, or it may be a specific response by cells of colorectal origin, given the lack of other observations of nuclear localisation in other studies. This kind of translocation of proteins in cancer is not a rare event, however, and it can have direct or indirect effects on the function of other genes. The precise mechanism of the function of *FAM134B* in cancer

cells is unknown. It is possible that translocation to the nucleus or to the endoplasmic reticulum allows new interactions with growth signaling pathways in developing cancer. For example, both nuclear and cytoplasmic localizations of BARD1 have been observed and increased cytoplasmic localization of BARD1 protein was associated with apoptosis.¹² A similar mechanism may be attributed to FAM134B cytoplasmic localisation in human cancers. Alternatively, translocation of FAM134B away from its normal location may sequester it from its usual binding partners and prevent its normal activity.

Without knowing the precise functional partners of FAM134B, it is difficult to know whether it has a single function in normal tissues, or whether the protein has alternative effects in some tissue types, differentiation stages, or developmental programs. It is possible that nuclear localisation of FAM134B is normal in undifferentiated cells. In cancer, this may represent the kind of reversion to stem-like characteristics seen for other mechanisms. Thus, further investigation with in-depth *in-vitro* and *in-vivo* analyses should be used to study the detailed mechanisms of this translocation in both normal and malignant cells and its functional consequences.

One of the other results shown in this study on *FAM134B* and our previous work on the gene is that its expression seems to be stage dependent. In human colorectal adenocarcinoma, eukaryotic translation initiation factor 4E (*eIF4E*) has showed stage dependent expression.¹³ In the early stage (T1) of colorectal cancer, *eIF4E* protein is expressed significantly higher than in late stage (T3) cancers.¹³ The reduced expression of *FAM134B* protein in advanced stage colorectal cancer when compared to early stage in this and our earlier study implies that the protein is involved in the molecular pathways of colorectal cancer pathogenesis, and it might act as a tumour suppressor in colon cancer development. In our previous study, patients with colorectal adenoma (pre-invasive lesion) expressed a higher amount of *FAM134B* protein and mRNA than non-cancer tissues.¹¹ On the

other hand, lower expression of *FAM134B* protein and mRNA were noted in advanced stages colorectal cancers.¹¹ The high expression (protein and mRNA) levels of *FAM134B* in colorectal adenomas, combined with later changes in malignant tissues, suggests that *FAM134B* plays an important role in cancer initiation and progression. The low expression levels of *FAM134B* in advanced colorectal adenocarcinomas, combined with results from the cellular experiments in this research, indicate that *FAM134B* acts as a tumour suppressor gene in colorectal adenocarcinoma.

The *in vitro* analyses of this study demonstrated that *FAM134B* protein and mRNA expression was down regulated in cell lines derived from advanced tumour stages. The most advanced colon cancer cell line used in this research, derived from stage IV colon cancer (HCT116), showed the lowest *FAM134B* expression (both protein and mRNA level) when compared to the colon cancer cell line derived from stage II colon cancer (SW-480) and the non-neoplastic colon epithelial cell line (FHC). This stage dependent expression of *FAM134B* further reinforced our hypothesis that *FAM134B* would act as a tumour suppressor gene in colon cancer pathogenesis and as a prognostic marker in colon cancer.

The molecular causes of the expression reduction of this novel gene both at the mRNA and protein levels in colon cancer are not clear, however. In part, this is due to our lack of knowledge of the how the gene is being regulated and what its function is. Several mechanisms may explain the loss. Mutation (deletion or point mutation) in the gene could be the possible cause of reduced expression, something common for tumour suppressors, but other mechanisms such as gene methylation may also be involved. Additionally, the loss may be caused by different mechanisms in different colorectal cancer subtypes, depending on specific predilections. For example, the hyper-methylator phenotype may tend to deactivation of *FAM134B* via methylation. As this is the first comparative study of *FAM134B* expression in different cancer cell lines and normal epithelial cell lines, further

studies are necessary to confirm exactly how *FAM134B* expression is altered in cancer progression. These findings of changes in *FAM134B* expression noted in cancer cell lines were similar to the results obtained from colorectal cancer tissues. This further strengthens the hypothesis that *FAM134B* possesses tumour suppressor properties in pathologically advanced colorectal cancer cells, both in solid tumours and viable cancer cells. Additionally, the fact that the trend from cancer tissues is repeated in several colorectal cancer cell lines indicates that *FAM134B* alteration may be a central event in the pathology of many colorectal cancers.

Functional assays with *FAM134B* suppressed colon cancer cells used in this study further support the tumour inhibitory properties of *FAM134B* in a colon cancer model. Compared to control shRNA treated and non-transfected wild type colon cancer cells, *FAM134B* shRNA treated cells showed increased cell proliferation, increased colony formation and higher migration and wound healing capacity in this study. A previous study from our group also noted that higher *FAM134B* expression reduced migration and invasion of colon cancer cells.¹⁰ Although the statistical test was not significant, *FAM134B* knockdown also decreased the apoptotic rate of cancer cells from stage II and stage IV colon cancers (SW-480 and SW-48) in comparison to that of control and un-transfected cells. The difference of apoptosis observed was approximately a 50% reduction, and from 5% to 2% for SW480 cells and 6% to 3% for SW48 cells (Figure 7). The relatively high error rates in these apoptosis measures may have prevented the accurate detection of this reduced apoptosis. Further experiments using cell lines with higher resting apoptosis rates, or following challenge with apoptosis triggers may determine whether the trend for decreased apoptosis seen in this study is experimental error, a direct suppression of apoptotic pathways or a manifestation of the increased proliferation observed following *FAM134B* knockdown.

A study from our group noted that knockdown of *FAM134B* expression increased the migration and invasion capacity of colon cancer cells.¹⁰ These observations strongly suggest that *FAM134B* negatively regulates colon cancer growth. In contrast to these results, Tang and co-workers reported that *FAM134B* promotes cancer cell proliferation and tumour growth in a model derived from oesophageal squamous cell carcinoma.¹ It is worth nothing that other cancer related genes such as cell surface mucin gene family members 11, 12 and 13 (MUC 11, MUC 12 and MUC 13) are also expressed in different ways in colorectal and oesophageal cancers.^{14,15} Lower mRNA expressions of MUC 11, MUC 12 and MUC 13 were noted in colon cancer whereas higher mRNA expressions of these genes were found in oesophageal cancer.^{14,15} Thus, *FAM134B* may act as both oncogene and tumour suppressor gene in human cancer. The function of *FAM134B* could depend on the genes that it interacts with in a given cell, which may also be an explanation for altered localisation of FAM134B as previously discussed. Researches to examine the mechanism of *FAM134B* action and identify its binding partners to more completely understand the significance of its expression in different cancer types.

Gene manipulation can modulate and interfere with cell cycle progression. In this study, alteration of cell cycle kinetics following downregulation of *FAM134B* using shRNA lentiviral particles was investigated. It was noted that *FAM134B* knockdown caused accumulation of cells in S phase of the cycle. This result indicated that FAM134B might be involved in the regulation of DNA synthesis and down regulation of *FAM134B*, triggered DNA synthesis via modulating molecules associated cell cycle progression. Such triggering is most likely through the loss of FAM134B suppression of the activity of another protein, causing the G1/S phase checkpoint to be opened. It has been reported, for example, that megakaryoblastic leukemia 1 (MKL1) downregulation causes S phase arrest of the cell cycle via modulating p21 activity, *FAM134B* may have similar effects, perhaps via interaction with

golgi proteins to promote stability or delivery of protein complexes that restrict DNA synthesis, enable ongoing protein production to prepare for DNA replication later on.¹⁶ Further studies to explore the mechanism by which the *FAM134B* gene causes alterations in the cell cycle kinetics are needed.

Results from our xenotransplantation animal model and previous studies provide further demonstration of the functional role of *FAM134B* in colon cancer pathogenesis [9-10]. Tumour weight and size in mice from the *FAM134B* suppressive group were much higher than that of the control group for both cell lines. Higher-grade tumour formation in mice receiving *FAM134B* knockdown cancer cells further implied a tumour inhibitory role of *FAM134B*, and potentially a role in development of other advanced cancer phenotypes. Results from the *in vivo* study thus validate and translate our *in vitro* studies. These correlations of *in vitro* and *in vivo* findings confirm the tumour suppressive function of *FAM134B* in colon cancer.

This study confirmed the presence of cytoplasmic and nuclear localization of the *FAM134B* protein in colon cancer tissues and cell lines. Expression of *FAM134B* mRNA and protein showed significant changes in cells derived from different pathological stages of colon cancer, matching previous data from human tumours. Knockdown of *FAM134B* also results in similarly advanced cancer behavior, including increased proliferation, wound healing, colony formation, higher grade tumours and altered cell cycle kinetics. The differential expression of *FAM134B* can therefore be used as a prognostic marker in colon cancer. Reduced expression in more advanced colon cancers indicate that it acts as a tumour suppressor in colon cancer pathogenesis. This is confirmed by the results of our *in vivo* and *in vitro* studies in colon cancer. It is also possible that *FAM134B* loss is a central driver of progression in many colorectal cancers, and the protein or gene may be a potential treatment target. New research to identify the mechanism of action of *FAM134B* in control of cellular

growth is needed to fully unravel the role of this gene and enable its effective use in clinical contexts.

Materials and methods

Cell culture

Three colon cancer cell lines and one non-cancer colon epithelial cell line were used in this study. The colon cancer cell lines included SW-480 (ATCC CCL228; stage 2, colonic adenocarcinoma), SW-48 (ATCC CCL-231; stage 3 colonic adenocarcinoma) and HCT116 (ATCC CCL-247; stage 4 colonic carcinoma). The non-neoplastic colon epithelial cell line used was FHC (ATCC CRL-1831). All the cell lines were maintained according to ATCC guidelines. The three colon cancer cell lines (SW-480, SW-48 and HCT 116) were maintained in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin at 37°C in a CO₂ incubator. The cells in FHC were cultured in DMEM: F-12 medium supplemented with 10 ng/ml cholera toxin, 0.005mg/ml insulin, 100ng/ml hydrocortisone, 0.005 mg/ml transferrin and 10% FBS 37°C in a CO₂ incubator.

Immunocytochemistry

Immunocytochemistry was performed to identify the cellular localisation of *FAM134B* protein in the recruited cell lines. The cells were fixed with 70% ice-cold ethanol for 10 minutes and were permeabilised with 0.1% Triton X-100 for 5 minutes. Following protein block with 5% BSA (bovine serum albumin), cells were incubated with anti-rabbit FAM134B antibody (Santa Cruz, Dallas, TX, USA) for overnight at 4°C. They were incubated with secondary antibody (1:1000; anti-rabbit IgG-poly- horseradish peroxidase) in 5% BSA for one hour at 37°C. A brown positive staining was obtained by developing the peroxidase activity with freshly prepared 3, 3'-diaminobenzidine (DAB) and substrate chromogen solution (Leica, North Ryde, NSW, Australia). After washing with phosphate

buffered saline (PBS), cells were visualized under light microscopy (Olympus CKX41, Macquarie Park, NSW, Australia). Each staining was run in triplicate on a 24 well plate.

Immunofluorescence

To confirm the cellular localisation of *FAM134B* protein in different cell lines (SW-480, SW-48, HCT116 and FHC) immunofluorescence analysis was performed. The cells were seeded on cover slips at a density of $\sim 10,000/\text{cm}^2$ and were incubated at 37°C for 12 hours. Then, after being washed with PBS, cells were fixed with freshly prepared fixative (70% cold ethanol), for 30 minutes. Afterwards, cells were blocked with 5% BSA for one hour at room temperature. Cells were then washed with PBS and were incubated with anti-rabbit FAM134B (1:150) and anti-goat giantin, a cis-golgi marker protein antibody (Santa Cruz) (1:150) at 4°C overnight. This was followed by 2 hours of incubation with secondary antibody labelled with fluorescein isothiocyanate (FITC) and Texas red fluorophore (Sigma-aldrich, St Louis, MO, USA) at room temperature and was later mounted on glass slides, sealed with nail polish and then observed under a confocal microscope (Nikon A1R+, Nikon Inc., Tokyo, Japan).

Quantitative real time PCR (qPCR) analysis

For quantitative PCR, total RNA was extracted from cell lines using miRNeasy mini kits (Qiagen, Valencia, CA USA). First-strand cDNA was then generated using DyNAmo™ cDNA Synthesis Kits (Qiagen) as previously described.¹⁷ *FAM134B* mRNA expressions change in cell lines were examined using IQ5 multicolour real-time PCR detection system (Bio-Rad, Hercules, CA, USA). qPCR was performed in a total volume of 20 μL reaction mixture containing 10 μL of DyNAmo Flash SYBR green master mix (Bio-Rad), 1.5 μL of each 5 $\mu\text{mol/L}$ primer, 3 μL of cDNA at 50ng/ μL , and 4 μL of 0.1% diethylpyrocarbonate

(DEPC) treated water as previously described.^{18,19} The amplification efficiencies were normalized to that of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Primers that were used in this study are given in supplementary data 5.

Western blot analysis

Total proteins were extracted from cultured cells with lysis buffer (Bio-Rad, Gladesville, NSW, Australia) and subsequently were quantitated by absorbance spectrometry.¹⁰ Afterwards, total protein (30 µg) was separated by 15% SDS-PAGE (Bio-Rad) and transferred to nitrocellulose membranes (Bio-Rad). The membrane was blocked with 5% non-fat milk powder for 2 hours at room temperature. Then incubated with anti-rabbit FAM134B polyclonal antibody (at 1:150) and polyclonal GAPDH antibody (Santa Cruz) (1:2000) overnight at 4°C. Membranes were then washed three times with PBS-T and incubated with the rabbit secondary antibody (Santa Cruz) (1:5000) at room temperature for 2 hours. Protein bands were developed and detected with a chemiluminescence HRP detection kit (Bio-Rad, USA). Images were taken with the ChemiDoc MP Imaging system (Bio-rad). Expression of FAM134B protein was quantified and normalized to GAPDH with Image J 1.48 software.²⁰

***FAM134B* shRNA transfection**

FAM134B gene knockdown in colon cancer cells (SW-480 and SW-48) was performed using transduction ready lenti-viral particles (shRNA) and a transfection reagent, polybrene (Santa Cruz). Control cells were prepared using a scrambled control shRNA (Santa Cruz). A GMO (genetically modified organism) project approval was obtained from Griffith University Institutional Biosafety Committee (NLRD/006/15). Selection of stable clones expressing *FAM134B* shRNA and control shRNA were carried out with puromycin as

previously described.¹⁰ Post transfected reduction of FAM134B mRNA and protein expression were analysed using qPCR and Western blot respectively.

Cell proliferation assay

A cell proliferation assay using a cell counting kit-8 (Sigma-aldrich) was performed to observe the changes of proliferation in *FAM134B* knockdown cells. Both SW-480 and SW-48 cells were first seeded in flat-bottom 96-well plates at 1×10^4 cells/well. Cell proliferation was determined on days 1 to 5 after the initial seeding with CCK-8 (cell counting kit-8) following manufacturer instructions.

Wound healing assay

A wound healing assay was performed to determine the effect of *FAM134B* knockdown on the capacity of cells to migrate. *FAM134B* shRNA treated cells, wild type (non-transfected) and control cells were cultured in appropriate medium until 80-90% confluence as a monolayer. Then scratches were made with a 200 μ l pipette tip across the centre of culture plates. Fresh media was then replaced and incubated for five days of investigation. Images were taken to monitor the changes among the cells type on day 1 to day 5. Wound areas on different days of all cell types were measured and compared with Image J 1.48 software.²⁰

Colony formation assay

Approximately 250 cells were seeded in 6-well plates with 2 ml of complete medium. The cells were then cultured at 37°C in 5% CO₂ and saturation humidity. After 16 days, when microscopic clones were noted in the plate, growing of the cells was stopped. At that point, the media was discarded and cells washed with PBS. Then, the cells were fixed with

70% cold ethanol for 15 min. Afterwards, the clones were stained with crystal violet (0.5%) for 2 hours at room temperature. Finally, after being air-dried, images were taken from the plates and clone formation rates were calculated.

Apoptosis assay

FAM134B related apoptotic changes following knockdown in the colon cancer cells was assayed by using nuclear stain, Hoechst 33342 and DAPI (4',6-diamidino-2-phenylindole). In short, cells were fixed in 4% paraformaldehyde for 10 minutes and permeabilised with 0.1% Triton-X-100 for 5 minutes at room temperature. After washing with PBS, the cells were stained with Hoechst 33342 (10µg/ml) and DAPI (50µg/ml) for 30 minutes at 37 °C. Finally, the cells were observed under fluorescence microscope (Olympus) and percentages of apoptotic cells recorded in three independent experiments from different plates.

Apoptosis assays on *FAM134B* shRNA transfected, control shRNA treated and wild type cells were also performed using an annexin V-FITC apoptosis detection kit (Invitrogen, Carlsbad, CA, USA). The numbers of annexin V and propidium-iodide positive cells were detected using BDFACS Calibur (BD Biosciences, Franklin Lakes, NJ, USA) and data were processed with FlowJo v10 software (FlowJo LLC, Ashland, OR, USA).

Cell cycle analysis

FAM134B knockdown and control/non-transfected wild type cells were fixed with cold 70% ethanol for one hour as previously described.²¹ After washing with cold PBS, 5µl of RNase A (10mg/ml) was added to the cells and incubated for one hour at 37 °C. Finally, 10 µl of propidium iodide solution (1 mg/ml) was added to the cell suspension and analysis was performed with flow cytometry (BD FACSCalibur, BD Biosciences).

In vivo mouse model

Four groups (n=3 in each group) of severe combined immunodeficiency (SCID) mice (3 week old males) were used for this study. Animal ethics approval for this study was from the University Animal Ethics Committee (Approval number: 45005204-10-0).

Mice in group I and group II received SW-480 and SW-48 *FAM134B* knockdown cells, respectively, whereas group III and group IV mice received SW-480 and SW-48 control cells, respectively. Approximately 1×10^6 cancer cells were injected subcutaneously into the flanks of the mice using a 1 ml syringe. After 21 days of cancer cell implantation, the mice from all groups were sacrificed using standard operation procedures.²² The weight and size of the tumours noted in the mice were recorded according to published protocol.²² The tumours were collected, fixed in 10% formalin and embedded in paraffin.²² Histopathological examinations of the specimen were performed after haematoxylin and eosin staining of the sections taken from the paraffin blocks. The grade and differentiation of tumours from the blocks were analysed.

Conflicts of interest Statement

The authors declare no conflict of interest.

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Figure legends

Figure 1. Localization of FAM134B in colon cancer cells. FAM134B co-stained with cis-golgi marker giantin in three colon cancer (SW-480, SW-48 and HCT116) and normal colon epithelial (FHC) cell lines (A). Cytoplasmic and nuclear localizations were also noted in all cell lines with immunocytochemistry (B).

Figure 2. Expression of *FAM134B* in colon cancer cell lines. Stage dependent expression of FAM134B was found in colon cancer cell lines. Expression level of FAM134B in four different cell lines (A). Expression of FAM134B showed notable reduction in cancer cell (SW-480, SW-48 and HCT116) when compared to normal colon cell (FHC). With the advancement of cancer stages, inverse ratio of *FAM134B* expression reduced remarkably (B). The results are expressed as mean $\pm$ standard deviation. Level of significance $**p < 0.01$ was noted when compared with that of FHC cells. The mRNA expression analysis using fold change also showed the similar reduction of *FAM134B* mRNA in colon cancers with advanced pathological stages (C). Protein expression of *FAM134B* reduced remarkably in cancers with advanced stages (HCT116 & SW-48) when compared to non-neoplastic colon (FHC) and cells from early stage colon cancer (SW480). There was no change of GAPDH expression in four different cell lines (D). Protein was quantified and normalized with GAPDH for Western blot analysis (E). Data present as mean $\pm$ standard deviation of three independent measurements. $P < 0.031$ when compared with control (FHC) cell line.

Figure 3. Downregulation of *FAM134B* in colon cancer cell lines with shRNA lentiviral particles. *FAM134B* mRNA expression in the knockdown SW-480 cell line was four fold less when compared to the non-transfected and control shRNA treated cells (A). Similarly, in SW-48 cells, *FAM134B* shRNA treatment showed more than threefold reduction of *FAM134B* mRNA expression (B). Results are shown as mean $\pm$ SD (standard deviation). In Western blot analysis, protein level of *FAM134B* was remarkably reduced in transfected SW-

480 (C lane 3) and SW-48 cells (D lane 3) when compared to non-transfected wild type (lane 1) and control scramble shRNA treated (lane 2) cell respectively. Proteins from both cells (SW-480 and SW-48) were quantified and normalized with GAPDH for Western blot analysis (F). The results are expressed as mean $\pm$ standard deviation. Level of significance ** $p < 0.01$ and * $p < 0.05$ when compared to control scramble shRNA.

Figure 4. Effect of *FAM134B* downregulation on proliferation of colon cancer.

FAM134B knockdown cells of both SW-480 (A-B) and SW-48 (B-C) exhibited higher proliferation in comparison to control and non-transfected cells on different days after transfection. Results are shown as mean $\pm$ SD (standard deviation). Level of significance * $p < 0.05$ and ** $p < 0.01$ when compared with that of control and untransfected cells.

Figure 5. Impact of *FAM134B* knockdown on wound healing potential of colon cancer cells.

Knockdown of *FAM134B* in SW-480 cells showed more migration capacity, healing the wound faster than the control and non-transfected cells (A). Similarly, *FAM134B* suppressed SW-48 cells healed the wound early compared to control and un-transfected cells (B). In both cells, wounds were completely repaired on day 4 after the initial scratch. Wound areas of all experimental cell types on different days (day-0 to day-5) were recorded from three independent measurements and expressed as mean $\pm$ SD (C-D). Level of significance * $p < 0.05$ when compared with that of control and untransfected cells.

Figure 6. Effect of *FAM134B* suppression on clonogenic capacity of colon cancer cells.

FAM134B down-regulation in SW-480 (A-B) and SW-48 (C-D) by shRNA treatment remarkably increases the colony formation capacity in comparison to the control and non-transfected colon cancer cells. Data in bar graphs represents mean $\pm$ SD from three independent measurements. Level of significance * $p < 0.05$ when compared with that of control and untransfected cells.

Figure 7. Effect of *FAM134B* downregulation on apoptosis of colon cancer cells.

Apoptosis levels of colon cancer cell lines (SW-480 and SW-48) were determined following *FAM134B* knockdown using annexin V fluorescein isothiocyanate and propidium iodide. *FAM134B* downregulation decreased the total (late + early) number of apoptotic cells in both cell lines. Representative scatter plots of apoptosis analysis in cells in the untransfected, control and *FAM134B* knockdown groups of SW-480 (A) and SW-48(C) cell double-labelled with annexin V fluorescein isothiocyanate and propidium iodide. Quantitative analysis of annexin V and propidium iodide stained SW-480 (B) and SW-48 (D) cells. Percentages of apoptotic cells were determined from three independent experiments. Results are shown as mean $\pm$ SD.

Figure 8. Impact of *FAM134B* knockdown on cell cycle kinetics of colon cancer cells.

Cell cycle analysis with flow cytometry revealed that *FAM134B* downregulation caused S phase accumulation of colon cancer cell and triggered the DNA synthesis. Representative histograms of cell cycle analysis of cell in the un-transfected, control and *FAM134B* down regulated groups of SW-480 (A) and SW-48 (C) cell. The bar graphs presented the percentage of cells in different phase. *FAM134B* knockdown notably changed the cell cycle kinetics in SW-480 (B) and SW-48 (D) cells. The results represented as mean $\pm$ SD from three independent experiments. Level of significance * $p < 0.05$ when compared with that of control and untransfected cells.

Figure 9. In vivo impact of *FAM134B* knockdown on colon cancer. A) Implantation of colon cancer cells (SW-480 and SW-48) in mice produced tumours. Mice receiving control SW-480 cells generated a small tumour (a), but mice treated with *FAM134B* knockdown SW-480 cells produced noticeably larger tumours (b). Mice treated with control SW-48 cells produced small tumours (c), whereas mice receiving SW-48 *FAM134B* knockdown cells produced large tumours (d). B) Tumour weight (in grams) of mice receiving control and

FAM134B knockdown colon cancer cell lines (SW-480 and SW-48). Mice treated with *FAM134B* downregulated cancer cells produced significantly larger tumours than that of corresponding control cell treated mice (B). Results are shown as mean $\pm$ SD and levels of significance were * $p < 0.05$ and ** $p < 0.01$ when compared with that of control. Representative images of histopathological section obtained from tumour of mice (C-F). Low-grade adenocarcinomas were produced in mice receiving control SW-480(C) and SW-48 (E). High-grade adenocarcinomas were formed in mice treated with *FAM134B* knockdown colon cancer cells (D; SW-480, F; SW-48).

Supplementary data 1. Localization of FAM134B in colon cancer and non-neoplastic colonic epithelial cell lines. Cytoplasmic and nuclear localizations were noted in all cell lines with immunofluorescence microscopy.

Supplementary data 2. Expression of *FAM134B* in *FAM134B* shRNA treated, scrambled control and wild type cells. The results represented as mean $\pm$ SD from three independent experiments.

Supplementary data 3. Apoptosis analysis of colon cancer cells with Hoechst 33342 staining. Nuclear staining of cells in un-transfected, control, and *FAM134B* knockdown groups did not exhibit significant differences in apoptotic features (nuclear condensation, fragmentation, apoptotic body formation). a) Un-transfected SW-480, b) Control SW-480, c) *FAM134B* downregulated SW-480, d) Un-transfected SW-48, e) Control SW-48 and f) *FAM134B* downregulated SW-48.

Supplementary data 4. DAPI staining of colon cancer cells for apoptosis analysis. Cells from different experimental groups (un-transfected, control and *FAM134B* knockdown) stained with DAPI did not show significant differences in apoptotic features (nuclear condensation, fragmentation, apoptotic body formation). a) Un-transfected SW-480, b) Control SW-480, c) *FAM134B* downregulated SW-480, d) Un-transfected SW-48, e) Control SW-48 and f) *FAM134B* downregulated SW-48.

Supplementary data 5. Primer pairs of *FAM134B* and *GAPDH*.