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Cartón-García, Fernando; Brotons, Bruno; Anguita, Estefanía; Dopeso, Higinio; Tarragona, Jordi; Nieto, Rocio; García-Vidal, Elia; Macaya, Irati; Zagyva, Zsuzsanna; Dalmau, Mariona

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ARTICLE

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Fernando Cartón-García[1], Bruno Brotons[1,2], Estefanía Anguita[1,2], Higinio Dopeso[1], Jordi Tarragona[3,4], Rocio Nieto[1], Elia García-Vidal[1], Irati Macaya[1], Zsuzsanna Zagyva[1], Mariona Dalmau[1,2], Manuel Sánchez-Martín[5,6,7], Sven C. D. van Ijzendoorn[8], Stefania Landolfi[9,10], Javier Hernandez-Losa[9,10], Simo Schwartz Jr[11,12], Xavier Matias-Guiu[3,4,10], Santiago Ramón y Cajal[9,10], Águeda Martínez-Barriocanal[1,2,13] and Diego Arango[1,2,13]✉

Colorectal cancer causes >900,000 deaths every year and a deeper understanding of the molecular mechanisms underlying this disease will contribute to improve its clinical management and survival. Myosin Vb (*MYO5B*) regulates intracellular vesicle trafficking, and inactivation of this myosin disrupts the polarization and differentiation of intestinal epithelial cells causing microvillous inclusion disease (MVID), a rare congenital disorder characterized by intractable life-threatening diarrhea. Here, we show that the loss Myosin Vb interfered with the differentiation/polarization of colorectal cancer cells. Although modulation of Myosin Vb expression did not affect the proliferation of colon cancer cells, *MYO5B* inactivation increased their migration, invasion, and metastatic potential. Moreover, *Myo5b* inactivation in an intestine-specific knockout mouse model caused a >15-fold increase in the number of azoxymethane-initiated small intestinal tumors. Consistently, reduced expression of Myosin Vb in a cohort of 155 primary colorectal tumors was associated with shorter patient survival. In conclusion, we show here that loss of Myosin Vb reduces polarization/differentiation of colon cancer cells while enhancing their metastatic potential, demonstrating a tumor suppressor function for this myosin. Moreover, reduced expression of Myosin Vb in primary tumors identifies a subset of poor prognosis colorectal cancer patients that could benefit from more aggressive therapeutic regimens.

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INTRODUCTION

Colorectal cancer represents a major health concern with over 1.9 million new cases diagnosed and about 900,000 patients dying of this disease in 2020 [1]. A deeper understanding of the molecular mechanisms underlying the tumorigenic process will significantly contribute to improve the management of these patients, and eventually reduce colorectal cancer mortality.

Myosins constitute a diverse family of ATP-dependent molecular motors that move along actin filaments [2]. This versatile family of proteins has diverse functions that in addition to their well-established role in muscle contraction include cell motility, tension maintenance, cytokinesis, and intracellular trafficking [2]. Several myosins are found in the apical brush border of intestinal epithelial cells, an intricate structure formed by cellular projections supported by actin bundles known as microvilli. Unconventional Myosin Vb (*MYO5B*) is expressed at high levels in the brush border of intestinal epithelial cells and participates in recycling endosome trafficking and the establishment and maintenance of cell polarity [3–5]. *MYO5B* inactivation has been shown to cause microvillus inclusion disease (MVID), a rare congenital intestinal disorder causing persistent neonatal watery diarrhea that can be life-threatening [6]. At the histological level, MVID is characterized by the loss of polarization of intestinal epithelial cells with a characteristic microvillus atrophy and mislocalization of apical and basolateral transporter proteins [7].

Although the relevance of the loss of polarity and epithelial architecture during late tumor progression and metastasis has been thoroughly documented [8, 9], its role during premetastatic tumorigenesis is not well understood. We have previously demonstrated that brush border Myosin IA (*MYO1A*) is frequently inactivated in colorectal cancer, causing loss of cell polarity and dedifferentiation, contributing to the early stages of colorectal tumorigenesis [10]. Interestingly, *MYO5B* is located in chromosome 18q21, the genomic region most frequently deleted in colorectal tumors [11]. Moreover, *Myo5b* knockout mouse models have shown that the loss of Myosin Vb in the intestinal epithelium leads to increased proliferation and crypt hyperplasia [5]. However, the role of Myosin Vb in colorectal cancer initiation and progression has not been thoroughly investigated.

[1]Group of Biomedical Research in Digestive Tract Tumors, Vall d'Hebron University Hospital Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain. [2]Group of Molecular Oncology, Biomedical Research Institute of Lleida (IRBLleida), Lleida, Spain. [3]Department of Pathology and Molecular Genetics, Hospital Universitari Arnau de Vilanova, University of Lleida, Lleida, Spain. [4]Group of Oncological Pathology, Institut de Recerca Biomedica de Lleida (IRBLleida), Lleida, Spain. [5]Instituto de Investigación Biomédica de Salamanca (IBSAL), Salamanca, Spain. [6]Servicio de Transgénesis, Nucleus, Universidad de Salamanca, Salamanca, Spain. [7]Departamento de Medicina, Universidad de Salamanca, Salamanca, Spain. [8]Department of Biomedical Sciences of Cells and Systems, Section Molecular Cell Biology, University of Groningen, University Medical Center Groningen, Groningen, The Netherlands. [9]Translational Molecular Pathology, Vall d'Hebron Research Institute (VHIR), Universitat Autònoma de Barcelona (UAB), Barcelona, Spain. [10]Centro de Investigación Biomédica en Red de Cáncer (CIBERONC), Madrid, Spain. [11]Group of Drug Delivery and Targeting, Vall d'Hebron University Hospital Research Institute (VHIR), Universitat Autònoma de Barcelona, Barcelona, Spain. [12]Clinical Biochemistry Department, Vall d'Hebron Hospital, Barcelona, Spain. [13]These authors contributed equally: Águeda Martínez-Barriocanal, Diego Arango. ✉email: darango@irblleida.cat

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Here, we used isogenic cell line systems, a conditional *Myo5b* knockout mouse model, and large tumor collections from colorectal cancer patients to demonstrate that Myosin Vb is frequently lost or reduced during colorectal cancer progression, and that this interferes with the differentiation of colon cancer cells and enhances its metastatic capacity. In addition, although *Myo5b* inactivation in the murine intestine did not efficiently initiate tumorigenesis, it significantly accelerated intestinal tumor progression. Moreover, reduced levels of expression of Myosin Vb in primary colorectal tumors were associated with shorter patient survival. Altogether, these results demonstrate a tumor suppressor role for Myosin Vb in colorectal tumorigenesis.

RESULTS

Myosin Vb regulates the differentiation of colon cancer cells

Inactivation of *MYO5B* interferes with the differentiation of normal intestinal epithelial cells, and causes microvillus inclusion disease (MVID), a rare inherited intestinal disorder [12]. To investigate the role of Myosin Vb in the differentiation of colorectal cancer cells, we used a CRISPR/Cas9 approach to inactivate *MYO5B* in CACO2-BBE cells, a colon cancer cell line with endogenous Myosin Vb expression (Supplementary Fig. S1) that spontaneously differentiates along the absorptive cell lineage and that has been frequently used as a model of normal intestinal epithelium to investigate the effects of *MYO5B* inactivation in MVID [6, 13] (Supplementary Fig. S2A, B). We found that when CACO2-BBE$^{MYO5B\text{-}KO}$ cells were grown for 21 days in confluence, they showed impaired differentiation compared to parental CACO2-BBE cells, as revealed by the reduced activity of alkaline phosphatase and sucrase-isomaltase, two digestive hydrolases normally expressed in the brush border of intestinal epithelial cells (Fig. 1A–B). Moreover, the number of domes, semicyst structures formed due to vectorial electrolyte transport and fluid accumulation under the differentiated monolayer, was significantly reduced in CACO2-BBE$^{MYO5B\text{-}KO}$ compared to parental CACO2-BBE cells (Fig. 1C–E). Next, we found that while parental CACO2-BBE cells grown in 3D culture conditions formed cystic structures with a polarized monolayer of cells showing apical accumulation of actin towards the lumen of the cyst, CACO2-BBE$^{MYO5B\text{-}KO}$ cells invaded the lumen of the cysts and completely lost the polarized growth pattern (Fig. 1F–J). CACO2-BBE$^{MYO5B\text{-}KO}$ cysts were also found in reduced numbers compared to parental cells (Fig. 1K).

Next, we used an shRNA approach to downregulate Myosin Vb in LS174T-W4, a colon cancer cell line with high endogenous levels of Myosin Vb (Supplementary Fig. S1) that undergoes polarization upon doxycycline-dependent activation of liver kinase B1 (LKB1; Supplementary Fig. S2C) [14]. The percentage of polarized cells following LKB1 activation was significantly reduced when Myosin Vb was downregulated by an shRNA against *MYO5B* (shMYO5B), compared to control cells expressing a non-targeting shRNAs (shNT; Fig. 1L–N). Finally, using a cohort of 155 Dukes C colorectal cancer cases, we found that the levels of Myosin Vb expression assessed by immunohistochemistry using a thoroughly validated antibody (Supplementary Fig. S3E, F/K, L, Supplementary Fig. S5B–E, Supplementary Fig. S6B, C, Supplementary Fig. S8E–G and Supplementary Fig. S9) were significantly lower in poorly differentiated tumors (grade 3) compared to moderately differentiated (grade 2) and well differentiated colorectal tumors (grade 1; Fig. 1O). Collectively, these results demonstrate that the loss of Myosin Vb results in a significant reduction in the capacity of colon cancer cells to differentiate. Importantly, it has been shown that reduced differentiation can significantly contribute to the progression of colorectal cancer [10, 15].

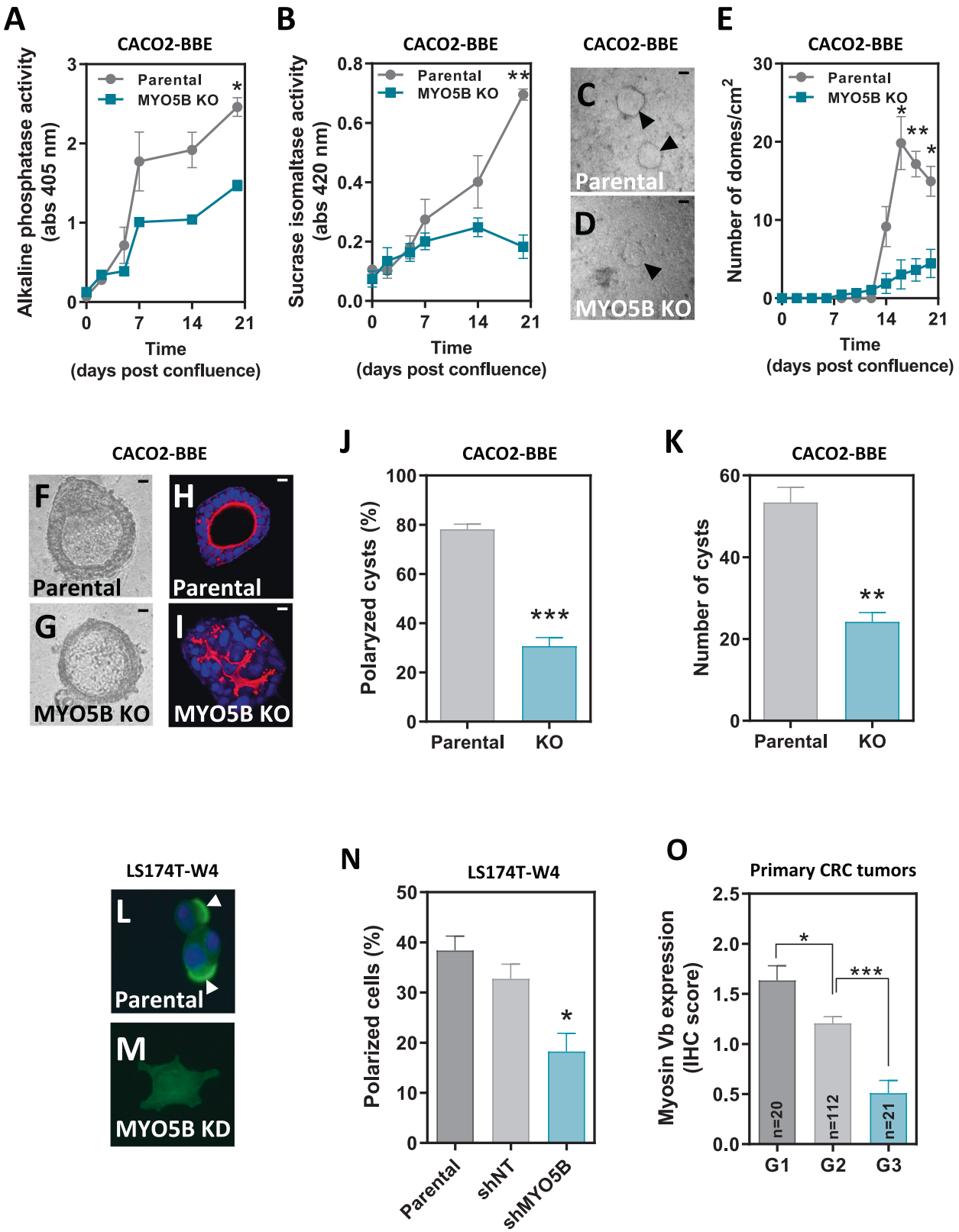
Myosin Vb regulates the metastatic capacity of colon cancer cells

Dedifferentiation of cancer cells can lead to increased motility, invasion capacity, and metastatic potential. To investigate the possible role of Myosin Vb in the motility of colon cancer cells, we used a transwell cell migration assay and assessed the number of cells reaching the underside compartment. We generated isogenic cell line models with doxycycline-dependent overexpression of Myosin Vb in RKO and SW837 cells (Supplementary Fig. S2D–G), two colon cancer cell lines with low endogenous levels of this myosin (Supplementary Fig. S1A, B). Although forced overexpression of Myosin Vb in RKO cells did not affect their motility (Fig. 2A), a >2-fold reduction in the migration of SW837 cells was observed when Myosin Vb was overexpressed (Fig. 2B). We also investigated the possible effect of Myosin Vb overexpression on the invasive capacity of RKO and SW837 cells using a Matrigel Boyden chamber assay. We found that overexpression of Myosin Vb impaired the capacity of RKO cells to invade through a complex extracellular matrix (Fig. 2C), although it did not significantly affect the invasion of SW837 (Fig. 2D).

Next, we used an experimental model of metastasis by injecting control RKO^{EV} or SW837^{EV} cells or the isogenic derivative cell lines with Myosin Vb overexpression (RKO^{MYO5B} or SW837^{MYO5B}) into the tail vein of NOD/SCID immunodeficient mice. Under the experimental protocol used, metastatic lesions were observed in the limbs of mice (popliteal and axillary fossae) starting 6 or 15 weeks after injecting control RKO^{EV} or SW837^{EV} cells, respectively. Overexpression of Myosin Vb resulted in a significant delay in the formation of macroscopic metastasis in RKO cells (Fig. 2E and Supplementary Fig. S3A–F), although this difference was not statistically significant for SW837 cells (Fig. 2F and Supplementary Fig. S3G–L). In good agreement, when we assessed the levels of Myosin Vb expression in a cohort of 68 primary locally advanced (stage III) colorectal tumors and 13 lymph node metastases by immunohistochemistry, we found that Myosin Vb expression was significantly lower in lymph node metastases compared to primary colorectal tumors (Fig. 2G). Collectively, these results indicate that reduced Myosin Vb expression enhances the metastatic potential of colorectal cancer cells.

Role of Myosin Vb in the proliferation of colorectal cancer cells

Increased proliferation and occasional hyperplasia have been reported in MVID patients [16]. Consistently, *Myo5b* inactivation in the normal murine intestinal epithelium has been shown to result in increased proliferation [5]. To further investigate the role of Myosin Vb in the proliferation of intestinal epithelial cells, first we used a constitutive *Myo5b* knockout mouse model [17]. Studies were conducted on mouse embryos, since this model displays a perinatal mortality with complete penetrance within the first 12 h of life [17]. Pregnant females at day 20 of gestation were intraperitoneally injected with 100 mg/kg of the nucleoside analog bromodeoxyuridine (BrdU) two hours before being sacrificed. Embryos were then dissected and the number of cells in S-phase (BrdU-positive) in the small and large intestinal epithelium was determined by anti-BrdU immunostaining. The results showed a significant increase in the percentage of proliferating cells in both small and large intestine of *Myo5b* knockout E20 embryos compared to the wild-type littermates (Fig. 3A and Supplementary Fig. S4A). Next, we generated a mouse model in which exon 5 of *Myo5b* was flanked by LoxP sites (*Myo5b*$^{lox/lox}$; Supplementary Fig. S5A), and crossed *Myo5b*$^{lox/lox}$ animals with *Vil-CreERT2* mice expressing a tamoxifen-inducible form of Cre recombinase (CreERT2) under the control of the intestine-specific villin 1 promoter [18]. Administration of 160 mg/kg (i.p.) of tamoxifen to *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice resulted in the loss of Myosin Vb expression in the small and large intestine of animals within 5 days (Supplementary Fig. S5B–E), and closely recapitulated the phenotype of patients with microvillous inclusion disease (MVID; Supplementary Fig. S5F, G). Intestine-specific *Myo5b* knockout mice showed significant hyperproliferation in the crypts of both small and large intestine, compared to control mice



(*Myo5*$^{lox/lox}$;*Vil-CreERT2*$^{-}$; Fig. 3B and Supplementary Fig. S4B). Importantly, increased proliferation was also detected at the crypt base where the intestinal stem cells are located (Supplementary Fig. S4C). Because all animals die within the first week after the administration of 160 mg/kg of tamoxifen, to assess the long-term effects of *Myo5b* inactivation on the murine small intestine, a separate group of animals received 40 mg/kg of tamoxifen. In this experimental setting, approximately 75% of the *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice survived the high level of intestinal *Myo5b* inactivation observed (>90%; Supplementary Fig. S6A–E).

Fig. 1 Role of Myosin Vb on the differentiation and polarization of colon cancer cells. The relative activity of the digestive hydrolases alkaline phosphatase (**A**) and sucrase-isomaltase (**B**) was assessed in CACO2-BBE cells and CACO2-BBE$^{MYO5B\text{-}KO}$ cells over the indicated times. Representative domes formed by parental CACO2-BBE cells (**C**) and CACO2-BBE$^{MYO5B\text{-}KO}$ cells (**D**) and quantification of the number of domes (mean ± SEM) formed over the indicated time in confluent cultures (**E**). Arrowheads indicate individual domes. **F–K** The capacity of CACO2-BBE cells and CACO2-BBE$^{MYO5B\text{-}KO}$ cells to form polarized cysts when grown in Matrigel was assessed. Representative examples of cysts under a phase contrast microscope (**F–G**) and stained with DAPI (nuclei; blue) and phalloidin (F-actin; red) (**H–I**) are shown. Scale bar: 100 µm. The percentage of polarized cysts (**J**) and the total number of cysts formed (**K**) by CACO2-BBE cells and CACO2-BBE$^{MYO5B\text{-}KO}$ cells was quantified. **L–N** Representative polarized parental LS174T-W4 (**L**) and unpolarized LS174T-W4 MYO5B knockdown (KD) cells (**M**) are shown (DAPI: blue; F-actin: green). The capacity of LS174T-W4 colon cancer cells to polarize after activation of LKB1 was quantified in parental cells and MYO5B knockdown cells (**N**). **O** Relative levels (mean ± SEM) of Myosin Vb expression (immunohistochemistry, IHC) in primary colorectal tumors that are well (grade 1; G1), moderately (grade 2; G2) and poorly (grade 3; G3) differentiated. The number of analyzed patient samples is indicated. Panels **A**, **B**, **E**, **J**, **K**, and **N** show the mean ± SEM of three independent experiments run in triplicate. Student's *T*-test $*p < 0.05$; $**p < 0.01$; $***p < 0.001$.

Myo5b$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice maintained hypertrophic, hyperproliferative crypts for one year compared to control animals (*Myo5*$^{lox/lox}$;*CreERT2*$^{-}$; Supplementary Fig. S4D–G).

To investigate the role of Myosin Vb in the proliferation of colon cancer cells, we next used our human cancer cell line systems with modulation of Myosin Vb expression. Inactivation of *MYO5B* in CACO2-BBE cells, or overexpression of this myosin in RKO and SW837 cells did not affect their growth under standard adherent conditions (Fig. 4A–C), their clonogenic potential when seeded at low density on solid substrate (conventional tissue culture plastic plates; Fig. 4D–F), or under anchorage-independent growth conditions (soft agar; Fig. 4G, H). Consistently, modulation of the expression of Myosin Vb in these colon cancer cell lines had no effect on their growth when implanted subcutaneously in NOD/SCID immunodeficient mice (Fig. 4I–K).

Finally, no associations were observed between Myosin Vb expression at the mRNA or protein level and the growth (doubling time) [19] of a collection of 41 colorectal cancer cell lines (Supplementary Fig. S7A, B). Moreover, no correlation was observed between the mitotic index of 30 primary tumors from the TCGA (assessed in hematoxylin-eosin stained sections, and used as a surrogate marker of tumor growth), and the expression of *MYO5B* (mRNA) (Supplementary Fig. S7C). These results indicate that although Myosin Vb regulates the proliferation of normal intestinal epithelial cells, it does not have a major role in the proliferation of colorectal cancer cells.

Inactivation of *Myo5b* enhances intestinal tumorigenesis in vivo

As shown before, treatment of *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice with a sublethal dose of tamoxifen (40 mg/kg) resulted in the inactivation of Myosin Vb in >90% of the intestinal epithelium (Supplementary Fig. S6), and sustained hyperproliferation in the murine intestinal epithelium for >1 year (Supplementary Fig. S4D–G). However, no difference in the number of intestinal tumors was observed between control animals and intestine-specific knockout mice even 18 months after sublethal tamoxifen administration (Supplementary Fig. 8A), indicating that the loss of Myosin Vb does not efficiently initiate intestinal tumorigenesis.

To assess the role of Myosin Vb in the progression of intestinal tumors, first we initiated the tumorigenic process genetically by introducing mutations in the *Apc* tumor suppressor gene [20]. We crossed *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice with *Apc*^{min};*Myo5b*$^{lox/lox}$ animals, carrying an inactivating mutation in the *Apc* tumor suppressor gene which results in the formation of multiple intestinal tumors within 12 weeks of age [20]. Three-month-old *Apc*^{min} mice carrying a *Myo5b* floxed gene and the tamoxifen-inducible CreERT2 recombinase (*Apc*^{min};*Myo5b*$^{lox/lox}$;*CreERT2*$^{+}$) or control mice without CreERT2 (*Apc*^{min};*Myo5b*$^{lox/lox}$;*CreERT2*$^{-}$) were administered 160 mg/kg of tamoxifen and their intestine was dissected out 4 days later to assess changes in the proliferation rate of intestinal tumor cells and in tumor size due to *Myo5b* inactivation. Surprisingly, although Myosin Vb expression was completely lost in the normal intestinal epithelium of these mice after tamoxifen administration, intestinal tumors of *Apc*^{min};*Myo5b*$^{lox/lox}$;*CreERT2*$^{+}$ mice retained Myosin Vb expression, probably due to the loss of villin 1 expression in the *Apc*^{min}-triggered tumors (Supplementary Fig. S8B, C). Therefore, the role of Myosin Vb in intestinal tumorigenesis could not be assessed using this model.

Next, we initiated the tumorigenic process with the intestine-specific carcinogen azoxymethane (AOM). *Myo5b*$^{lox/lox}$ animals with or without the *Vil-CreERT2* allele were treated with a sublethal dose of tamoxifen (40 mg/kg) at 9 weeks of age, and 9 weeks later all animals received weekly i.p. AOM injections for 12 weeks to initiate the tumorigenic process. Animals were sacrificed 13 weeks after the last AOM injection and the number of intestinal tumors counted (Fig. 5A–D and Supplementary Fig. S8D–G). In Myosin Vb expressing control animals, most of the tumors formed in the large intestine, and *Myo5b* inactivation in >90% of the colonic epithelium had no effect on the number of these tumors (Fig. 5D). However, *Myo5b* inactivation resulted in a >15-fold increase in the number of tumors found in the small intestine, compared to control mice (Fig. 5D), indicating that the loss of Myosin Vb significantly contributes to the progression of small intestinal tumors in this mouse model.

Low expression of Myosin Vb is associated with shorter patient survival

It has been shown before that reduced mRNA expression of Myosin Vb in non-metastatic colorectal tumors (stage I–II) is associated with shorter patient survival [21]. Here, we used a tissue microarray (TMA) containing triplicate tumor samples from 155 patients with locally advanced (stage III) colorectal cancer to investigate possible associations between Myosin Vb expression and clinicopathological features as well as patient survival. Myosin Vb expression in these tumors was determined by immunohistochemistry (Supplementary Tables S1 and S2). Normal colonic epithelial cells displayed high levels of Myosin Vb expression, which was retained in colonic adenomas (Fig. 6A, F). Contrary, the average expression of Myosin Vb was significantly lower in primary colorectal tumors compared to the normal epithelium (Figs. 6B–E and F). As mentioned before, lower levels of expression were observed in lymph node metastasis compared to the primary colorectal tumors (Fig. 2G), and reduced Myosin Vb expression was associated with higher tumor grade (Fig. 1O). A significant correlation between Myosin Vb mRNA and protein expression was observed using a cohort of 96 colorectal tumors from the Clinical Proteomic Tumor Analysis Consortium (CPTAC; Supplementary Fig. S10) [22]. No correlation was observed between the expression of Myosin Vb and other clinicopathological or molecular features, including the expression of Myosin Ia or the loss of heterozygosity in chromosome 18q21 (Supplementary Tables S1 and S2).

We next investigated whether Myosin Vb expression was associated with patient survival and found that when patients were dichotomized according to their levels of Myosin Vb, low

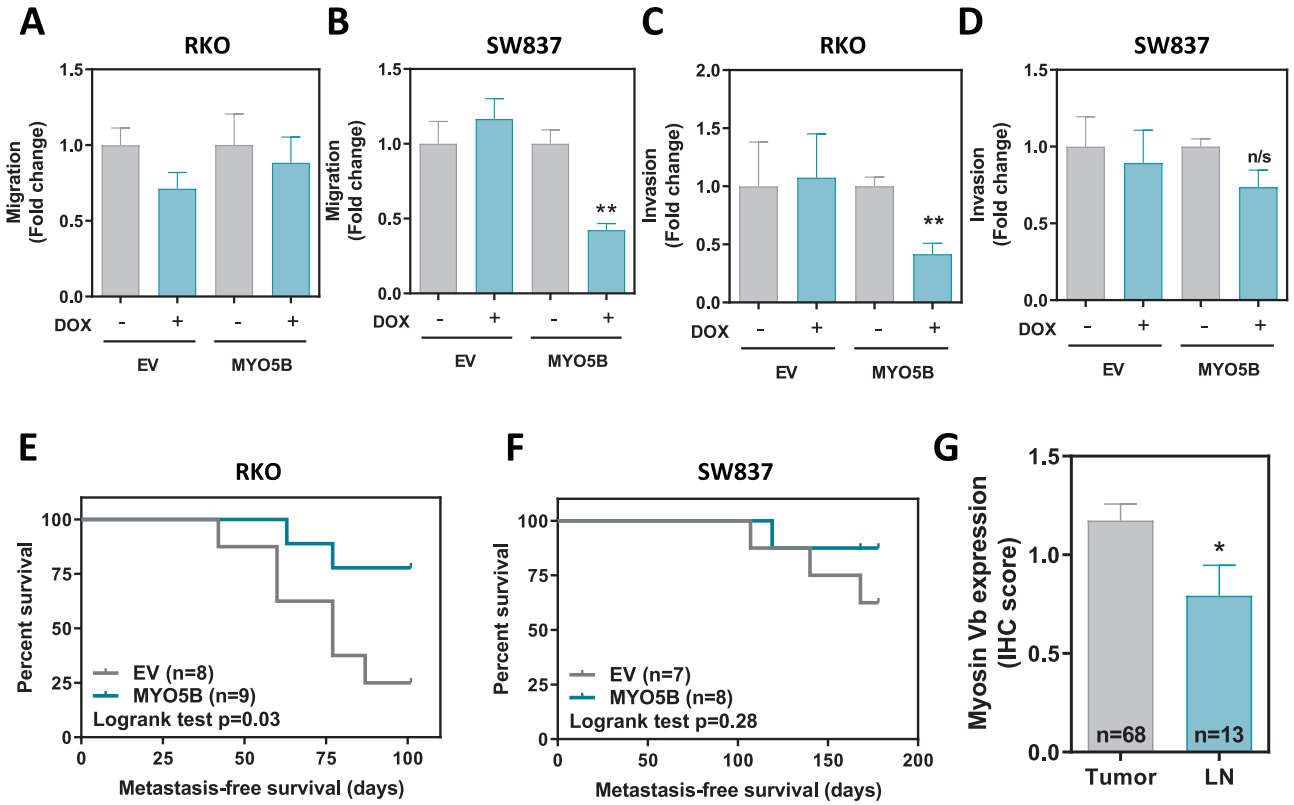


Fig. 2 Myosin Vb regulates the metastatic potential of colon cancer cells. The motility of RKO (**A**) and SW837 (**B**) cells was assessed in control (EV; mCherry) and mCherry-MYO5B overexpressing cells (MYO5B) with and without doxycycline (DOX) treatment. Matrigel invasion capacity of RKO (**C**) and SW837 (**D**) cells was assessed in control (EV; mCherry) and mCherry-MYO5B overexpressing cells (MYO5B) with and without doxycycline (DOX) treatment. Kaplan–Meier plots of metastasis-free survival of mice that were tail vein injected with control (mCherry; EV) or mCherry-MYO5B overexpressing (MYO5B) RKO (**E**) and SW837 (**F**) cells, and received doxycycline in the drinking water. The number of animals in each group and the Log-rank test *p* value are shown. **G** Relative levels (mean ± SEM) of Myosin Vb expression (immunohistochemistry, IHC) in locally advanced (Stage III) primary colorectal tumors and lymph node metastases. The number of analyzed patient samples is indicated. Panels **A**–**D** show the mean ± SEM of three independent experiments each run in triplicate. Student's *T*-test $*p < 0.05$; $**p < 0.01$; n/s non-significant.

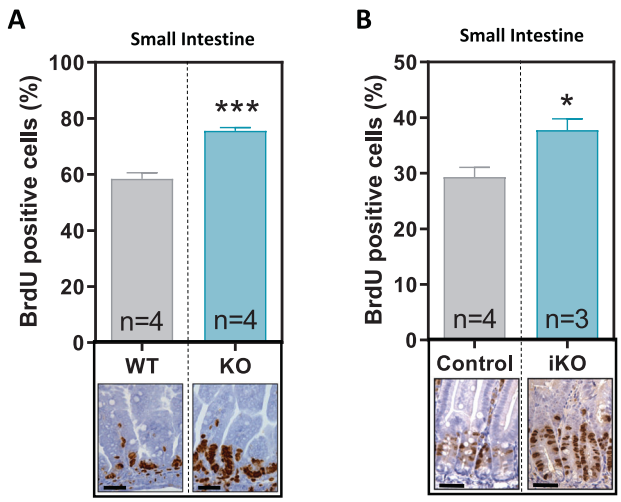


Fig. 3 Loss of Myosin Vb enhances the proliferation of normal intestinal epithelial cells. A The number of proliferating (BrdU positive) cells was quantified in the small intestine of E20 embryos that are wild type (WT) or constitutively knockout for *Myo5b* (KO). **B** Number of proliferating cells in the crypt of the small intestine of adult *Myo5b* wild type or conditionally inducible knockout mice (iKO). Representative images of anti-BrdU immunostainings are shown. The number of animals in each group is indicated. Scale bar: 25 μm. Student's *T*-test $*p < 0.05$; $***p < 0.001$.

expression of this myosin was associated with both shorter disease-free (Log-rank test $p < 0.001$) and overall survival (Log-rank test $p < 0.019$) (Fig. 6G–H). Moreover, this association was confirmed when the tumor levels of Myosin VB were considered as a continuous variable (Cox regression $p = 0.034$ and $p = 0.05$ for overall and disease-free survival, respectively), demonstrating that the levels of expression of this myosin can be used to identify a group of patients with locally advanced colorectal cancer with poor prognosis.

DISCUSSION

Mutations in *MYO5B* have been shown to cause microvillus inclusion disease (MVID), a rare genetic syndrome characterized by the onset of intractable life-threatening watery diarrhea shortly after birth [12]. The cell line CACO2 [23] and the derivative line known as CACO2-BBE [24] have been widely used as a model of the small intestinal barrier, due to its ability to spontaneously differentiate into a monolayer of cells with properties typical of absorptive enterocytes [25, 26]. However, CACO2 cells are derived from a colon adenocarcinoma [23] and downregulation of Myosin Vb in these cells has been shown to interfere with its differentiation and polarization [6, 7, 27]. Consistently, we found that *MYO5B* inactivation resulted in reduced differentiation and polarization in CACO2-BBE cells, as revealed by lower activity of brush border digestive hydrolases, and limited capacity to form domes and polarized cystic structures when grown in two or three dimensions, respectively. Moreover, downregulation of Myosin Vb

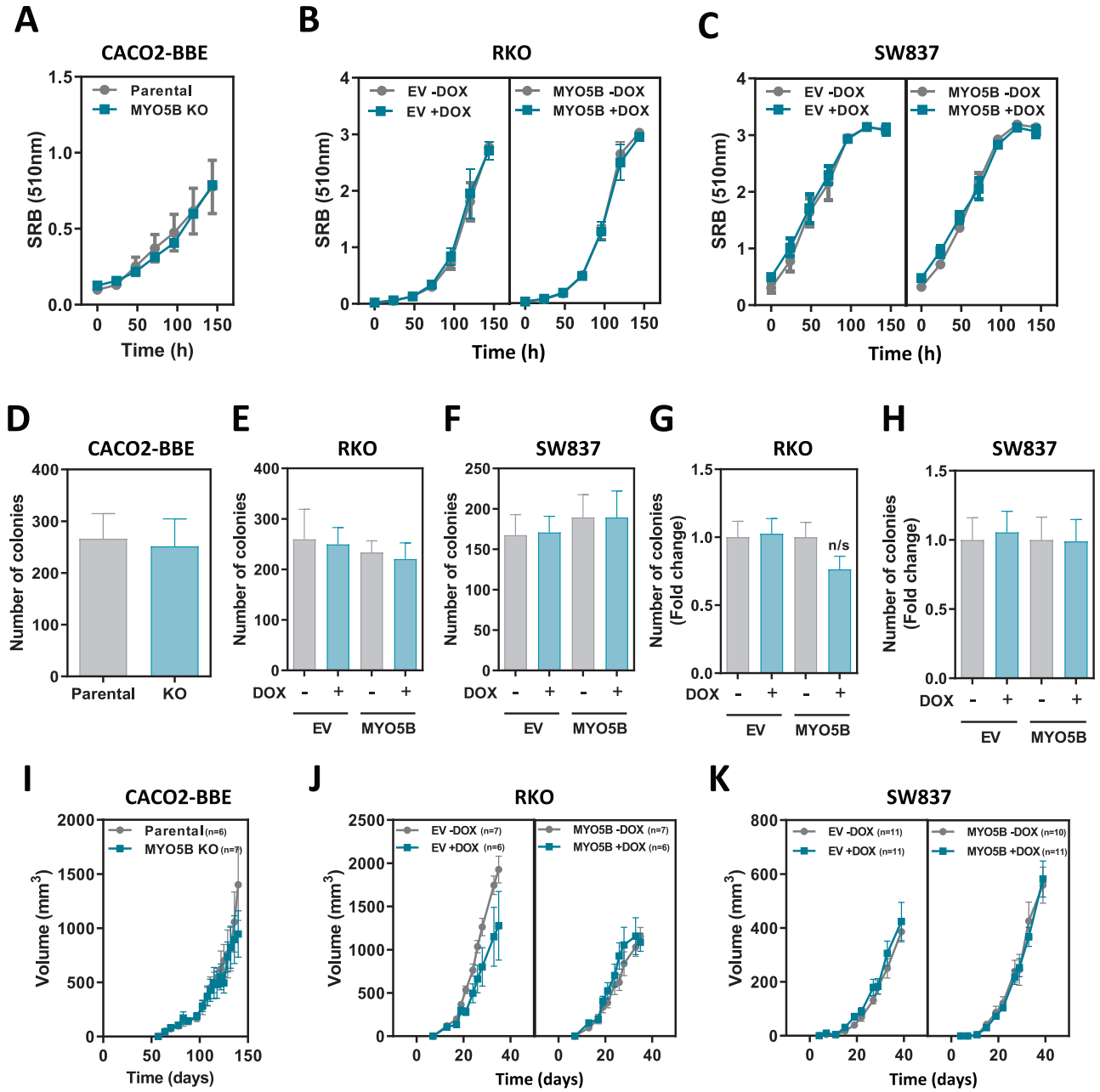


Fig. 4 Myosin Vb does not regulate the growth of colon cancer cells. The proliferation of parental CACO2-BBE and CACO2-BBE$^{MYO5B\text{-}KO}$ cells (**A**), and RKO (**B**) or SW837 (**C**) with doxycycline-dependent overexpression of mCherry (EV; control) or mCherry-MYO5B (MYO5B), was assessed using sulforhodamine B staining. Clonogenic capacity of parental CACO2-BBE and CACO2-BBE$^{MYO5B\text{-}KO}$ cells (**D**), and RKO (**E**) or SW837 (**F**) with doxycycline-dependent overexpression of mCherry (EV) or mCherry-MYO5B (MYO5B). Anchorage-independent growth (soft agar colony formation) was assessed in RKO (**G**) or SW837 (**H**) with doxycycline-dependent overexpression of mCherry (EV) or mCherry-MYO5B (MYO5B). The growth of parental CACO2-BBE and CACO2-BBE$^{MYO5B\text{-}KO}$ cells (**I**), and RKO (**J**) or SW837 (**K**) with doxycycline-dependent overexpression of mCherry (EV) or mCherry-MYO5B (MYO5B), was assessed in a subcutaneous xenograft model using immunodeficient NOD/SCID mice. The number of animals in each group is shown. The mean ± SEM of three independent experiments each run in triplicate is shown in panels **A**–**H**. ns non-significant.

in LS174T-W4 colon cancer cells significantly reduced its capacity to polarize upon LKB1 activation, and consistent with an earlier observation [21], we found that Myosin Vb expression is significantly reduced in poorly differentiated primary colorectal tumors compared to moderately or well differentiated tumors, further confirming that *MYO5B* inactivation leads to reduced differentiation and polarization of colon cancer cells. The correct localization of proteins on the apical and basolateral compartments is dependent on intracellular vesicle trafficking [28, 29], which is regulated by Myosin Vb and RAB small GTPases [29, 30]. Indeed, RAB8A and RAB11A deficient mice display MVID-like phenotypes, highlighting the role of vesicle trafficking in cell polarity [31, 32].

Loss of apical-basal polarity is an early event in epithelial cancers and can occur at preinvasive stages [9]. Indeed, reduced expression of the key polarity regulators Lgl, Dlg, and Scrib is associated with tumor progression [33–35], and we have previously shown that the loss of Myosin Ia, a structural brush border myosin, causes decreased differentiation and polarization and promotes colorectal tumorigenesis [10]. Moreover, we and

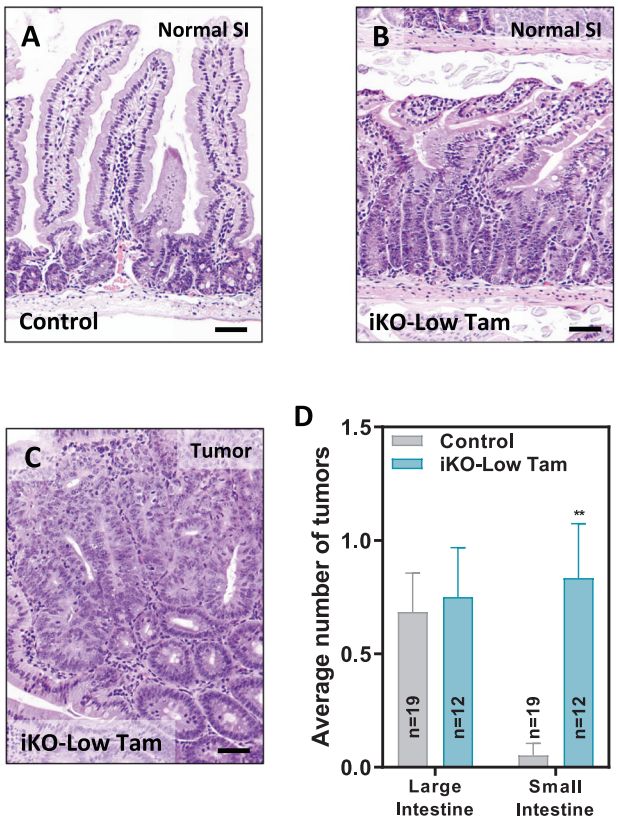


Fig. 5 *Myo5b* inactivation enhances intestinal tumorigenesis in a mouse model. A, B Examples of the histology (H&E staining) of the normal small intestinal epithelium of control (*Myo5b*$^{lox/lox}$;*CreERT2*$^{-}$; Control; **A**) and mice with targeted inactivation of *Myo5b* in > 90% of the intestinal epithelium (*Myo5b*$^{lox/lox}$;*CreERT2*$^{+}$; iKO-Low Tam; **B**) after receiving 40 mg/kg of tamoxifen and treatment with the intestinal carcinogen azoxymethane (AOM). **C** A representative example of tumors in the small intestine of iKO-Low Tam mice caused by AOM treatment. **D** The number of tumors in the small and large intestine was quantified. The number of animals in each group is shown. The mean ± SEM is represented. Scale bar: 50 µm. Student's *T*-test $^{**}p < 0.01$.

others have shown that the disruption of a polarized epithelial architecture can lead to increased proliferation and thus further contribute to tumor formation and development [10, 36, 37]. Therefore, we investigated the possible role of Myosin Vb on the proliferation of normal intestinal epithelial cells in our mouse models of *Myo5b* inactivation and colon cancer cell lines.

Inactivation of *MYO5B* in the murine intestinal epithelium causes crypt hyperplasia and increased proliferation [5]. A similar phenotype is observed in mice that are knockout for the small GTPase Cdc42 [38], which also develop gross hyperplasia, crypt enlargement and microvillus inclusions. Consistently, we demonstrate here hyperproliferation in the intestinal epithelium of constitutive *Myo5b* knockout E20 embryos as well as in the epithelium of adult intestine-specific *Myo5b* knockout mice. Although the molecular mechanisms underlying the increased proliferation in normal intestinal epithelial cells remain to be fully elucidated, it does not seem to be secondary to feeding and subsequent villus atrophy, since hyperproliferation is observed in E20 embryos [17], and could be related with subtle changes in the orientation of the mitotic spindle of dividing crypt cells [13], or changes in Hippo signaling, as reported before [39]. However, *MYO5B* inactivation had no effect on the proliferation of colon cancer cell lines, possibly due to additional oncogenic events activating similar signaling pathways in fully transformed cells. Moreover, no increase in the number of intestinal tumors was observed even 18 months after *Myo5b* inactivation, indicating that the loss of Myosin Vb is not sufficient to initiate the tumorigenic process. Importantly, modulation of the levels of expression of Myosin Vb in three different colon cancer cell lines had no impact on their growth under standard in vitro conditions, low-density conditions (colony formation), anchorage-independent growth (soft agar) or in a subcutaneous mouse xenograft model.

Reduced cell polarity and differentiation have been shown to increase the motility, invasion and metastatic potential of epithelial cells [29, 40]. The loss of cell polarity can lead to inappropriate delivery of degradative metalloproteinases (MMP) to the cell surface, promoting cell invasion and transformation [41]. In addition, defective intracellular trafficking may cause the redistribution of junctional components, such as integrins, potentially leading to changes in motility [42]. Here we found that reintroduction of Myosin Vb into colon cancer cells with low levels of endogenous Myosin Vb could significantly reduce their motility and their capacity to invade through a complex extracellular matrix. These results are in agreement with previous observations in gastric cancer showing that *MYO5B* inactivation increases the motility and invasion of gastric cancer cells [43]. Consistently, using a model of experimental metastasis we found that mice tail vein injected with colon cancer cells with forced overexpression of Myosin Vb had fewer metastases and longer metastasis-free survival compared to mice injected with control cells. Moreover, consistent with earlier findings [21], we found reduced Myosin Vb levels in lymph node metastasis compared to primary colorectal tumors.

The loss of individual structural cell polarity components does not generally lead to carcinogenesis and additional perturbations are required to reveal their role on the oncogenic process. To study the role of Myosin Vb in vivo, we used a mouse model of conditional intestinal inactivation of this myosin. However, the investigation of the role of Myosin Vb in intestinal tumorigenesis is complex due to the fully penetrant perinatal lethality caused by *Myo5b* inactivation in the intestine [5, 17, 44]. Here, we used the intestine-specific carcinogen azoxymethane (AOM) to initiate the tumorigenic process in the intestine of mice that received a sublethal concentration of tamoxifen resulting in the depletion of Myosin Vb in >90% of the intestinal epithelium. *Myo5b* inactivation resulted in a > 15-fold increase in the number of tumors in the small intestine following AOM treatment, convincingly demonstrating the tumor suppressive activity of this myosin. Myosin Vb interacts with several Rab GTPases, including RAB8A [45, 46], and RAB11A [47], which have been shown to have tumor suppressor activity in mice and humans, likely though the deregulation of the Hippo-YAP1/TAZ pathway, although the detailed mechanisms remain to be fully elucidated. Interestingly, *Myo5b* inactivation did not have an effect on the number of AOM-initiated tumors in the large intestine. Similarly, mutations in the tumor suppressor gene *APC*, believed to be the initiating event in >80% of the human colorectal tumors [48], cause mostly small intestinal tumors in mouse models [20]. Moreover, in old mice, spontaneous tumors are found mostly in the small intestine rather than in the large intestine [49], as often observed in humans, likely reflecting differences in the physiology of the intestinal tract of mice and humans.

We have previously shown that reduced expression of Myosin Ia, also a brush border myosin that regulates the polarization and differentiation of normal intestinal cells and colorectal tumors, is associated with poor prognosis of colorectal cancer patients [10]. Similarly, we show here that reduced protein expression of Myosin Vb in colorectal tumors is associated with shorter disease-free and overall survival of patients with locally advanced (Stage III) colorectal cancer. This is consistent with an earlier report showing that mRNA levels of *MYO5B* are associated with the survival of colorectal cancer patients [21]. Although *Myo5b* inactivation did not efficiently initiate the tumorigenic process in our mouse model and other genetic or epigenetic events are needed for the

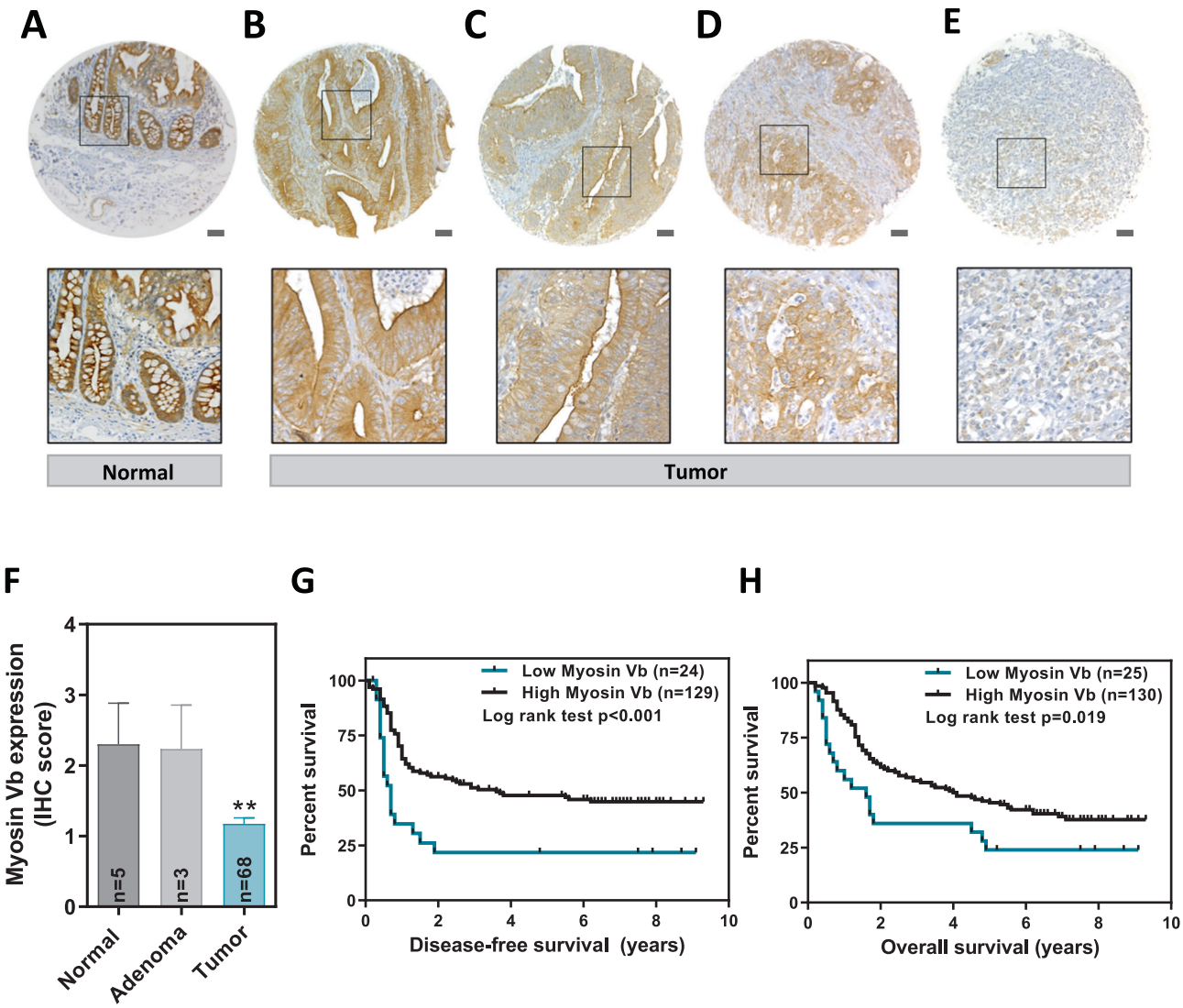


Fig. 6 Low levels of Myosin Vb are associated with poor patient prognosis. Representative immunohistochemistry images of the normal colonic mucosa (**A**) and primary colorectal tumors (**B–E**) stained with anti-Myosin Vb, and showing a gradient of Myosin Vb expression. **F** Relative levels (mean ± SEM) of Myosin Vb immunostaining in the normal colonic epithelium, colon adenomas and primary colorectal tumors. The number of analyzed patient samples is indicated. Student's *T*-test $^{**}p < 0.01$. Disease-free (**G**) and overall (**H**) survival of patients with locally advanced (Stage III) colorectal tumors as a function of Myosin Vb protein expression. Scale bar: 50 μm. The Log-rank test *p* value is shown.

oncogenic potential of the loss of Myosin Vb to be unleashed, no associations were observed between Myosin Vb expression and other clinicopathological or molecular features investigated, such as microsatellite instability or mutations in *KRAS* or *TP53*.

In conclusion, our findings demonstrate that the loss of Myosin Vb results in reduced polarization and differentiation of colorectal cancer cells and contributes to increased motility, invasion and metastatic potential. Moreover, *Myo5b* inactivation in a mouse model of intestinal tumorigenesis resulted in a >15-fold increase in the number of intestinal tumors observed after treatment with the intestinal carcinogen AOM, and reduced expression of this myosin in primary colorectal tumors is associated with shorter patient survival. When considered together, our results demonstrate a tumor suppressor function for Myosin Vb during colorectal tumorigenesis.

MATERIALS AND METHODS

Cell lines and isogenic in vitro models

CACO2-BBE, RKO, and SW837 cell lines were obtained from the ATCC. LS174T-W4 cells were a kid gift of Dr. Hans Clevers (Hubrecht Institute, Utrecht, The Netherlands) [14]. The main molecular features of the cell lines used in this study can be found in Supplementary Table S3. All cell lines were maintained on Dubelco's Modified Eagle's Medium (DMEM; Gibco) containing 10% fetal bovine serum (FBS; Sigma) and 1× antibiotic-antimycotic (Gibco) at 37 °C and 5% CO_2. *MYO5B* was inactivated in CACO2-BBE cells using a CRISPR/Cas9 approach, and conditionally down-regulated in LS174T-W4 cells with the pINDUCER10-shMYO5B vector. The sgRNA and shRNAs used are described in Supplementary Table S4. Myosin Vb was overexpressed in RKO and SW837 colon cancer cells using the doxycycline-inducible vector pINDUCER20-mCherry-MYO5B. More details can be found in the Supplementary Materials and Methods.

Proliferation, clonogenic, soft agar, migration, and invasion assays

Myosin Vb overexpression was induced with 0.1 μg/mL of doxycycline (Dox) at time of seeding, unless otherwise indicated. For Myosin Vb downregulation, cells were pretreated with or without Dox (1 μg/mL) for 72 h before seeding to ensure efficient knockdown. The assays for assessing proliferation, the clonogenic potential, anchorage-independent growth, migration and Matrigel invasion were carried out as previously

described [10, 50], and detailed in the Supplementary Materials and methods section.

Differentiation and polarization assays

Briefly, parental CACO2-BBE and CACO2-BBE$^{MYO5B\text{-}KO}$ derivative cells were grown for 21 days in confluence to induce spontaneous differentiation. The number of domes was directly counted at different time points indicated. Enzymatic assays for alkaline phosphatase (AP) and sucrase-isomaltase (SI) were performed as previously described [50]. Cyst formation and analysis was performed as described in the Supplementary Materials and methods. For polarization, parental LS174-W4 and derivative cells expressing control shRNA (NT) or shRNA4 targeting Myosin Vb were pretreated with 1 µg/mL of Dox for 72 h. Cells were paraformaldehyde-fixed and stained with Alexa Fluor 488-labeled phalloidin (0.1 µM; Cytoskeleton) and DAPI (Sigma). At least 500 cells were counted under the microscope (×10 magnification), blinded from the sample identity.

Subcutaneous xenografts and experimental metastasis mouse model

All animal experiments were carried out according to procedures approved by the Ethics Committee for Animal Experimentation at Vall d'Hebron Research Institute. ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines from the National Centre for the Replacement, Refinement and Reduction of Animals in Research were used to determine experimental sample sizes. NOD/SCID mice (Charles River) 7-8 weeks old and from both sexes were used. For xenograft experiments, animals were injected with RKO, SW837, CACO2-BBE or the corresponding derivative lines in both flanks. Tumor size was measured over the indicated time blinded from the cell line identity. For the experimental metastasis model, animals were injected with RKO, SW837 or the corresponding derivative cell lines in the tail vein. Animals carrying inducible cell line systems, were randomized in two groups after injection, one receiving Dox in the drinking water (1 mg/mL Dox + 2.5% sucrose; Sigma) or a control group (2.5% sucrose). More details can be found in the Supplementary Materials and Methods section.

Generation of *Myo5b* knockout mice

Myo5b$^{tm1a(KOMP)Wtsi}$ mouse model was previously described [17]. *Myo5b*^{tm1c} (*Myo5b* floxed) mice with a conditional *Myo5b* knockout allele were generated by crossing *Myo5b*$^{tm1a(KOMP)Wtsi}$ and C57BL/6 Tg(CAG-Flpo)1Afst (Flp deleter) mice [51]. For intestinal epithelium-specific deletion of Myosin Vb, *Myo5b*$^{tm1c/tm1c}$ (henceforth *Myo5b*$^{lox/lox}$) mice were crossed to a C57BL/6 Tg(Vil-cre/ERT2)23Syr (*Vil-Cre*^{ERT2}) mice [52]. Homozygous *Myo5b*$^{lox/lox}$ mice lacking the *Cre*^{ERT2} transgene (*Vil-CreERT2*$^{-}$) were used as control animals. Cre recombinase was induced in animals from both sexes by a single intraperitoneal (i.p.) injection of either 160 or 40 mg/kg, as indicated.

Intestinal carcinogenesis mouse models

Treatment of *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ mice with 160 mg/kg of tamoxifen (i.p.) resulted in complete ablation of intestinal Myosin Vb and death of the animals within 5 days. To investigate the long-term effects of *Myo5b* inactivation in the intestinal epithelium, animals from both sexes were treated with a sublethal dose of tamoxifen of 40 mg/kg (i.p.), causing deletion of Myosin Vb in >90% of intestinal epithelial cells. The weight and the presence of intestinal tumors in *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{+}$ and *Myo5b*$^{lox/lox}$;*Vil-CreERT2*$^{-}$ control mice was monitored at the indicated time points. To initiate the intestinal tumorigenic process, a genetic (*Apc*^{min}) or a carcinogenic (azoxymethane; AOM, Sigma) approach was used (Supplementary Materials and methods). All animals received 100 mg/kg of bromodeoxyuridine (BrdU) two hours before being sacrificed.

Histology and immunohistochemistry

Subcutaneous xenograft tumors, metastatic lesions in NOD/SCID mice and murine intestinal samples were processed as previously reported [10, 50] and described in detail in the Supplementary Materials and Methods section. The primary antibodies used are listed in Supplementary Table S5.

Clinical samples and TMA analysis

Samples from colorectal cancer patients with locally advanced disease were collected at collaborating medical institutions as previously described [53]. All experimental protocols were approved by Ethics Committee for Human Investigations at the appropriate Institution. Informed consent was obtained from all patients. A total of 155 patients with Duke C colorectal tumors were used for immunohistochemical assessment of Myosin Vb levels.

Statistical analysis

All experiments were performed three times each using triplicate repeats and data present means ± SEM, unless otherwise stated. Statistical significance was evaluated using Student's *t*-test or the Log-rank test, and *P* values were reported as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$.

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AUTHOR CONTRIBUTIONS

Study concept and design: DA, FC-G, AM-B. Acquisition of data: FC-G, BB, EA, HD, JT, RN, EG-V, IM, ZZ, MD, MS-M, SCDvl, SL, JH-L, SS, XM-G, SRC, AM-B. Analysis and interpretation of data: DA, FC-G, AM-B. Drafting of the manuscript: DA, FC-G, BB, AM-B.

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COMPETING INTERESTS

The authors declare no competing interests.

ADDITIONAL INFORMATION

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Correspondence and requests for materials should be addressed to Diego Arango.

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