Brush border Myosin Ia has tumor suppressor activity in the intestine

Rocco Mazzolini[a,b], Higinio Dopeso[a,b,1], Silvia Mateo-Lozano[a,b,1], Wakam Chang[c], Paulo Rodrigues[a,b], Sarah Bazzocco[a], Hafid Alazzouzi[a], Stefania Landolfi[d], Javier Hernández-Losa[d], Elena Andretta[a,b], Pia Alhopuro[e], Eloy Espín[f], Manel Armengol[f], Josep Tabernero[g], Santiago Ramón y Cajal[d], Matthias Kloor[h], Johannes Gebert[h], John M. Mariadason[i], Simo Schwartz, Jr.[b,j], Lauri A. Aaltonen[e], Mark S. Mooseker[c], and Diego Arango[a,b,2]

[a]Group of Molecular Oncology, and [j]Group of Drug Delivery and Targeting, Centro de Investigaciones en Bioquímica y Biología Molecular-Nanomedicine, Vall d'Hebron University Hospital Research Institute, Universitat Autònoma de Barcelona, 08035 Barcelona, Spain; [b]Centro de Investigación Biomédica en Red de Bioingeniería, Biomateriales y Nanomedicina, 50018 Zaragoza, Spain; [c]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, 06520-8103 CT; [d]Department of Pathology, [f]Department of Surgery, and [g]Department of Medical Oncology, Vall d'Hebron University Hospital, Universitat Autònoma de Barcelona, 08035 Barcelona, Spain; [e]Department of Medical Genetics, Genome-Scale Biology Research Program, Biomedicum Helsinki, University of Helsinki, 00014 Helsinki, Finland; [h]Department of Applied Tumor Biology, Institute of Pathology, University Hospital Heidelberg, 69120 Heidelberg, Germany; and [i]Ludwig Institute for Cancer Research, Melbourne Centre for Clinical Sciences, Austin Health, Heidelberg, Victoria 3084, Australia

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The loss of the epithelial architecture and cell polarity/differentiation is known to be important during the tumorigenic process. Here we demonstrate that the brush border protein Myosin Ia (MYO1A) is important for polarization and differentiation of colon cancer cells and is frequently inactivated in colorectal tumors by genetic and epigenetic mechanisms. *MYO1A* frame-shift mutations were observed in 32% (37 of 116) of the colorectal tumors with microsatellite instability analyzed, and evidence of promoter methylation was observed in a significant proportion of colon cancer cell lines and primary colorectal tumors. The loss of polarization/differentiation resulting from MYO1A inactivation is associated with higher tumor growth in soft agar and in a xenograft model. In addition, the progression of genetically and carcinogen-initiated intestinal tumors was significantly accelerated in *Myo1a* knockout mice compared with *Myo1a* wild-type animals. Moreover, MYO1A tumor expression was found to be an independent prognostic factor for colorectal cancer patients. Patients with low MYO1A tumor protein levels had significantly shorter disease-free and overall survival compared with patients with high tumoral MYO1A (logrank test $P = 0.004$ and $P = 0.009$, respectively). The median time-to-disease recurrence in patients with low MYO1A was 1 y, compared with >9 y in the group of patients with high MYO1A. These results identify MYO1A as a unique tumor-suppressor gene in colorectal cancer and demonstrate that the loss of structural brush border proteins involved in cell polarity are important for tumor development.

Loss of cell polarity, differentiation, and tissue architecture are hallmarks of advanced metastatic carcinomas and strongly correlate with poor patient prognosis (1). The mechanisms regulating this epithelial-to-mesenchymal transition are well characterized (2). However, the importance of the loss of cell polarity and differentiation during premetastatic epithelial tumorigenesis is not well understood. Recent evidence from model organisms indicates that genes regulating cell polarity and differentiation in epithelial cells can have tumor-suppressor activity, although the role of polarity regulators in human cancer has not been thoroughly investigated (1, 3, 4). The Ser/Thr kinase 11 (STK11; also known as LKB1) regulates cellular metabolism and proliferation and has recently been shown to be a master regulator of polarity in epithelial cells (5, 6). Germ-line *STK11/LKB1* mutations are responsible for Peutz-Jeghers syndrome, an autosomal dominant genetic disease characterized by the development of benign hamartomatous intestinal polyps and predisposition to different types of cancer, including colorectal cancer (7–9). Importantly, the suppressor activity of STK11/LKB1 in intestinal epithelial cells seems to be associated with the regulation of cell polarity and differentiation rather than its role in cell cycle and metabolic control (10). However, *STK11/LKB1* mutations are rare in sporadic colorectal tumors and epigenetic inactivation is not frequently observed (11–13), suggesting that alternative genetic/epigenetic mechanisms exist in intestinal tumor cells to disrupt epithelial cell polarity and differentiation during the earlier stages of the tumorigenic process.

The intestinal epithelium is among the most rapidly proliferating tissues in the human adult body. This cell monolayer lining the inner surface of the intestine is in constant renewal, with cells rapidly proliferating in the lower part of the intestinal crypts of Lieberkuhn. These immature cells migrate toward the top of the crypts and into the villi of the small intestine or the flat colonic mucosa as they differentiate, and are eventually shed into the intestinal lumen (14). The stem cells of the system are located in the bottom of the crypts (15, 16) and give rise to all of the cell types of this epithelium, namely, absorptive, goblet, enteroendocrine, and Paneth cells. Differentiated intestinal epithelial cells are highly polarized with a well-defined apical and basolateral domain. Differentiated cells of the absorptive lineage show a characteristic apical brush border consisting of a tight array of microvilli that significantly increase the contact surface with the intestinal contents. The core of each microvillus is maintained by a dense bundle of actin filaments cross-linked by fimbrin, villin, and espin. Myosin Ia (MYO1A) is a major component of the cytoskeleton that underlies and supports the apical brush border of the enterocytes (17). MYO1A forms a spiral array of bridges that links the microvillar actin core to the membrane (18–20). The loss of structural proteins supporting the complex cytoskeleton of the brush border is generally believed to be the result of the de-differentiation and loss of polarization occurring during tumor progression. A causative role for these proteins in the tumorigenic process has never been observed. Here we show that the gene encoding Myosin Ia (*MYO1A*) regulates the polarization/differentiation of colorectal cancer cells, is frequently mutated/methylated in colorectal tumors, and has tumor-suppressor activity.

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[1]H.D. and S.M.-L. contributed equally to this work.

[2]To whom correspondence should be addressed. E-mail: diego.arango@vhir.org.

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Results

Frequent MYO1A Mutations in Colorectal Tumors with Microsatellite Instability. A subset of colorectal tumors accumulates mutations mainly in microsatellite repeats throughout the genome because of defects in mismatch repair mechanisms. Frame-shift mutations in coding microsatellites of tumor-suppressor genes may confer a growth advantage to cancer cells and are therefore observed at high frequency in colorectal tumors with microsatellite instability (MSI). We found frame-shift mutations in an A8 microsatellite repeat located in the last exon (Ex 28) of *MYO1A* in 44.4% (16 of 36) and 31.3% (42 of 134) of MSI colorectal cancer cell lines and primary tumors, respectively (*SI Appendix*, Fig. S1 *A* and *B*, and Tables S1 and S2). No mutations were observed in the matching normal DNA of these cases. As a control, a noncoding A8 microsatellite in intron 22–23, located 4.7-Kb upstream of the coding A8 in exon 28, showed no mutations in a subset of 23 MSI primary colorectal tumors and 11 MSI lines studied (Fisher's exact test, $P < 0.0001$). Moreover, the frequency of mutations in *MYO1A* observed was above the 95% prediction interval expected for A8 repeats when plotted against the mutation frequency in a series of 62 intronic A/T repeats in MSI colorectal tumors (*SI Appendix*, Fig. S1*C*), collectively suggesting that the mutations observed in *MYO1A* are clonally selected and confer a growth advantage to these tumors. The full *MYO1A* coding region of seven MSI colorectal cancer lines with heterozygous mutations in the A8 tract in Exon 28 was sequenced, and no additional mutations were found. To further investigate the role of *MYO1A* mutations in tumor progression, we also sequenced this region in 17 MSI adenomas and found 2 A8→A7 mutations (11.8%). A significant association was observed between mutation frequency and tumor stage (*SI Appendix*, Fig. S1*D*). *MYO1A* was mutated in 19% of the premalignant lesions (adenomas) and tumors that have not invaded through the intestinal wall (Dukes A) compared with 35% of the tumors that had invaded through the gut wall or metastasized to regional lymph nodes or distant organs (Dukes B–D; Fisher's exact test, $P = 0.04$), suggesting that *MYO1A* inactivation is important for local tumor invasion through the intestinal wall but does not confer an increased metastatic potential.

Fifty-five of the 58 mutations (95%) observed in the tumor lines and primary colorectal tumors studied were deletions of one A in the A8 repeat in exon 28 of *MYO1A* ($MYO1A^{A7MUT}$) (*SI Appendix*, Fig. S1*A*). This mutation causes a frame-shift and a premature stop codon and, as a result, the last 11 amino acids of the wild-type MYO1A are replaced by 7 different amino acids (*SI Appendix*, Fig. S2*A*). Previous studies have shown that the tail domain of MYO1A is necessary and sufficient for localization to the plasma membrane (21, 22). To investigate the effects of the observed *MYO1A* mutations on its function and subcellular localization, wild-type or mutant full-length MYO1A and MYO1A tail domain were transfected into Caco2 colon cancer cells and the derivative clone $Caco2_{BBe}$. Transfection of the full-length wild-type EGFP-MYO1A protein and the wild-type EGFP-tail domain (*SI Appendix*, Fig. S2*B*) showed membrane localization in both undifferentiated (Fig. 1*A*) and fully differentiated Caco2 cells (Fig. 1*E*). In contrast, the EGFP/ERFP-tagged full-length $MYO1A^{A7MUT}$ and $tail^{A7MUT}$ domain mutant proteins failed to localize to the membrane and were instead mislocalized to the cytoplasm in both undifferentiated and differentiated Caco2 cells (Fig. 1 *B* and *H*), demonstrating that the frequently observed $MYO1A^{A7}$ mutations interfere with the subcellular localization of MYO1A.

Promoter Methylation Regulates MYO1A Expression. Although no mutations were found in the coding region of 10 microsatellite stable (MSS) colon cancer cell lines, we found frequent promoter hypermethylation in both MSI and MSS colon cancer cell lines. Despite the absence of a dense CpG island in the *MYO1A* promoter (*SI Appendix*, Fig. S3), quantitative assessment of the levels of methylation in two CpG dinucleotides located −154 bp and +271 bp relative to the transcription start site revealed

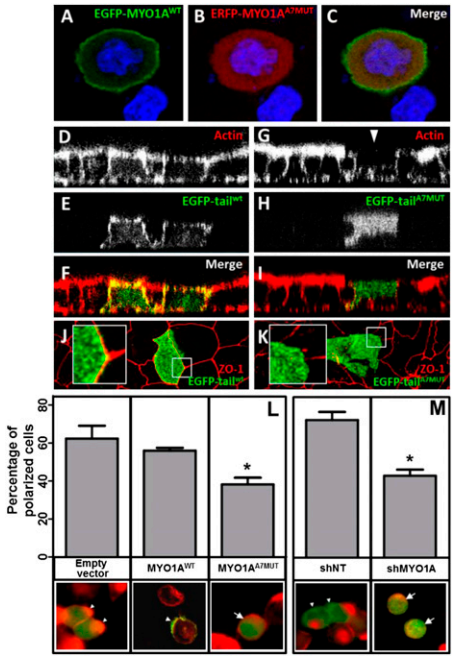

Fig. 1. MYO1A A8->A7 mutations affect the localization of the protein and the polarization of colon cancer cells. (*A–C*) Cotransfection of wild-type $EGFP\text{-}MYO1A^{wt}$ and mutant $ERFP\text{-}MYO1A^{A7MUT}$ demonstrated that the mutant protein mislocalized to the cytoplasm of undifferentiated Caco2 cells. (*D–I*) An orthogonal view of differentiated $Caco2_{BBe}$ cells. Alexa 568-labeled Phalloidin was used to visualize F-actin. Wild-type MYO1A $EGFP\text{-}tail^{wt}$ showed membrane localization (*D–F*) compared with the cytoplasmic localization of mutant MYO1A $EGFP\text{-}tail^{A7MUT}$ (*G–I*). F-actin was reduced in the apical membrane of MYO1A $EGFP\text{-}tail^{A7MUT}$-expressing cells (*G*, white arrowhead). ZO-1 immunostaining demonstrated that MYO1A $EGFP\text{-}tail^{A7MUT}$–expressing $Caco2_{BBe}$ cells exhibit loss of tight junctional integrity (*z*-axis stack; *J–K*). (*Inset*) Higher magnification of the indicated areas demonstrating loss of ZO-1 membrane staining in $EGFP\text{-}Tail^{A7MUT}$ transfected cells. Induction of LKB1/STK11 expression resulted in the polarization of most LS174T-W4 cells characterized by the apical accumulation of actin within 24 h (white arrowheads in *L* and *M*). The number of polarized cells 24 h after LKB1/STK11 activation was significantly reduced following transfection of either mutant $EGFP\text{-}MYO1A^{A7MUT}$ (*L*, mean ± SE; Student's *t* test, $*P = 0.03$) or cotransfection of EGFP and shMYO1A (*M*; mean ± SE; Student's *t* test, $*P = 0.01$), compared with the corresponding pEGFP-C3 empty vector, $EGFP\text{-}MYO1A^{WT}$ and nontarget shRNA (shNT) controls. Rhodamine-Phalloidin was used to visualize F-actin (red). White arrows show unpolarized transfected cells. The average (± SE) of three independent experiments is shown. Original magnification is 600×.

frequent methylation in a panel of 46 colorectal cancer cell lines (50% of the lines showed levels of methylation >50%) (*SI Appendix*, Fig. S3 and Table S2). Moreover, the levels of methylation in these two CpGs correlated positively with each other (Pearson's $r = 0.75$; $P < 0.0001$) (*SI Appendix*, Fig. S4*A*) and negatively with the levels of mRNA expression in these cell lines (Pearson's $r = -0.54$; $P = 0.005$) (*SI Appendix*, Fig. S4*B*). Although normal colonic mucosal samples typically contain high levels of contamination with nonepithelial cells, the average level of *MYO1A* promoter methylation at CpG −154 bp and +271 bp was significantly lower in normal colon samples compared with normal breast, stomach, ovary, lung, or kidney samples (Student's *t* test, $P < 2.6 \times 10^{-19}$) (*SI Appendix*, Fig. S4*C*). Analysis of a series of 122 primary colorrectal tumors demonstrated that the levels of methylation at these CpG dinucleotides were significantly correlated with the levels of MYO1A mRNA expression (Pearson's $r < -0.41$; $P < 0.0001$) (*SI Appendix*, Fig. S4 *D* and *E*). Methylation was confirmed by direct sequencing of bisulfite treated DNA in a subset of samples (*SI Appendix*, Fig. S3 and Table S2). The activity of cellular DNA methyltransferases was

reduced in cell lines with high *MYO1A* promoter methylation using either a pharmacologic (5-Aza-2′-deoxycytidine treatment in HCT116 and Co115 cells) or genetic approach (targeted deletion of the DNA methyltransferases *DNMT1* and *DNMT3b* in HCT116 cells) and resulted in a reduction in the levels of methylation in the *MYO1A* promoter (*SI Appendix*, Fig. S5 *A*, *C*, *E*, and *F*) that was associated with a significant increase in the levels of expression of MYO1A (*SI Appendix*, Fig. S5 *B*, *D*, and *G*). Collectively, these results demonstrate that *MYO1A* promoter methylation is a common event that regulates *MYO1A* expression in colorectal tumors.

MYO1A Regulates Differentiation and Polarization of Colon Cancer Cells. Because MYO1A is important for the apical localization of additional brush border proteins (22), we next assessed whether MYO1A can regulate cell polarity and differentiation of colon cancer cells. The apical accumulation of F-actin is a hallmark of epithelial differentiation along the absorptive cell lineage (5). Fully differentiated Caco2 cells, which express high levels of wild-type MYO1A protein (*SI Appendix*, Fig. S6*A*), when transfected with the mutant MYO1A EGFP-tail^{A7MUT} frequently failed to develop the characteristic accumulation of actin in the apical brush border (Fig. 1*G*). In addition, mutant EGFP-tail^{A7MUT}-expressing cells exhibit loss of tight junctional integrity as assessed by reduced levels of the junction-associated protein ZO-1 (Fig. 1 *J* and *K*). To further investigate the role of MYO1A in the polarization of colon cancer cells, we used an in vitro system where a fully polarized phenotype can be induced by constitutive overexpression of the pseudokinase STRAD and inducible expression of LKB1/STK11 in LS174T-W4 colon cancer cells (5) that express endogenous wild-type MYO1A (*SI Appendix*, Fig. S7 and Table S2). Transfection of either shMYO1A (which induced a 51% decrease in MYO1A protein expression) (*SI Appendix*, Fig. S8) or mutant EGFP-MYO1A^{A7MUT} into LS174T-W4 cells interfered with the polarization of these cells upon induction of LKB1/STK11, compared with the nontarget shRNA or empty vector/EGFP-MYO1A^{wt} controls, respectively (Fig. 1 *L* and *M*). Moreover, stable shRNA-mediated down-regulation of MYO1A in Caco2 cells (*SI Appendix*, Fig. S6*A*) resulted in significantly reduced activity of alkaline phosphatase, sucrose isomaltase, and dipeptidyl-peptidase 4, three markers of differentiation along the absorptive cell lineage (*SI Appendix*, Fig. S6 *B–D*). In addition, dome formation following 21 d of confluent culture, an additional marker of differentiation of Caco2 monolayers (23), was also significantly reduced in cells transduced with shMYO1A compared with parental and nontarget shRNA transduced Caco2 cells (*SI Appendix*, Fig. S6*E*). Moreover, although SW403 cells do not undergo significant differentiation under confluent culture conditions, shRNA-mediated MYO1A knockdown interfered with the capacity of these cells to differentiate after treatment with the short-chain fatty acid butyrate, a potent inducer of differentiation in colorectal cancer cells (*SI Appendix*, Fig. S9). Collectively, these experiments demonstrate that MYO1A regulates the polarization and differentiation of colon cancer cells.

MYO1A Inhibits Tumor Growth. To investigate the possible functional contribution of the loss of MYO1A to tumor progression, we next investigated whether MYO1A regulates the growth of colon cancer cells using Caco2 and SW403 cells stably expressing shMYOA. Knockdown of MYO1A in these two cell lines (Fig. 2 *A* and *D*) resulted in a significant increase in the number of colonies observed when cells were grown in soft-agar medium compared with either the parental cell line or the nontarget shRNA control (Fig. 2 *B* and *E*), demonstrating that reduced MYO1A levels significantly improved the anchorage-independent growth of colon cancer cells. Moreover, when these cell lines were grown as xenografts in immunocompromised mice, MYO1A knockdown sublines grew significantly faster than the nontarget shRNA control lines (Fig. 2 *C* and *F*), indicating that reduced MYO1A levels confer a growth advantage to colon cancer cells in vivo.

Loss of MYO1A Accelerates Tumor Progression. To further investigate the role of MYO1A on intestinal tumorigenesis, we used a mouse model where the first three exons of *Myo1a* have been replaced by a neo/ura selection cassette, resulting in the complete absence of Myo1a protein (24). Although *Myo1a* knockout (KO) mice show important structural and compositional defects in the brush border domain of the intestinal epithelium, inactivation of *Myo1a* alone is not sufficient to initiate intestinal tumorigenesis (24). We therefore used both a genetic (*Apc* mutations; Apc^{min} mouse model) and a pharmacological (azoxymethane, AOM) approach to initiate intestinal tumorigenesis in *Myo1a* KO mice. Using the genetic model of tumor initiation, we found that animals were born at Mendelian ratios (78/138/73 for $Myo1a^{+/+}$, $Myo1a^{+/-}$, and $Myo1a^{-/-}$, respectively) and the weight of the animals with different genotype at 153 or 214 d of age was not significantly different (Student's *t* test, $P > 0.1$). However, introduction of a heterozygous or homozygous deletion of *Myo1a* in mice bearing the Apc^{min} mutation resulted in a significant shortening of their lifespan (Fig. 3*A*). The median survival of the animals in the $Apc^{min/+};Myo1a^{+/+}$ group was 378 d, which was reduced to 329 d (13%; logrank test, $P = 0.02$) and 245 d (35%; logrank test, $P = 0.0003$) for the $Apc^{min/+};Myo1a^{+/-}$ and $Apc^{min/+};Myo1a^{-/-}$ groups, respectively. In good agreement, scoring of microscopically and macroscopically visible small intestinal tumors demonstrated a significantly higher number of tumors in $Apc^{min/+};Myo1a^{-/-}$ and $Apc^{min/+};Myo1a^{+/-}$ mice compared with $Apc^{min/+};Myo1a^{+/+}$ animals (Fig. 3*B*).

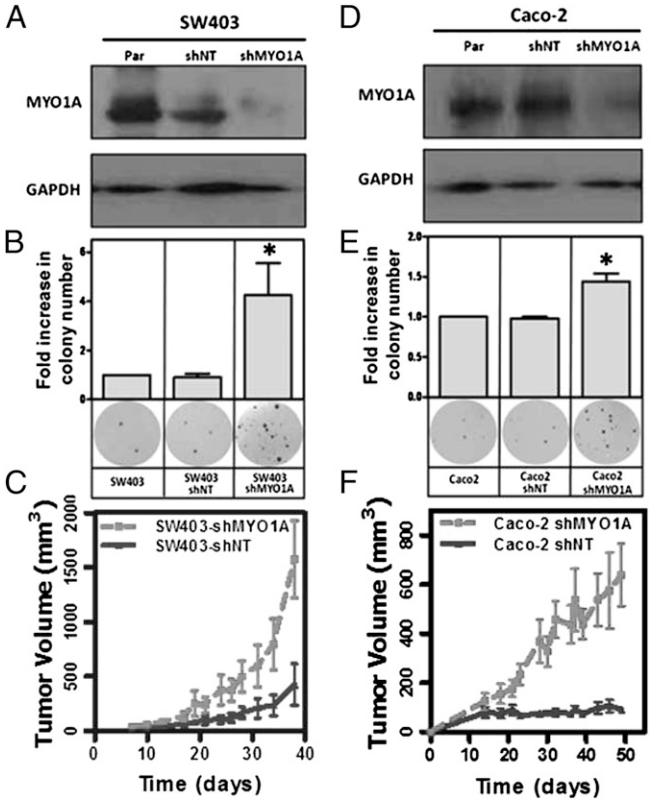

Fig. 2. MYO1A regulates the growth of colon cancer cells. Western blot analysis demonstrated that stable shRNA transduction resulted in reduced MYO1A levels in Caco2 and SW403 colon cancer cells (*A* and *D*) (Par, parental cells; shNT and shMYO1A, cells transduced with a nontarget shRNA or shMYO1A, respectively). MYO1A knockdown cells showed increased anchorage-independent growth in soft agar medium (*B* and *E*) (mean of three independent experiments run in triplicate; Student's *t* test $*P < 0.001$). A significantly faster growth of the shMYO1A SW403 (*C*) and shMYO1A Caco2 (*F*) cells was observed in a xenograft model compared with the corresponding nontarget shRNA sublines.

Although *Myo1a* inactivation did not affect the average tumor size, we found >twofold increase in the number of infiltrating adenocarcinomas in $Apc^{min/+}$;$Myo1a^{-/-}$ compared with $Apc^{min/+}$;$Myo1a^{+/+}$ animals (11.9% versus 5.6%, respectively; Student's *t* test, $P = 0.05$) (Fig. 3 *C–F* and *SI Appendix*, Fig. S10). Examination of the normal mucosa demonstrated no differences in the total number of cells per crypt/villus or the frequency of goblet, Paneth, or enteroendocrine cells between $Apc^{min/+}$;$Myo1a^{+/+}$ and $Apc^{min/+}$;$Myo1a^{-/-}$ mice (*SI Appendix*, Fig. S11). Similarly, no differences were observed in the number of proliferating cells either in the normal epithelium or intestinal tumors from $Apc^{min/+}$ mice that were $Myo1a^{+/+}$, or $Myo1a^{-/-}$ (*SI Appendix*, Fig. S12). As an alternative mechanism to initiate intestinal tumorigenesis we used AOM, an intestinal-specific chemical carcinogen. Consistent with findings made in the Apc^{min} model, we observed a significantly higher number of tumors in the small intestine of $Myo1a^{+/+}$ mice compared with $Myo1a^{+/+}$ animals (2.5 ± 1.3 vs. 0.8 ± 0.7 tumors per mouse, respectively; Student's *t* test; $P = 0.001$) (*SI Appendix*, Fig. S13). When taken together, these results demonstrate that inactivation of *Myo1a* is an important event contributing to intestinal tumor progression and may participate in the adenoma-to-carcinoma transition.

Low Levels of MYO1A Are Associated with Shorter Survival of Colorectal Cancer Patients. Because *Myo1a* inactivation accelerated the oncogenic process in mouse models of intestinal tumorigenesis, we next investigated the possible association between MYO1A tumor levels and prognosis of patients with colorectal cancer. For this purpose we assessed the tumor levels of MYO1A protein in a series of 155 colorectal cancer patients with locally advanced disease (Dukes C) using immunohistochemical staining of sections of a tissue microarray (Fig. 4 *A–F* and *SI Appendix*, Table S3). Patients with low MYO1A tumor protein levels had significantly shorter disease-free and overall survival compared with patients with high tumoral MYO1A (Fig. 4 *G* and *H* and *SI Appendix*, Fig. S14) (logrank test, $P = 0.004$ and $P = 0.009$, respectively). The median time-to-disease recurrence in Dukes C patients with low MYO1A was 1 y, compared with >9 y in the group of patients with high MYO1A. Multivariate analysis showed that MYO1A remained a good marker of prognosis of colorectal cancer patients (overall and disease-free survival, Cox regression, $P < 0.04$; covariates: patient age, sex, adjuvant treatment, tumor location, and grade). Twenty-one of the 155 tumors in the tissue microarray were MSI and MYO1A mutation data were available, allowing analysis of a possible association between $MYO1A^{7A}$ mutations and expression levels. A significant reduction in MYO1A protein levels was observed in MSI tumors with *MYO1A* mutations ($n = 13$) compared with MSI tumors without mutations ($n = 8$; average score 2.1 ± 0.9 vs. 3.1 ± 0.9, respectively; Student's *t* test, $P = 0.031$). However, no difference in MYO1A protein expression was observed in primary Dukes C tumors and matched lymph node metastases ($n = 16$; average score 2.5 ± 0.6 vs. 2.2 ± 0.8 respectively; Student's *t* test, $P = 0.24$).

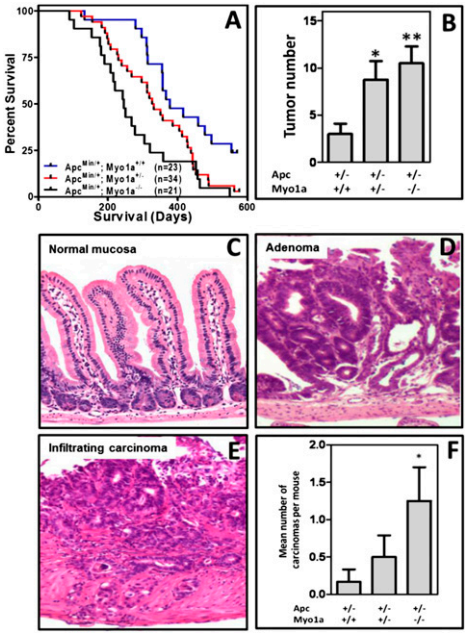

Fig. 3. Role of Myo1a in intestinal tumorigenesis using a murine KO model. The inactivation of one or two copies of *Myo1a* in mice bearing heterozygous mutations of the tumor suppressor gene *Apc* resulted in significantly reduced animal lifespan (*A*). The *P* values of the logrank tests for the comparison of the survival of $Myo1a^{+/-}$ and $Myo1a^{-/-}$ relative to $Myo1a^{+/+}$ animals were $P = 0.022$ and $P = 0.0003$, respectively. (*B*) The number of tumors observed in histological sections of the full small intestine of 30-wk-old $Apc^{Min/+}$;$Myo1a^{-/-}$ ($n = 15$; *t* test, $^{**}P = 0.004$) and $Apc^{Min/+}$;$Myo1a^{+/-}$ ($n = 7$; *t* test, $^{*}P = 0.05$) animals was significantly higher than in the $Apc^{Min/+}$;$Myo1a^{+/+}$ control mice ($n = 11$). Mean ± SD is shown. Examples of the normal small intestine as well as intestinal adenomas and adenocarcinomas are shown in *C*, *D*, and *E*, respectively. Original magnification was 200×. (*F*) The average number of infiltrating adenocarcinomas in 30-wk-old $Apc^{Min/+}$;$Myo1a^{-/-}$ mice was significantly higher than in $Apc^{Min/+}$;$Myo1a^{+/+}$ animals (mean ± SD; Student's *t* test; $^{*}P = 0.05$).

Discussion

Structural proteins of the brush border of the intestinal epithelium have been shown to be lost or reduced during tumor progression (18–20), and this is generally believed to be the result of the loss of differentiation/polarization accompanying this process. However, we show here that the loss of MYO1A drives the tumorigenic process in intestinal tumors. We found frame-shift *MYO1A* mutations in one-third of MSI colorectal tumors and the A8→A7 mutation frequently observed disrupts membrane targeting of MYO1A and interferes with polarization/differentiation of colon cancer cells. Although the promoter region of *MYO1A* does not contain a dense CpG island, methylation of promoter regions that are not within CpG islands has been shown to be important for the regulation of the transcriptional activity (25, 26). We therefore investigated the contribution of CpG methylation to *MYO1A* silencing in colorectal cancer tumor cells and found evidence of *MYO1A* promoter hypermethylation in a significant proportion of colorectal cancer cell lines and primary tumors tested. Moreover, there was an inverse correlation between MYO1A expression and promoter methylation, and treatment with a DNA methyltransferase inhibitor or genetic inactivation of DNA methyltransferase activity led to reduced levels of methylation in the *MYO1A* promoter and increased MYO1A expression in colon cancer cell lines that showed promoter methylation, indicating that promoter hypermethylation is an alternative mechanism of gene silencing in both MSS and MSI colorectal tumors. Ectopic expression of the mutant $MYO1A^{A7}$ form in colon cancer cells that express high endogenous levels of wild-type MYO1A (Caco2 and LS174T cells) leads to a loss of polarization/differentiation, suggesting a dominant-negative effect, probably through the sequestration of additional proteins necessary for the function of the wild-type MYO1A. In good agreement, overexpression of the mutant MYO1A and knockdown of the wild-type endogenous MYO1A phenocopy each other in vitro. However, the lower levels of expression observed in MSI tumors with $MYO1A^{A7}$ mutations suggest that the relevance of the dominant-negative activity of the mutant protein may be limited in primary colorectal tumors.

MYO1A Is Important for STK11/LKB1-Mediated Polarization/Differentiation of Colon Cancer Cells. STK11/LKB1 has been shown to be a key regulator of cell polarity in organisms ranging from yeast to humans. The role of STK11/LKB1 in maintaining the polarity of the intestinal epithelium is highlighted by the fact that germ-line

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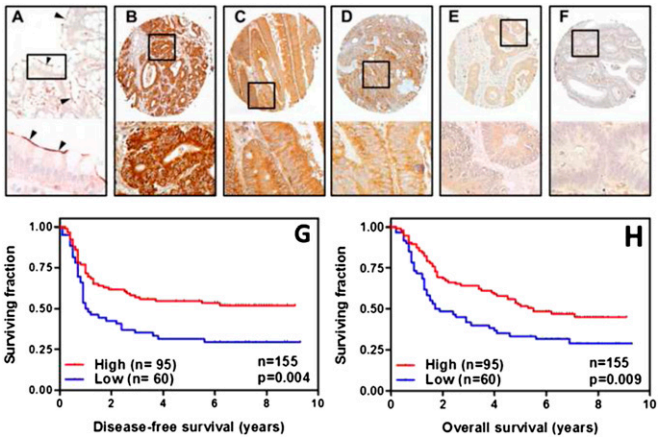

Fig. 4. MYO1A and prognosis of colorectal cancer patients. (*A*) MYO1A immunostaining showed the expected accumulation in the apical brush border of the normal human colonic mucosa (arrowhead). Colorectal human tumors showed variable staining intensity, ranging from levels similar to normal epithelial cells to undetectable levels (*B–F*). (*Lower*) Higher magnification of the indicated regions. Magnification for the upper and lower panels was 200× and 600×, respectively. Kaplan-Meier plots show that patients with low tumor MYO1A levels have shorter disease-free (*G*) (log-rank test, $P = 0.004$) and overall (*H*) (logrank test $P = 0.009$) survival than patients with high tumor MYO1A levels.

mutations of this kinase in humans result in the presence of intestinal hamartomatous polyps and colorectal cancer predisposition (7–9). Although STK11/LKB1 has important functions as a regulator of multiple biological processes and signaling pathways, such as energy metabolism and Wnt or TGF-β signaling, *STK11/LKB1* mutations that do not disrupt its kinase activity but affect its capacity to induce polarization of intestinal epithelial cells are sufficient to cause tumor initiation/progression (10), indicating that the role of STK11/LKB1 in cell polarity is important for intestinal tumor suppression. Here, we demonstrate that MYO1A is important for the STK11/LKB1-dependent polarization/differentiation program of colon cancer cells. Ectopic expression of the mutant MYO1A^{A7} or shRNA-mediated knockdown in LS174T colon cancer cells significantly interferes with their capacity to polarize following STK11/LKB1 activation. Moreover, transfection of MYO1A tail^{A7} into Caco2 cells also interferes with the differentiation program of these cells when grown in confluence, which has also been shown to be STK11/LKB1-dependent (5). Unlike most tumor-suppressor genes that were initially identified as the underlying genetic cause of hereditary cancer predisposition and then found to be frequently mutated in sporadic cancer cases, STK11/LKB1 mutations are rare in sporadic colorectal tumors and epigenetic inactivation is not frequently observed (11–13). Therefore, genetic/epigenetic inactivation of *MYO1A* and other genes downstream of STK11/LKB1 may account for the limited number of STK11/LKB1 mutations observed in sporadic colorectal tumors.

Strict regulation of protein trafficking and vesicle targeting are required for apical-basal polarization of epithelial cells (1, 27). Because Myosin I members have been shown to be important for the targeted delivery of proteins in polarized cells (22, 28, 29), the loss of MYO1A may directly interfere with the subcellular localization of key mediators of cell polarization. Although the detailed molecular mechanisms underlying the role of MYO1A in polarization downstream of STK11/LKB1 remain to be fully elucidated, STK11/LKB1 has been shown to activate AMP-activated protein kinase, which in turn phosphorylates myosin regulatory light chain 2 (MYL12B/MRLC2) (30). MRLC2 phosphorylation seems to be necessary and sufficient for polarization of colon cancer cells (30). Although this myosin light chain has not been reported to directly regulate Myosin Ia, both MRLC2 (30) and MYO1A colocalize with the brush border actin cytoskeleton, providing a possible mechanistic link between MYO1A and polarization of intestinal epithelial cells. Alternatively, the loss of MYO1A-dependent localization of calmodulin to the brush border membrane (24) may affect the phosphorylation state of MRLC2 through aberrant regulation of myosin light-chain kinase.

Loss of MYO1A Is Important for Intestinal Tumorigenesis. Although the molecular mechanisms of the epithelial-to-mesenchymal transition associated with increased metastatic potential of advanced tumors are well characterized, the importance of the loss of polarity/differentiation in tumor cells before the acquisition of metastatic potential is not well understood. Studies in *Drosophila* have convincingly demonstrated that inactivation of genes involved in epithelial cell polarity is important for tumor progression beyond the hyperproliferative stage (1, 3, 4, 31). However, examples of the role of polarity genes in early tumorigenesis of higher vertebrates are scarce. Mice lacking the Lethal giant larvae 1 (*Lgl1*) gene show loss of neural progenitor cell polarity and severe brain dysplasia (32). Furthermore, reduced expression of the key polarity regulators Lgl, Dlg, and Scrib is associated with tumor progression in humans (33–35).

The loss of Myo1a leads to important defects in the differentiation of normal intestinal epithelial cells. As reported previously, in the normal intestine, the loss of Myo1a in the KO mouse causes defects in enterocyte apical membrane tethering, loss of intramicrovillar calmodulin, and the resultant secondary effects of impaired Ca^{2+} homeostasis, mis-targeting of sucrase-isomaltase, destabilization of lipid rafts, and loss of intermicrovillar MYO6 and MYO1E (24). As discussed above, in intestinal tumor cells, the loss of MYO1A interferes with their ability to polarize and differentiate in response to either cell-cell contact or STK11/LKB1 activation. Moreover, in the mouse models used, *Myo1a* inactivation appears to participate in the adenoma-to-carcinoma transition and this is illustrated by a significantly higher incidence of locally invasive carcinomas in the intestine of *Myo1a* KO mice compared with *Myo1a* wild-type mice following tumor initiation by *Apc* mutations. However, the inactivation of *Myo1a* in *Apc*$^{Min/+}$ mice did not lead to a metastatic phenotype. In human tumors, the loss of MYO1A seems to contribute to the acquisition of local invasive capacity. This finding is consistent with the observation that mutation frequency significantly increases as tumors progress from a benign adenoma to a locally invasive carcinoma (Dukes B) (*SI Appendix*, Fig. S1*D*). However, no further increase in mutation frequency was observed in late-stage tumors that have metastasized to either regional lymph nodes (Dukes C) or distant organs (Dukes D) compared with locally invasive tumors (Dukes B). Moreover, we found no differences in the levels of MYO1A protein expression in Dukes C primary tumors and matched lymph node metastases, further indicating that the loss of MYO1A does not significantly contribute to tumor metastasis.

MYO1A Is an Independent Marker of Prognosis for Colorectal Cancer Patients. The prognosis of colorectal cancer patients is largely determined by tumor staging based on the degree of penetration of the tumor through the intestinal wall and the presence of metastasis in regional lymph nodes or distant organs. However, patients diagnosed with histopathologically indistinguishable tumors of the same stage can widely vary in their survival, highlighting the need for additional markers allowing stratification of patients with different prognosis. Here we show that the tumor levels of MYO1A in patients diagnosed with Dukes C colorectal cancer were significantly associated with patient survival. The disease-free survival of patients that retained high tumor levels of MYO1A was >ninefold longer than patients with low MYO1A tumor levels (>9.1 vs. 1.0 y respectively), indicating that MYO1A levels can be used to predict the probability of patient survival.

In conclusion, these results demonstrate that structural proteins of the intestinal brush border, previously believed to be passive differentiation markers in colorectal tumors, can actively

contribute to tumor progression. Therefore, our results indicate that further investigation of the oncogenic effects of the loss of additional putative differentiation markers is warranted in colorectal tumors and possibly other tumor types. The results of the mouse KO models and the genetic data from human colorectal tumors significantly contribute to elucidate the emerging role of genes involved in epithelial cell polarity/differentiation on epithelial tumorigenesis.

Materials and Methods

Cell lines and clinical samples: All cell lines were maintained in DMEM medium supplemented with 10% FBS and 1× antibiotic antimycotic (Invitrogen). The clinicopathological data of the 134 MSI patients used for the *MYO1A* mutation screening and the 155 patients in the tissue microarray used for MYO1A immunostaining are summarized in Tables S1 and S3, respectively. Sequencing: *MYO1A* mutations were assessed by direct sequencing of *MYO1A* exon 28. Fluorescence microscopy: Caco2 and LS174T-W4 cells were grown on gelatinized coverslips and transfected (Lipofectamine 2000) with the indicated vectors. Images were captured with a confocal microscope (Olympus FV1000). Enzymatic activity assays: The activity of alkaline phosphatase (AP), sucrase isomaltase (SI), and dipeptidyl peptidase-4 (DPP4) in Caco-2 cells that were confluent for 0, 2, 5, 7, 14, or 21 days was assessed as previously described. Xenograft model: Caco2 and SW403 cells transduced with shMYO1A and control nontarget shRNA (shNT) were s.c. injected in athymic nude mice. Tumor growth was monitored for 7 wk. Mouse knockout strains: *Myo1a* KO mice were used to investigate the role of Myo1a in tumor progression initiated by Apc mutations ($Apc^{min/+\ model}$) or azoxymethane treatment (nine weekly i.p. injections of 10 mg/kg). Immunohistochemistry and Western blotting: Immunohistochemical staining of formalin-fixed, paraffin embedded samples following antigen retrieval with 10 mM citrate buffer pH 6.0 [anti-BrdU, Developmental Studies Hybridoma Bank; anti-MYO1A (24)] or 1 mM EDTA (lysozyme, Dako). Anti-GAPDH (1:1000; clone 6C5; Santa Cruz) was used as a loading control in Western blot analysis. CpG methylation assays and Real-Time RT-PCR: The Infinium quantitative methylation assay (Illumina) was used following manufacturer's instructions. For bisulfite sequencing, DNA was treated with bisulfite and sequenced. MYO1A promoter methylation and expression levels of primary colorectal tumors were obtained from The Cancer Genome Atlas (http://cancergenome.nih.gov/). For qPCR, total RNA was extracted and reverse transcribed, and relative MYO1A mRNA levels assessed by real-time PCR using SYBR Green Master Mix (Applied Biosystems). 18S rRNA was used as a standardization control for the 2-ΔΔCt method. Primer sequence and PCR conditions can be found in Table S5. Additional materials and methods are described in *SI Materials and Methods*.

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