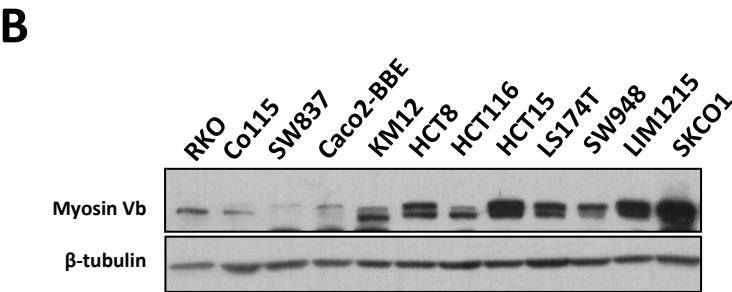
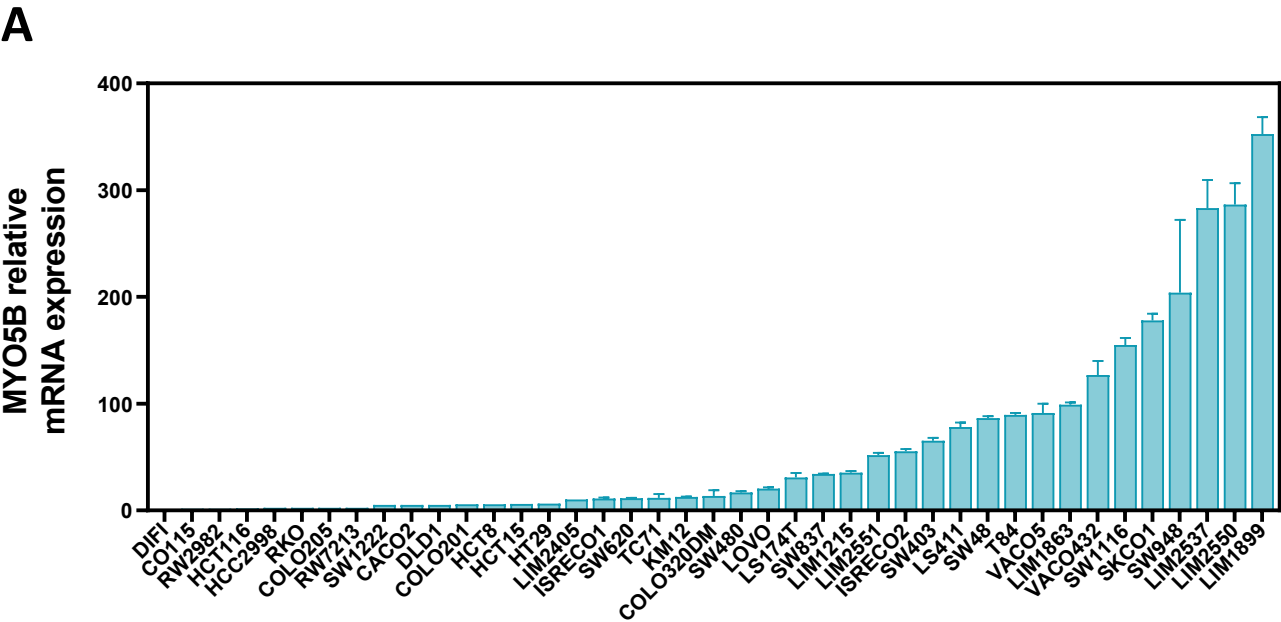
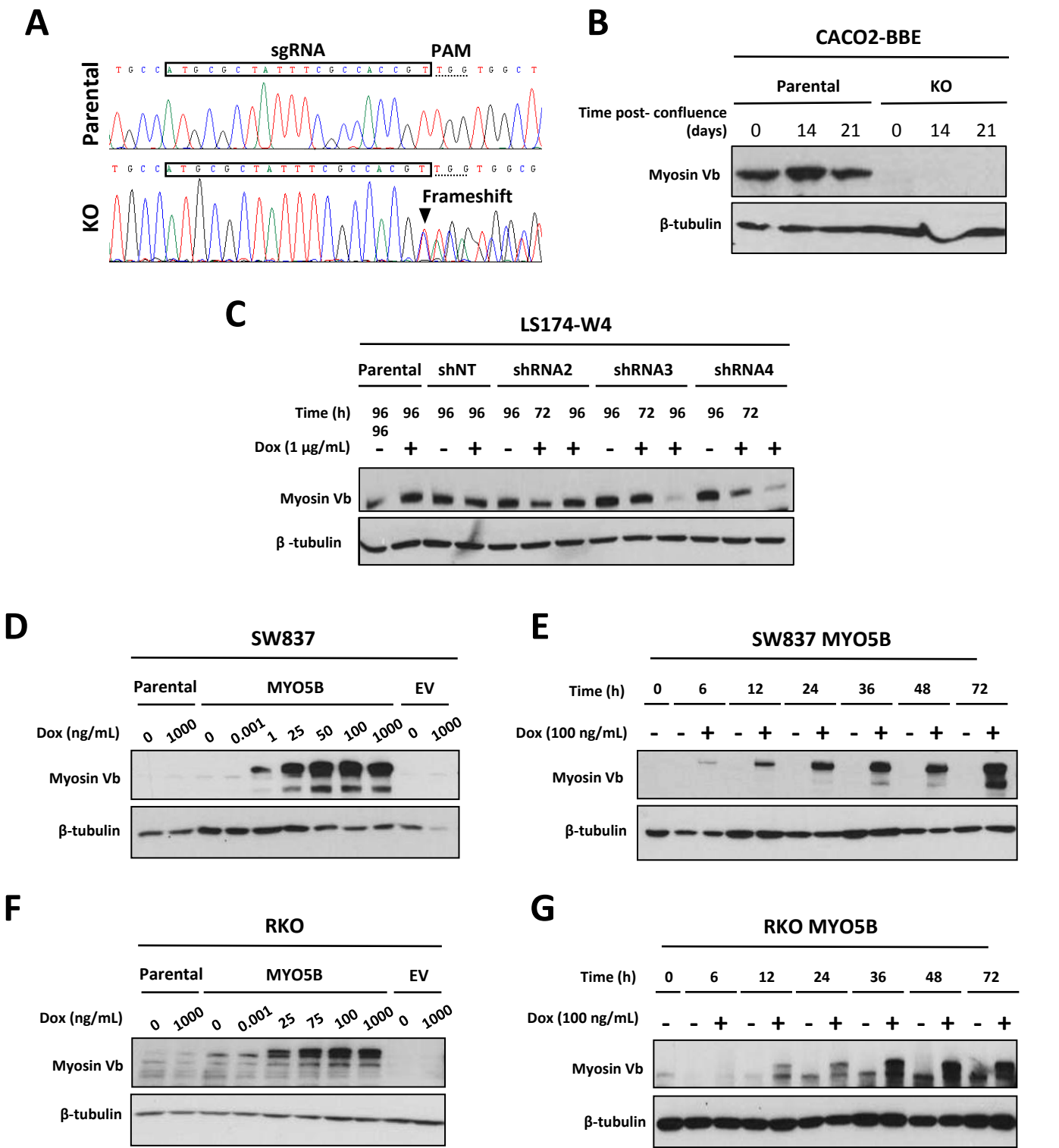


Supplementary Figure S1



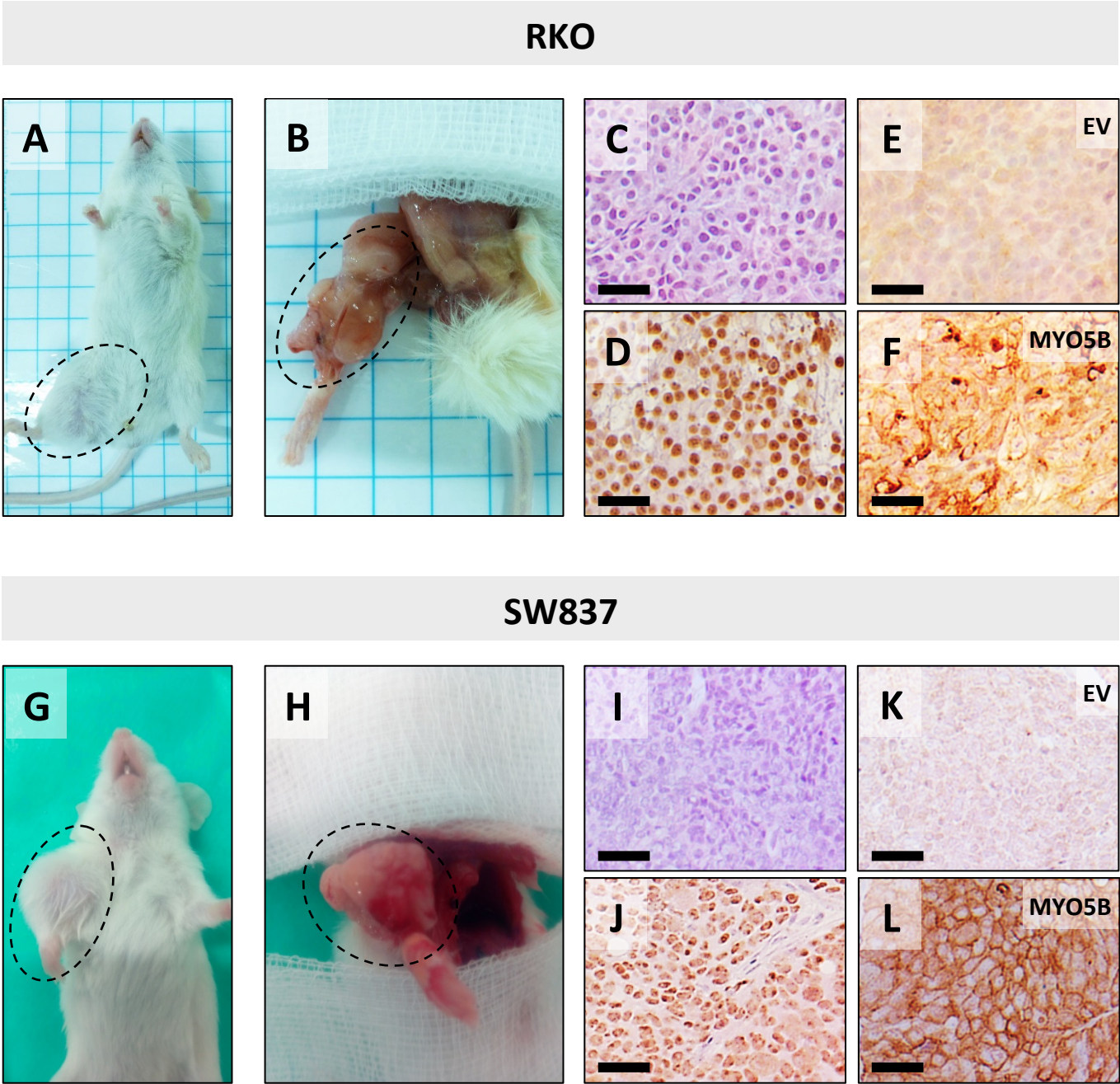
Supplementary Figure S1: Myosin Vb expression levels in a panel of colorectal cancer cell lines. **A)** The average ($\pm$ SD) *MYO5B* expression (mRNA) in a panel of 40 colorectal cancer cell lines was assessed by quantitative RT-PCR. 18S was used for normalization. **B)** Western blot showing the relative levels of expression of Myosin Vb (protein) in 12 different colon cancer cell lines. β -Tubulin was used as a loading control.

Supplementary Figure S2



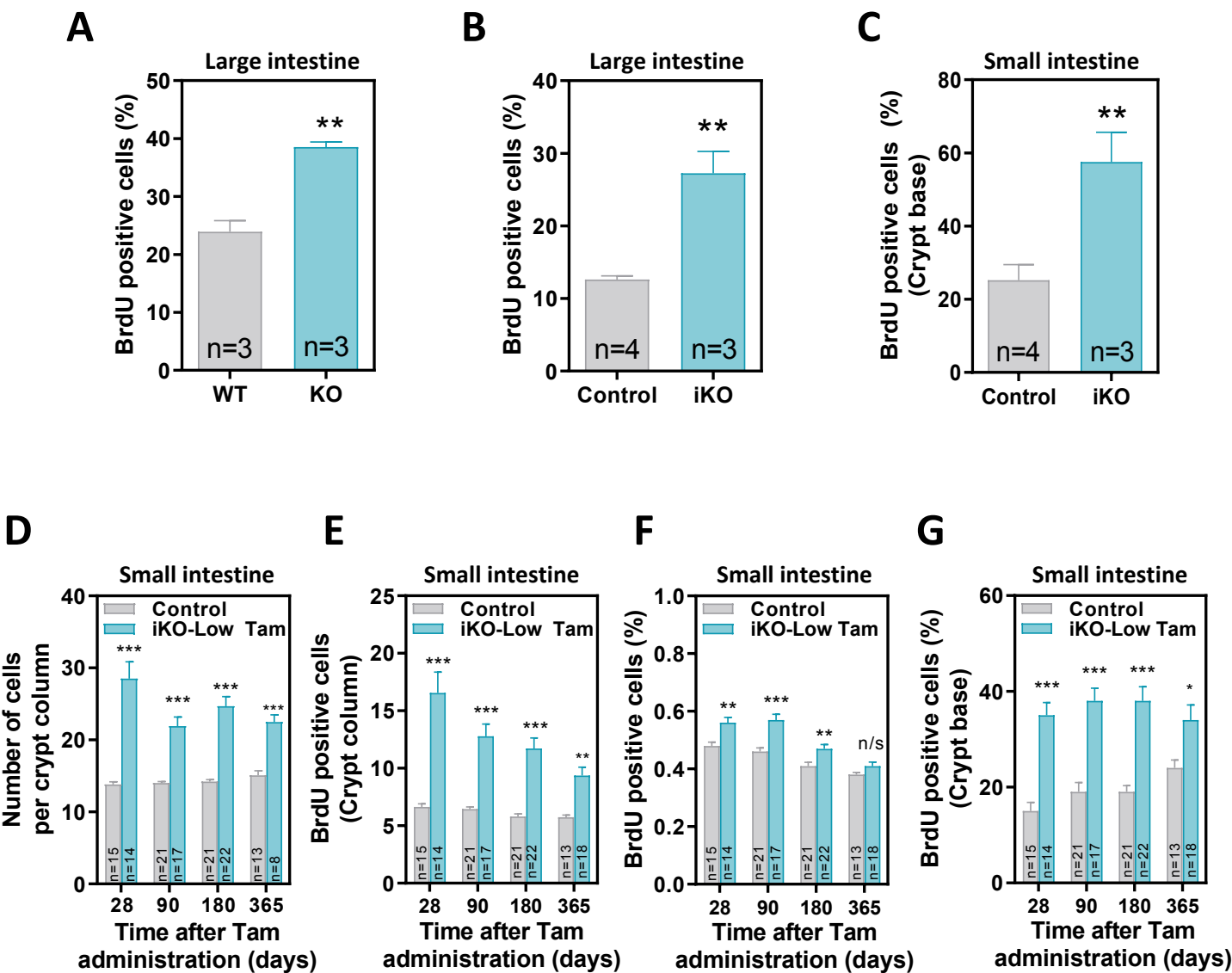
Supplementary Figure S2. Isogenic colorectal cancer cell line systems. **A)** Sanger sequencing data showing an example of a frameshift mutation observed after CRISPR/Cas9 targeting of *MYO5B* in CACO2-BBE cells. **B)** Western blot showing *MYO5B* expression in parental and *MYO5B* knockout CACO2-BBE cells after the indicated time points in confluent culture. **C)** Levels of expression of Myosin Vb by Western blot in LS174T-W4 cells showing doxycycline-dependent downregulation of *MYO5B* using an shRNA approach. **D)** Western blot showing Myosin Vb expression in parental SW837 cells and SW837 cells transduced with mCherry-*MYO5B* (MYO5B) or control mCherry (EV) after exposure to the indicated concentrations of doxycycline for 48 h. **E)** Expression of Myosin Vb in SW837 cells transduced with mCherry-*MYO5B* after exposure to 100 ng/ml doxycycline for the indicated time points. **F)** Western blot showing *MYO5B* expression in parental RKO cells and RKO cells transduced with mCherry-*MYO5B* (MYO5B) or control mCherry (EV) after exposure to the indicated concentrations of doxycycline for 48 h. **G)** Expression of Myosin Vb in RKO cells transduced with mCherry-*MYO5B* after exposure to 100 ng/ml doxycycline for the indicated times. β -Tubulin was used as a loading control.

Supplementary Figure S3



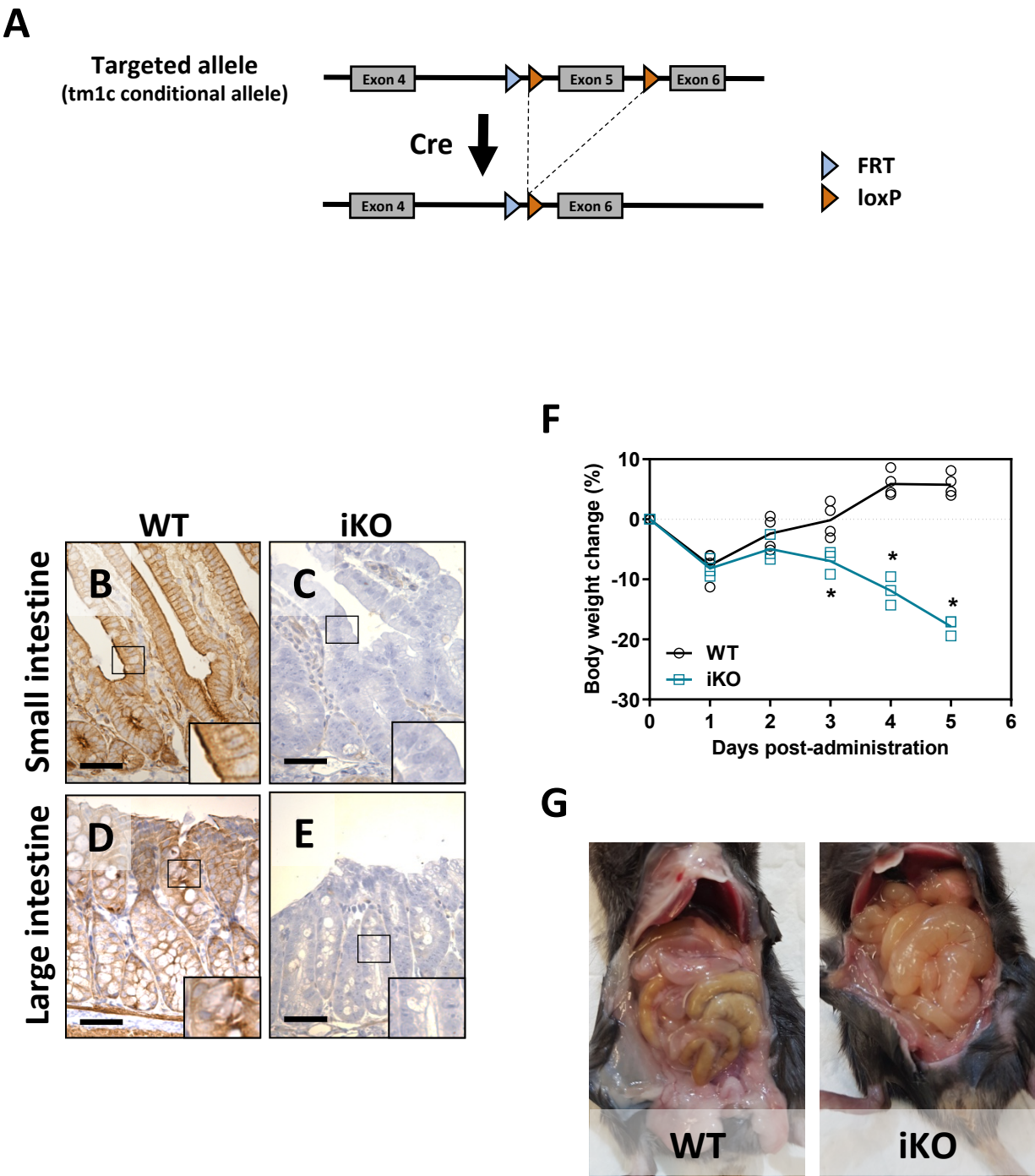
Supplementary Figure S3. Experimental model of metastasis. Control (mCherry, EV) and Myosin Vb (mCherry-Myosin Vb, MYO5B)-overexpressing RKO (A-F) or SW837 (G-L) were injected in the tail vein of NOD/SCID immunodeficient mice. Metastasis were macroscopically observed in the limbs of some of the mice (A-B and G-H). Panels C and I show hematoxylin and eosin staining of histological sections of the metastasis formed by RKO and SW837 cells, respectively. D, J) Immunostaining with a human-specific anti-Ku80 antibody confirming that the tumors were formed by the human RKO and SW837 colorectal cancer cell lines injected. E-F, K-L) The overexpression of Myosin Vb was confirmed by immunohistochemistry in the RKO (E-F) and SW837 (K-L) tumors. Scale bar 100 μ m.

Supplementary Figure S4



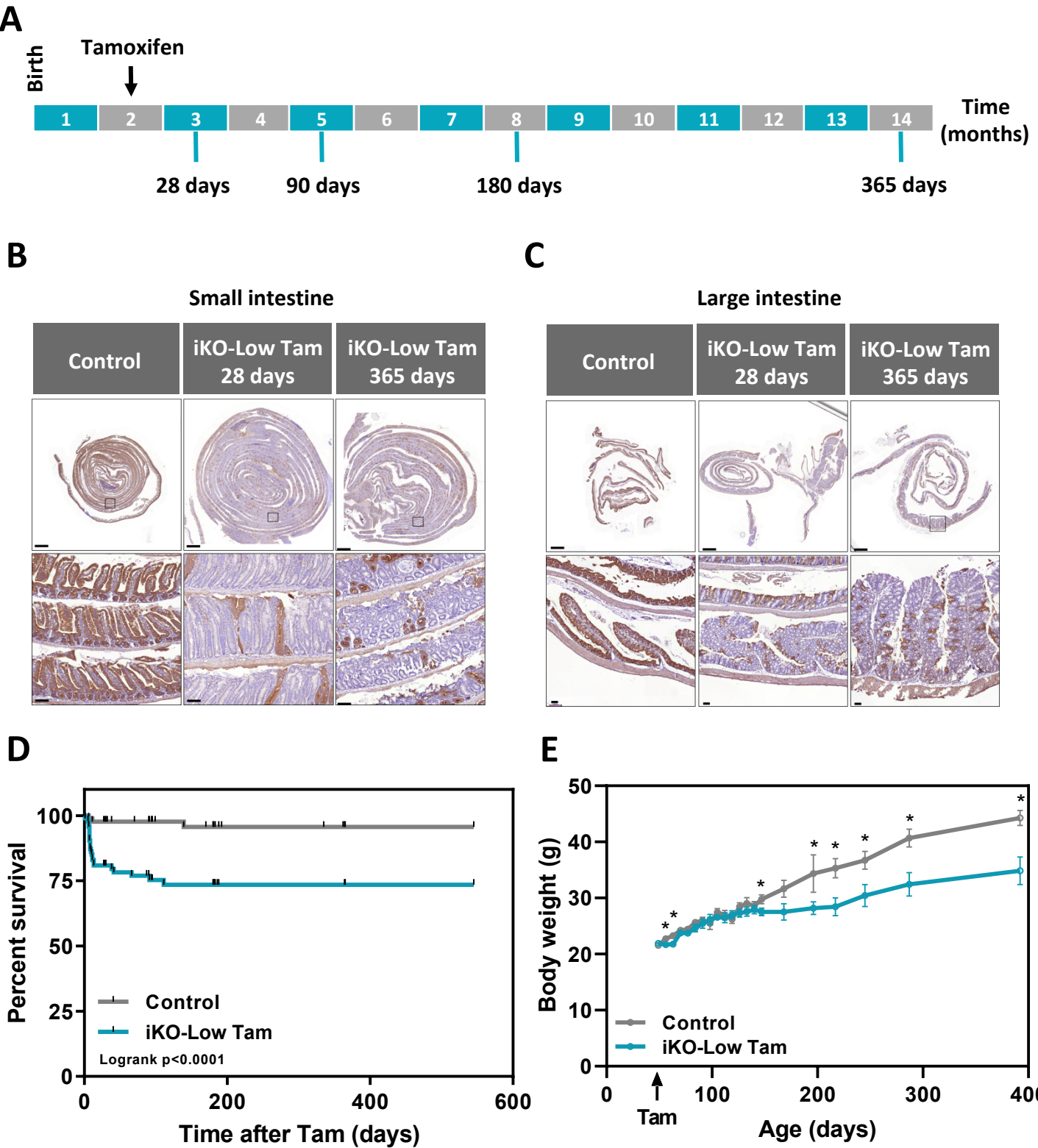
Supplementary Figure S4: Loss of Myosin Vb enhances the proliferation of normal intestinal epithelial cells. **A)** The number of proliferating (BrdU positive) cells was quantified in the large intestine of E20 embryos that are wild type (WT) or constitutively knockout for *Myo5b* (KO). **B-C)** The number of proliferating cells in the crypt of the large intestine (**B**) and in the lower crypt (positions 1–4 from the bottom of the crypt) of the small intestine (**C**) of control (*Myo5b^{lox/lox};CreERT2⁻*, Control) or conditionally inducible knockout mice (*Myo5b^{lox/lox};CreERT2⁺*, iKO). **D-G)** Cellularity in the small intestinal crypts of control animals (*Myo5b^{lox/lox};CreERT2⁻*, Control) and mice with inactivation of *Myo5b* in > 90% of the epithelial cells (*Myo5b^{lox/lox};CreERT2⁺*, iKO-Low Tam) was evaluated at different time points after administration of 40 mg/kg of tamoxifen: total (**D**) and BrdU positive (**E**) number of cells per crypt column, percentage of BrdU positive cells within crypts (**F**) and in the lower crypt (positions 1–4 from the bottom of the crypt) (**G**). The number of animals in each group is shown. The mean ± SEM is represented. Student's T-test *p<0.05; **p<0.01; ***p<0.001.

Supplementary Figure S5



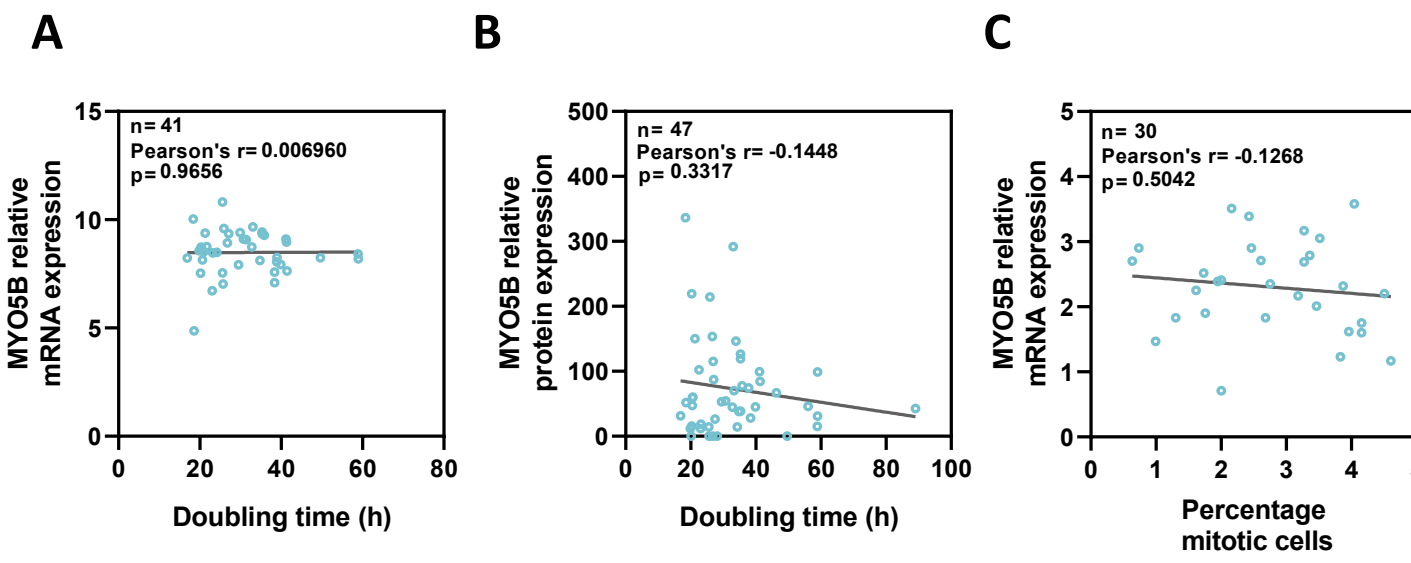
Supplementary Figure S5. Tamoxifen-dependent conditional inactivation of *Myo5b* in the murine intestine. **A)** Schematic representation of the *Myo5b* targeted allele. **B-E)** Immunohistochemistry with an anti-Myosin Vb antibody showing the complete loss of Myosin Vb expression in the epithelium of the small (**B-C**) and large (**D-E**) intestine of wild type (WT; *Myo5b*^{Flox/Flox}; *VilCreERT2*^{-/-}; **B** and **D**) and intestinal-specific and inducible *Myo5b* knockout (iKO; *Myo5b*^{Flox/Flox}; *VilCreERT2*^{+/+}; **C** and **E**) mice after i.p. administration of 160 mg/kg of tamoxifen. Insets show higher magnification micrographs of the indicated areas. **F)** Percentage change in body weight after i.p. administration of 100 mg/kg of tamoxifen to control and iKO *Myo5b* mice. **G)** Representative pictures showing the accumulation of fluids in the intestine of iKO *Myo5b* mice 5 days after administration of 160 mg/kg of tamoxifen. Scale bar: 50 μ m.

Supplementary Figure S6



Supplementary Figure S6. Myosin Vb downregulation in the murine intestine. **A)** Eight weeks-old animals received 40 mg/kg of tamoxifen (i.p.). Groups of animals were euthanized 28, 90, 180 or 365 days after tamoxifen administration. **B-C)** Myosin Vb immunohistochemistry in the small (**B**) and large (**C**) intestine of control animals (*Myo5b^{lox/lox}; CreERT2⁻*, Control) and mice with inactivation of *Myo5b* in > 90% of the epithelial cells (*Myo5b^{lox/lox}; CreERT2⁺*, iKO-Low Tam) 28 or 365 days after i.p. administration of tamoxifen. **D-E)** Survival (**D**) and body weight (**E**) of control and *Myo5b* iKO-Low Tam mice animals following tamoxifen treatment. Scale bar: 2 mm in the upper panels and 100 μm in the lower panels. *Student's T-test $p < 0.05$.

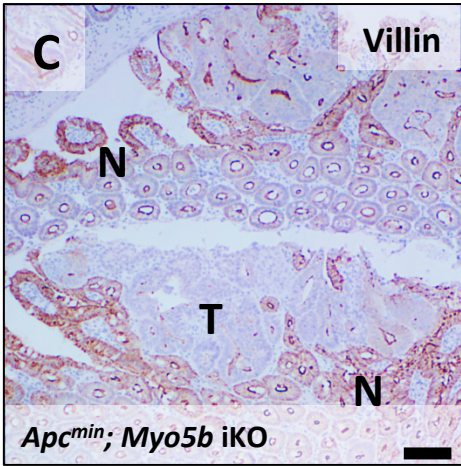
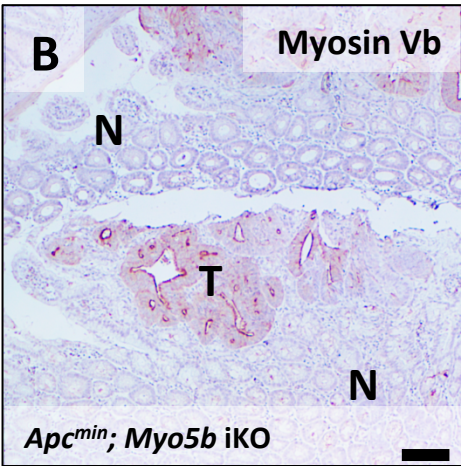
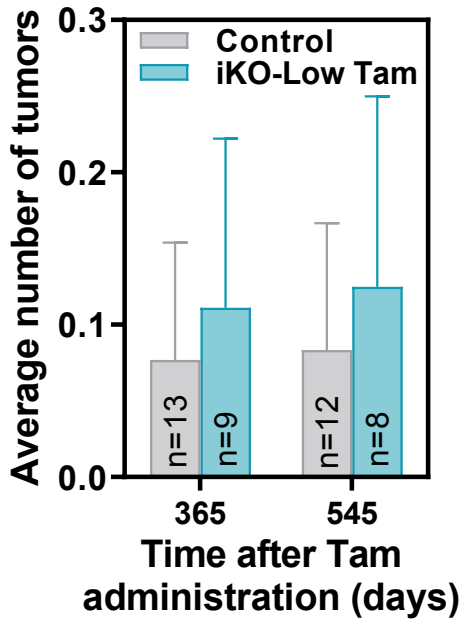
Supplementary Figure S7



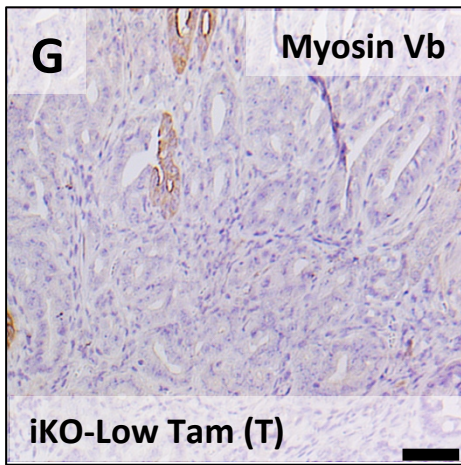
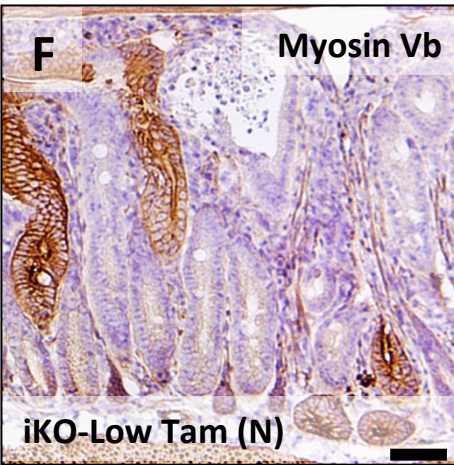
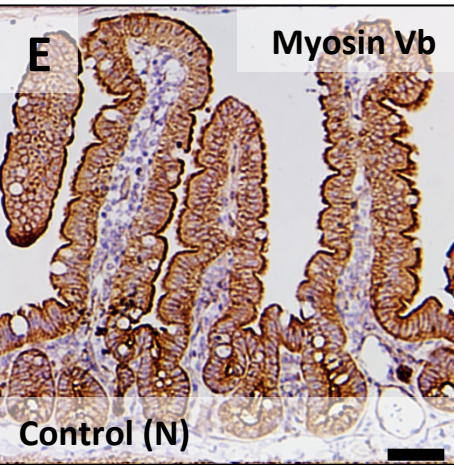
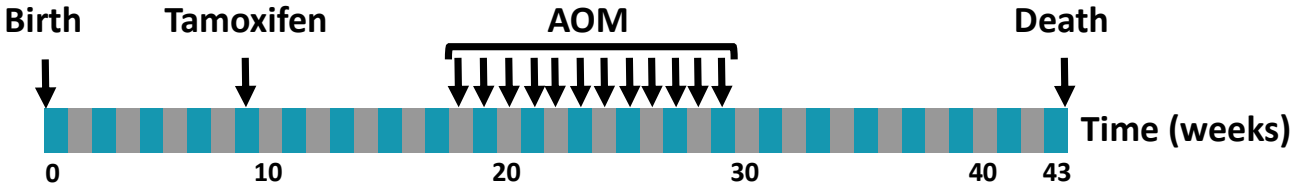
Supplementary Figure S7. Correlation between Myosin Vb expression and proliferation in human colon cancer cells. **A-B)** Correlation between the doubling time of different colorectal cancer cell lines and the levels of expression of Myosin Vb at the mRNA (**A**) and protein (**B**) level. **C)** Correlation between the mitotic index (ratio of mitotic cells and total number of tumor cells in hematoxylin and eosin stained sections) and the expression of MYO5B (mRNA) in a cohort of 30 colorectal tumors from the TCGA.

Supplementary Figure S8

A

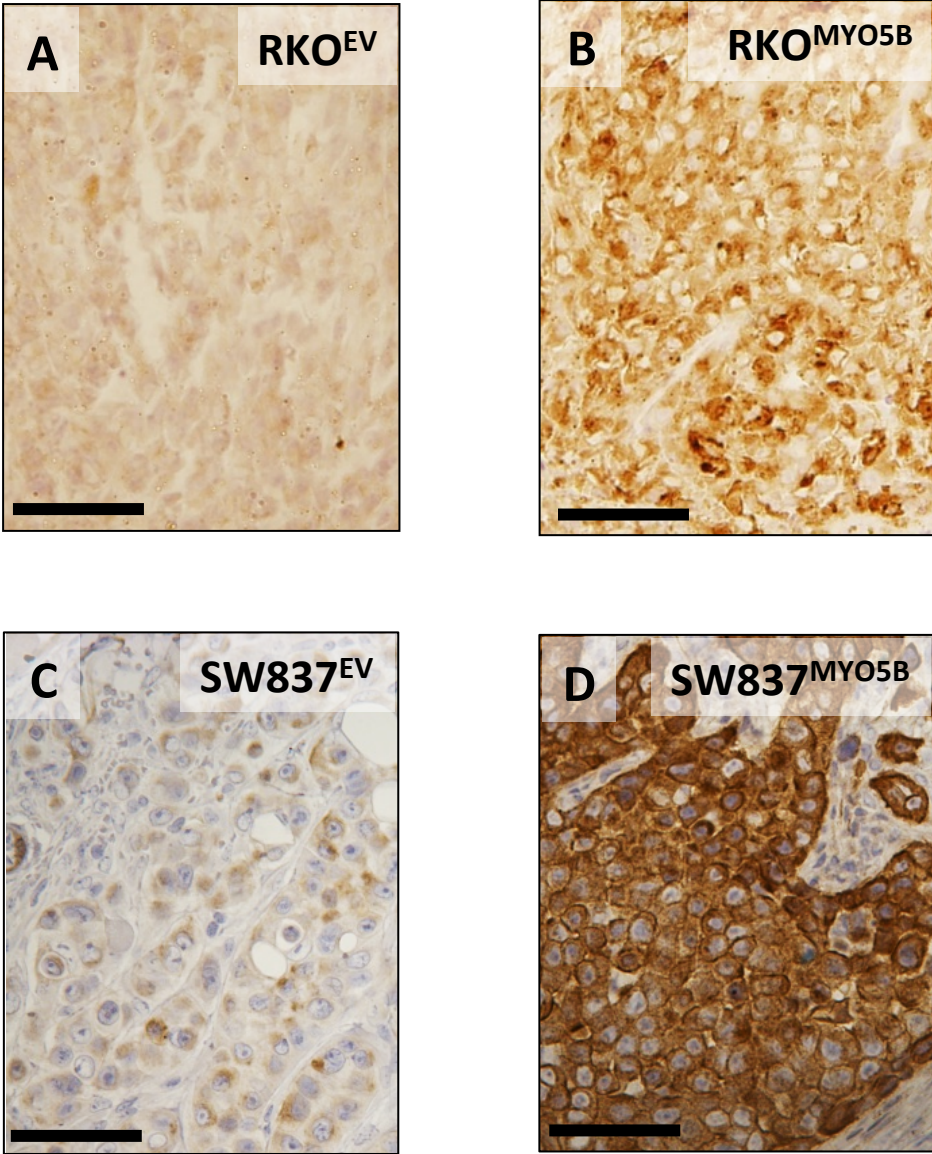


D



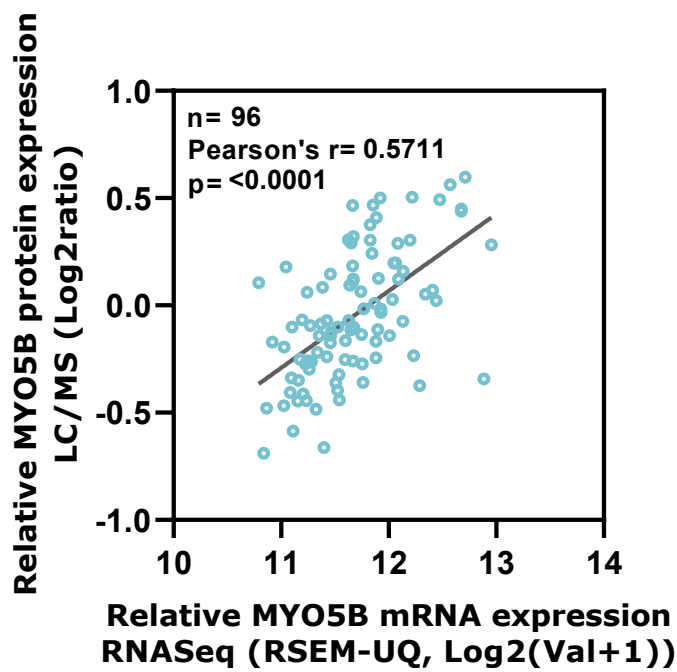
Supplementary Figure S8. Tumorigenesis in intestine-specific *Myo5b* floxed mice. A) Average number (± SEM) of intestinal tumors in control (*Myo5b^{lox/lox}; VilCreERT2^{-/-}*, Control) and *Myo5b* floxed (*Myo5b^{lox/lox}; VilCreERT2⁺*, *Myo5b* iKO-Low Tam) mice at the indicated time points after tamoxifen administration (40 mg/kg; i.p.). B-C) Myosin Vb (B) and Villin 1 (C) expression (immunohistochemistry) in the normal small intestinal epithelium (N) and tumors (T) of *Myo5b* inducible knockout mice carrying *Apc* mutation (*Apc^{min}; Myo5b^{lox/lox}; VilCreERT2⁺*, *Myo5b* iKO) four days after tamoxifen administration (160 mg/Kg; i.p.). D) Experimental design of the study of AOM-initiated intestinal tumorigenesis. Control animals (*Myo5b^{lox/lox}; CreERT2^{-/-}*, Control) and mice with inactivation of *Myo5b* in > 90% of the epithelial cells (*Myo5b^{lox/lox}; CreERT2⁺*, iKO-Low Tam) received 40 mg/kg of tamoxifen (i.p.) at 9 weeks of age and intestinal tumorigenesis was initiated by treatment with the carcinogen azoxymethane (AOM) 9 weeks later (12 weekly i.p. injections). Animals were sacrificed 13 weeks after the last AOM administration. E-F) Myosin Vb expression (immunohistochemistry) is shown in the normal small intestinal epithelium of control (E) and iKO-Low Tam (F) mice, as well as in a representative tumor in the small intestine of a iKO-Low Tam mouse (G). Scalebar: 10µm (B-C) and 50µm (E-G).

Supplementary Figure S9



Supplementary Figure S9. Myosin Vb expression in subcutaneous mouse xenografts. Expression of Myosin Vb (Immunohistochemistry) in control (mCherry, EV; **A** and **C**) and Myosin Vb (mCherry-Myosin Vb, MYO5B; **B** and **D**) in RKO (**A-B**) and SW837 (**C-D**) cells grown as subcutaneous tumors in NOD.SCID mice. Scale bar: 100 μ m.

Supplementary Figure S10



Supplementary Figure S10. Correlation of the expression of Myosin Vb at the mRNA and protein level. The expression of Myosin Vb in 96 colorectal tumors from the Clinical Proteomic Tumor Analysis Consortium (CPTAC) demonstrates a tight correlation between the mRNA (RNaseq) and protein (mass spectrometry) expression.