

## Original Article

# KLF5 mediates the hyper-proliferative phenotype of the intestinal epithelium in mice with intestine-specific endogenous K-Ras<sup>G12D</sup> expression

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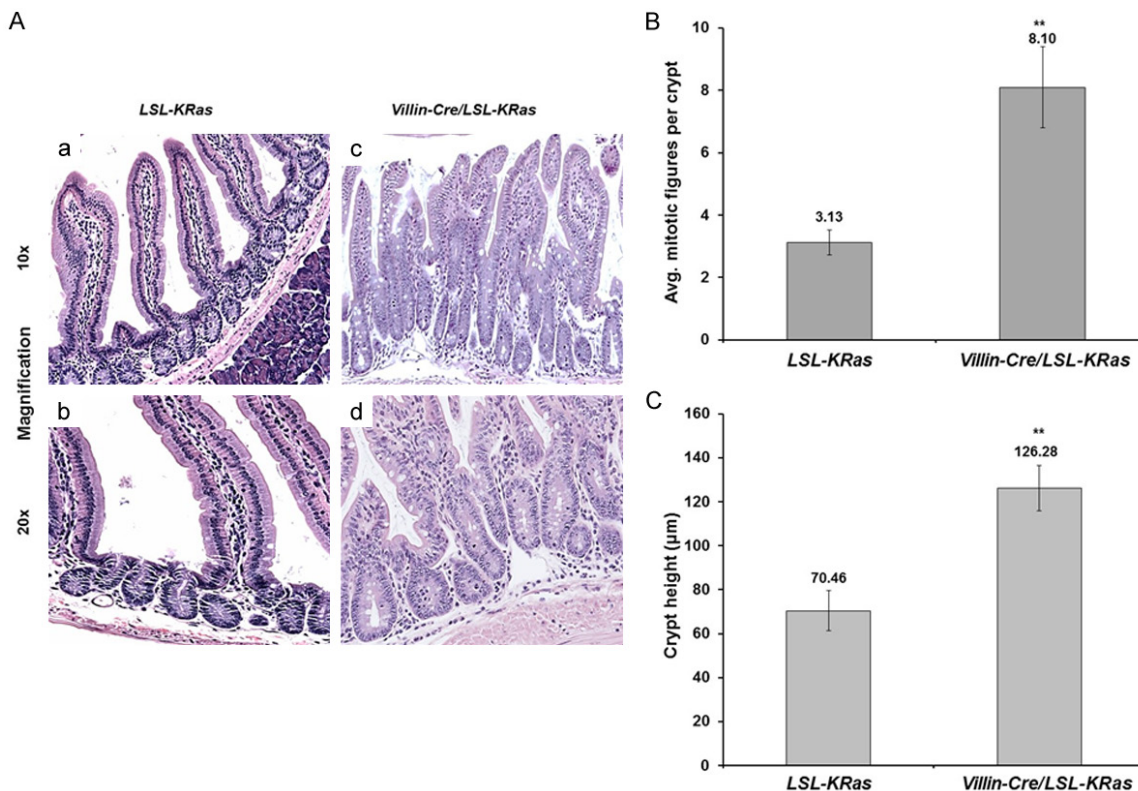
**Abstract:** Oncogenic *K-Ras* activation is a common mutational event in colorectal cancer. We previously showed that transcription factor, Krüppel-like factor 5 (KLF5), contributes to intestinal polyposis in mice with *K-Ras* activation. At 14 months of age, *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice developed small intestinal and colonic hyperplastic polyps while *LSL-K-Ras<sup>G12D</sup>* had none. The intestinal crypts of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice contained a higher number of mitotic figures and increased crypt heights compared to controls. The intestinal epithelium of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice showed prolific KLF5 expression throughout and above the elongated crypts. In contrast, KLF5 expression was limited to the upper crypt region in the controls. The levels of K-Ras effectors were significantly increased in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* as compared to controls. The *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice showed decreased survival upon treatment with azoxymethane (AOM) as compared to controls. Furthermore, loss of one of *Klf5* alleles reduced levels of K-Ras effector proteins and prevented mortality of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice upon AOM treatment. The *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice spontaneously develop hyperplastic intestinal polyps and display a hyper-proliferative intestinal phenotype with elongated crypts, increased numbers of mitotic figures, elevated expression of KLF5, and other pro-proliferative targets. Induction of colonic tumorigenesis with AOM is detrimental to *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice that is in part dependent of KLF5.

**Keywords:** KLF5, KRAS, intestinal hyper-proliferation, colonic tumorigenesis

## Introduction

Krüppel-like factor 5 (KLF5) is a pro-proliferative transcription factor highly expressed in the crypts of the intestinal epithelium [1]. In adults, KLF5 has a crucial role in regulating the rapidly dividing transient amplifying epithelial cells of the intestine [1]. Furthermore, KLF5 expression levels are increased in transformed cells and KLF5 was shown to be a target of oncogenic HRAS and KRAS [2-4]. Oncogenic mutations in the RAS gene have been shown to occur in 50% of colon cancer [5, 6] and are linked to formation of precancerous aberrant crypt foci. Previously, it was demonstrated that mice with intestine-specific over-expression of oncogenic KRAS (*Villin-KRAS<sup>V12G</sup>* mice; expressing KRAS<sup>V12G</sup> under the control of the intestine-specific villin promoter) develop intestinal adenomas by 9 months of age [7]. However, expression of oncogenic KRAS alone from its

endogenous promoter in mice up to one year of age is not sufficient to drive colon carcinogenesis as accompanying secondary mutations are necessary [8, 9]. We have previously shown that intestinal tumors derived from KRAS<sup>V12G</sup> mice and human primary colorectal cancers with mutated KRAS contain high levels of KLF5 [2, 10]. Conversely, genetic reduction of *Klf5* results in a significant reduction in intestinal tumor formation in mice harboring a germline mutation in the murine *Apc* gene (*Apc<sup>Min/+</sup>*) or combined *Apc<sup>Min/+</sup>* and KRAS<sup>V12G</sup> mutations [10, 11]. Importantly, KLF5 was shown to be both necessary and sufficient for the tumor initiating activity of  $\beta$ -catenin during intestinal adenoma formation in both *Apc<sup>Min/+</sup>* and *Apc<sup>Min/+</sup>/KRAS<sup>V12G</sup>* mice. In this study, we evaluated the role for KLF5 in mediating the long-term effects of oncogenic KRAS expression from the endogenous locus in the mouse intestinal epithelium.



**Figure 1.** Morphological assessment of small intestines tissues of *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. A. Histological staining of small intestines tissues from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Formalin-fixed, paraffin-embedded tissue sections of small intestines were stained with hematoxylin and eosin. (a and b) represent *LSL-K-Ras<sup>G12D</sup>*, (c and d) represent *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice, respectively. Images taken at 10x magnification (a and c) and 20x magnification (b and d). B. Average number of mitotic figures per crypt in *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Data represent mean  $\pm$  SEM (N=3), \*\*P<0.01. C. Crypt height (mm) in small intestinal tissues of *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Data represent mean  $\pm$  SEM (N=3), \*\*P<0.01.

**Materials and methods**

*Mouse strains and maintenance*

*LSL-K-Ras<sup>G12D</sup>* (*LSL*; for *LoxP-STOP-LoxP*) and *Villin-Cre* mice strains were purchased from The Jackson Laboratory. *LSL-K-Ras<sup>G12D</sup>* mice were bred into a C57BL/6 background through backcrossing for 7 generations. All animal studies were approved by Stony Brook University Institutional Animal Care and Use Committee (IACUC) and the methods were carried out in accordance with the relevant guidelines and regulations. *Villin-Cre* mice were crossed with *LSL-K-Ras<sup>G12D</sup>* mice to generate double heterozygous *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice with intestine-specific expression of *K-Ras<sup>G12D</sup>* from the endogenous promoter. Genotype analysis was performed as described previously [12]. Tail tips from mice were collected and processed using Red Extract-N-Amp kit according

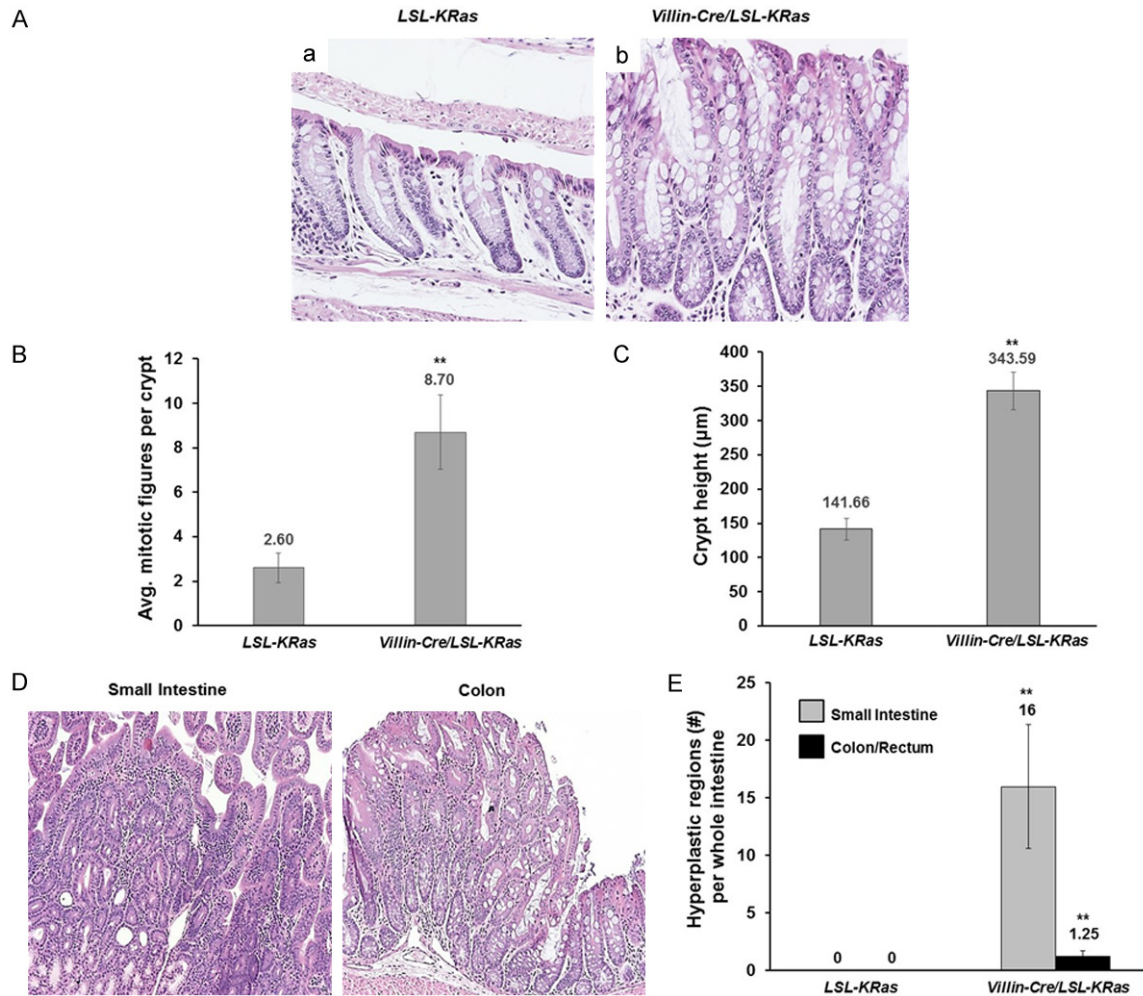
to manufacturer’s protocol (Sigma Aldrich). Allele-specific PCR analyses were performed using appropriate primers.

Azoxymethane (AOM) treatment. *LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fllox/+</sup>*, *Villin-Cre/Klf5<sup>fllox/+</sup>*, *Villin-Cre/LSL-K-Ras<sup>G12D</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fllox/+</sup>* mice were intraperitoneal injected (i.p.) with AOM (10 mg/kg), once a week for 4 consecutive weeks. Animals were observed for survival post treatment, and the small intestine and colon were removed for further analysis.

*Histology and immunohistochemistry*

Tissue collection and immunohistochemical analysis was performed as previously described [10]. Rabbit anti-phospho-MEK1 and anti-phospho-ERK1/2 antibodies (Cell Signaling Technology) were used at 1:100 dilution. Rabbit polyclonal antibody generated against amino

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**Figure 2.** Morphological assessment of colonic tissues of *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. A. Histological staining of small intestines tissues from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Formalin-fixed, paraffin-embedded tissue sections of small intestines were stained with hematoxylin and eosin. a represents *LSL-K-Ras<sup>G12D</sup>*, b represents *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Images taken at 10x magnification. B. Average number of mitotic figures per colonic crypt in *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Data represent mean  $\pm$  SEM (N=3), \*\*P<0.01. C. Colonic crypt height (mm) in intestinal tissues of *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Data represent mean  $\pm$  SEM (N=3), \*\*P<0.01. D. Representative images of hyperplastic regions of small intestine and colonic tissues of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. E. Quantitation of hyperplastic regions in small intestine and colon/rectum of *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. Data represent mean  $\pm$  SEM (N=3), \*\*P<0.01.

acid positions 95-111 of the KLF5 protein (SDIX/Origene) were used at 1:150 dilution. Mouse monoclonal antibody anti-whole- $\beta$ -catenin (Life Technologies) was used at 1:1,000 dilution. Rabbit monoclonal antibody anti-cyclin D1 (Biocare Medical) was used at 1:200 dilution. Rabbit polyclonal antibody against MKI67 (Leica Microsystems) was used at 1:400 dilution. Mouse monoclonal antibody against phosphorylated  $\beta$ -catenin Ser552 (Cell Signaling) was used at 1:200 dilution.

### Western blot analyses

Western Blot analyses were performed as described previously [3, 10]. Rabbit polyclonal antibody anti-KLF5 (SDIX/Origene) was used at 1:2,000. Rabbit anti-phospho-MEK1 and anti-phospho-ERK1/2 antibodies (Cell Signaling Technology) were used at 1:1,000 dilution. Mouse monoclonal antibody anti- $\beta$ -actin (Sigma-Aldrich) was used at 1:5,000 dilution.



### Statistical analysis

All statistical analysis was performed using Student's T- test method and Mantel-Cox test with Graph Pad Prism 5 Software.

### Data availability

All data generated or analyzed during this study are included in this published article.

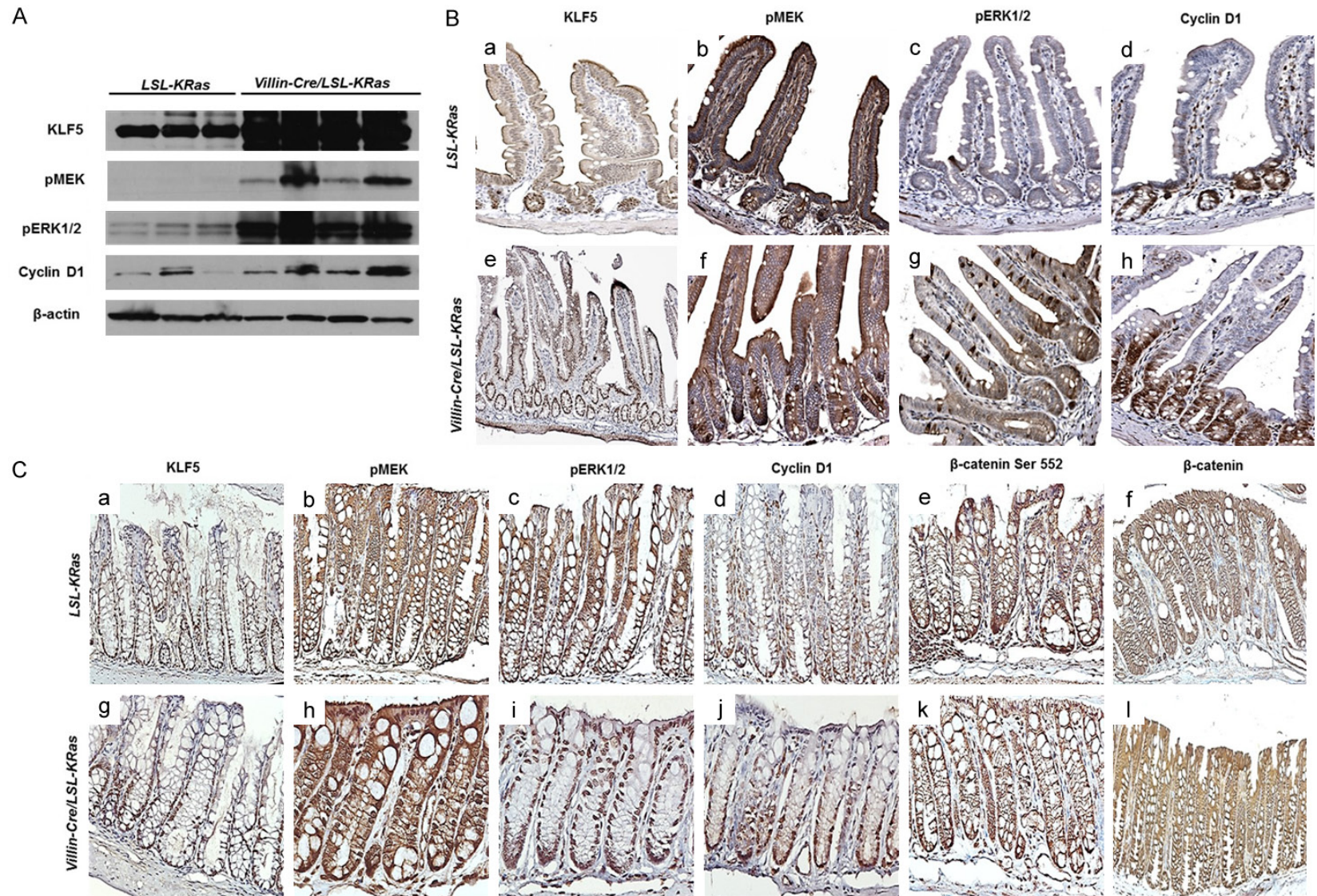
### Results

*Villin-Cre* mice [13] were crossed with *LSL-K-Ras<sup>G12D</sup>* (*LSL; for LoxP-STOP-LoxP*) mice [14] to generate double heterozygous *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice with intestine-specific expression of *K-Ras<sup>G12D</sup>* from the endogenous locus. These double heterozygous mice, along with age-matched control *LSL-K-Ras<sup>G12D</sup>* mice, were sacrificed at 14 months of age. *K-Ras<sup>G12D</sup>* mutation affects the architecture of the intestinal epithelium. As shown in the **Figures 1A and 2A**, numerous hyper-proliferative regions were observed in the small intestine and colon, respectively, of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>*, but not in the control mice (*LSL-K-Ras<sup>G12D</sup>*). In the small intestine, the number of mitotic figures per crypt (**Figure 1B**) and the crypt height (**Figure 1C**) were significantly higher in the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice when compared to control. Similar findings were observed for those in the colon (**Figure 2B and 2C**). Additionally, the small intestine and colon of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice contained many hyperplastic polyps that were absent from control mice (**Figure 2D and 2E**). We did not observe the development of dysplasia or neoplasia in either the small intestine or colon of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice by 14 months of age. These results are consistent with previous observations indicating that endogenous *K-Ras* mutations alone are not sufficient to initiate carcinogenesis [8, 9, 15]. Previous studies using *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice were mainly focused on the role of mutated *KRAS* within colonic tissue [8, 9]. These reports confirm higher levels of MEK activity in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice in proximal colon [8] and in colonic epithelium [9]. In our studies, we assessed the levels of *K-Ras* effector proteins, pMEK, pERK1/2, and cyclin D1 in normal-appearing small intestinal mucosa of the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* and control mice (**Figure 3A and 3B**). We observed elevated levels of pMEK, pERK1/2, and cyclin D1 in the small

intestinal epithelium in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* as compared to control mice by western blotting and immunohistochemistry (**Figure 3A and 3B**, respectively). It has been previously established that KLF5 is a downstream effector of MAPK pathway [3, 4, 10, 16, 17]. Furthermore, we showed increased levels of KLF5 protein in the tissues from human colorectal cancer and in tumors developed in mice harboring *KRAS<sup>V12G</sup>* mutation [10]. In the current study, KLF5 protein levels were elevated in the small intestinal epithelium in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* as compared to control mice by western blotting (**Figure 3A**) and in the normal-appearing small intestinal mucosa from *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice compared to controls (data not shown). Moreover, we observed prolific KLF5 expression not only along and above the elongated crypts marking the normal intestinal epithelia but also in the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* hyperplastic polyp sections (**Figure 3Be**). In contrast, KLF5 expression was limited to around the crypt-villus junction in the controls mice (**Figure 3Ba**). The levels of cyclin D1 (**Figure 3Bd and 3Bh**) and MKI67 (data not shown) were also significantly increased in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* intestinal tissues compared to controls. The analysis of effectors of *K-Ras* signaling pathway in the colon showed similar results as in small intestinal epithelium with increased expression levels of pMEK and pERK1/2 in colonic tissues of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice as compare to control (**Figure 3Cb, 3Cc, 3Ch and 3Ci**). We also observed increased levels of KLF5 and cyclin D1 in the colonic tissues of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* in comparison to control (**Figure 3Ca, 3Cd, 3Cg and 3Cj**). There was no appreciable difference in the levels of protein of either total (**Figure 3Cf and 3Ci**) or activated  $\beta$ -catenin (**Figure 3Ce and 3Ck**) between *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice and controls.

To address the role of KLF5 in the background of mutated *KRAS* in colonic tumorigenesis, we utilized established animal model for sporadic colon cancer development using azoxymethane (AOM) as a carcinogen [18]. *LSL-K-Ras<sup>G12D</sup>*, *Villin-Cre/Klf5<sup>fllox/+</sup>*, *Villin-Cre/LSL-K-Ras<sup>G12D</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fllox/+</sup>* mice were injected with AOM as described in Methods (**Figure 4A**). We showed that *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice treated with AOM exhibited decreased survival as compared to

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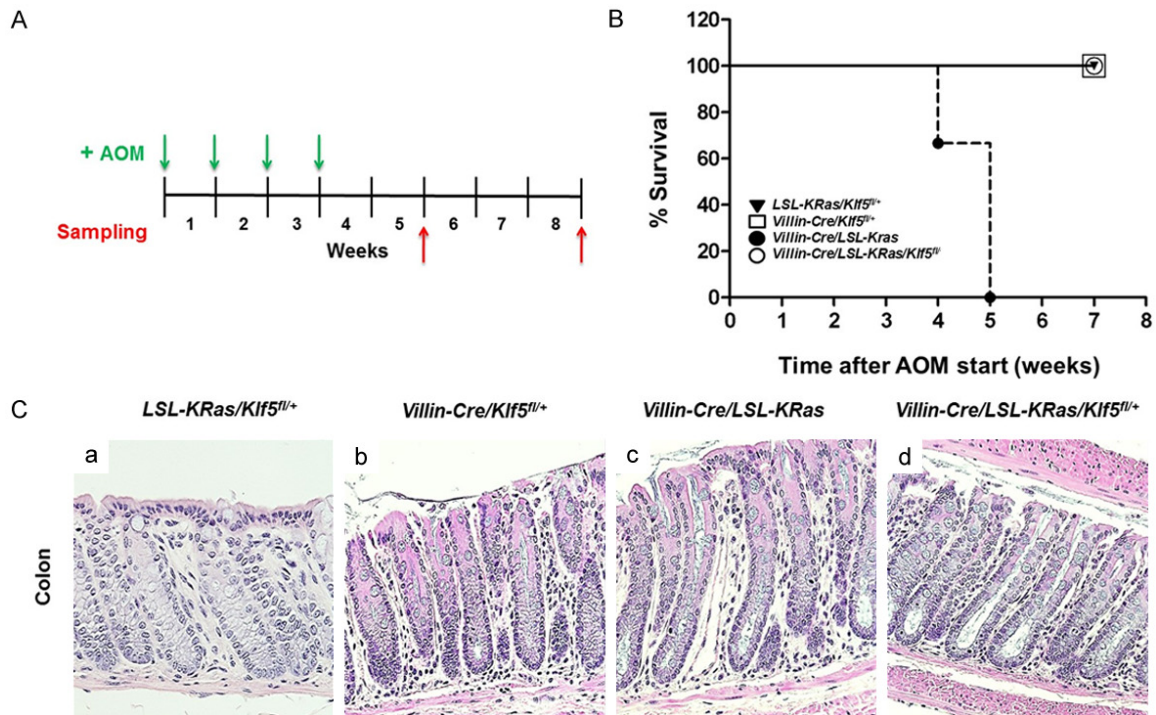


**Figure 3.** Upregulation of downstream targets of ERK pathway. **A.** Western Blot analysis of protein extract from normal appearing mucosa obtained from small intestines tissues from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice. 10 ug of total protein extracts from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* were analyzed by Western blotting using antibody against KLF5, pMEK, pERK1/2, cyclin D1 and β-actin (as a loading control). The images are shown separately as they were obtained from different gels or different parts of the same gel. **B.** Small intestinal tissues from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice were stained with antibody



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against KLF5 (a, e), pMEK (b, f), pERK1/2 (c, g) and cyclin D1 (d, h). C. Colonic tissues from *LSL-K-Ras<sup>G12D</sup>* and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice were stained with antibody against KLF5 (a, g), pMEK (b, h), pERK1/2 (c, i), cyclin D1 (d, j),  $\beta$ -catenin Ser552 (e, k), and whole  $\beta$ -catenin (f, l).



**Figure 4.** KLF5 increases survival of *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* in AOM-induced carcinogenesis murine model. A. Design of experiment: *LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>*, *Villin-Cre/Klf5<sup>fl/+</sup>*, *Villin-Cre/LSL-K-Ras<sup>G12D</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* mice were injected once a week for four weeks with AOM and tissues were collected 2 and 4 weeks after last AOM injection. B. Kaplan-Meier survival curves of *LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* (N=4), *Villin-Cre/Klf5<sup>fl/+</sup>* (N=4), *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* (N=3), and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* (N=4) mice after injections with AOM, P=0.0014 using Mantel-Cox test. C. H&E staining of colonic tissues from *LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* (a), *Villin-Cre/Klf5<sup>fl/+</sup>* (b), *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* (c), and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* (d) mice after AOM treatment.

the *LSL-K-Ras<sup>G12D</sup>*, *Villin-Cre/Klf5<sup>fl/ox/+</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/ox/+</sup>* mice, which did not exhibit any mortality at the end point of the experiment (Figure 4B, control mice are depicted as one lane;  $p$  value =0.0014 by Mantel-Cox test). The morphology of intestinal tissues obtained from the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice showed signs of necrosis and ischemia with sporadic regions of normal mucosa (data not shown). The H&E analysis of the normal mucosa of colonic tissues shows increased height of the crypts of the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice as compared to the *LSL-K-Ras<sup>G12D</sup>*, *Villin-Cre/Klf5<sup>fl/ox/+</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/ox/+</sup>* mice (Figure 4C). Our data suggest that loss of one allele of *Klf5* gene in the context of *K-Ras<sup>G12D</sup>* mutation is sufficient to neutralize oncogenic KRAS function upon carcinogen treatment. To further analyze the

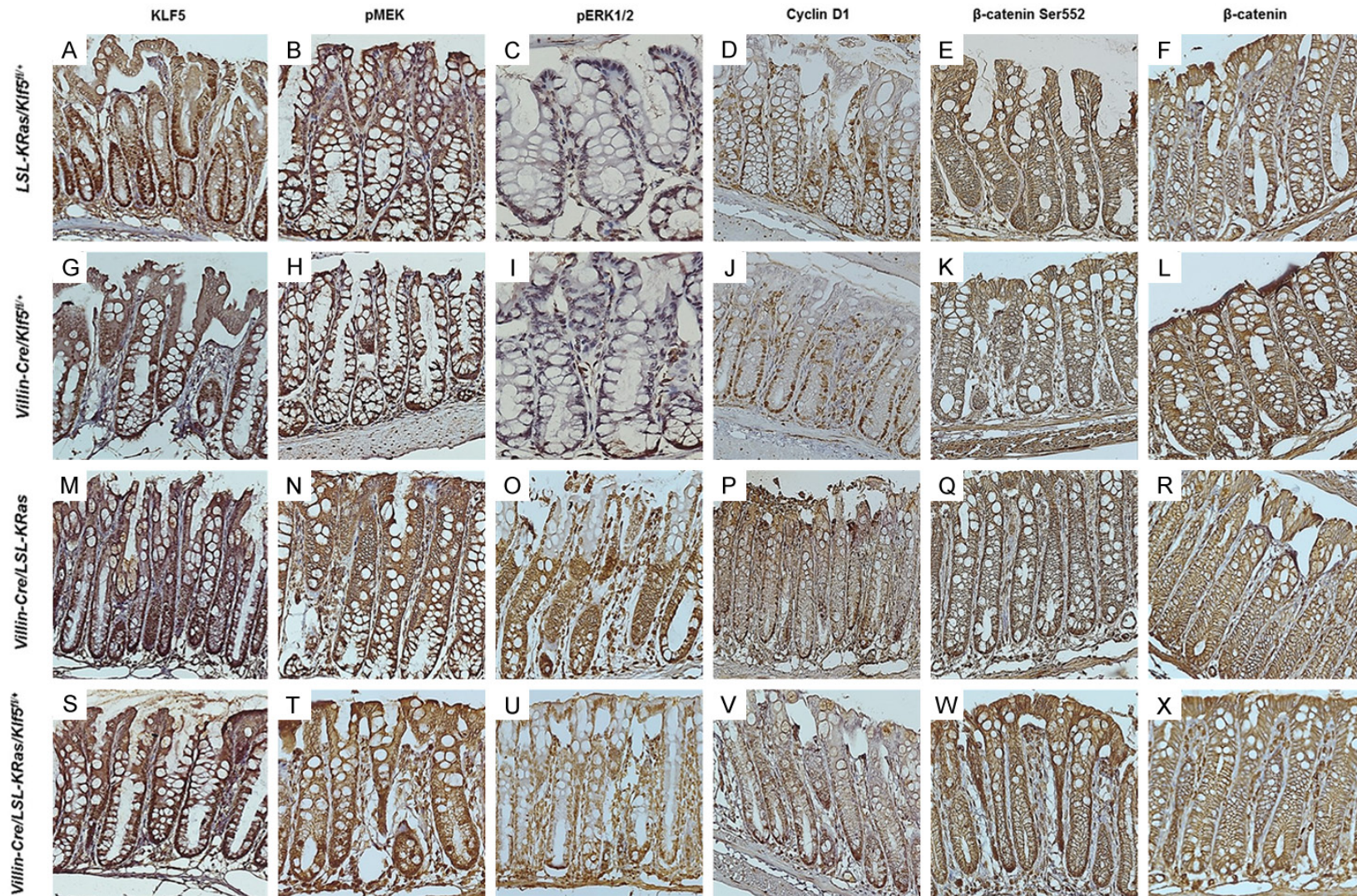
expression levels of components of KRAS and WNT signaling pathways we performed immunohistochemical staining of colonic tissues collected upon completion of the treatment. We show elevated levels of KLF5, pMEK, pERK1/2, and cyclin D1 in the colonic epithelium in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* as compared to the *LSL-K-Ras<sup>G12D</sup>*, *Villin-Cre/Klf5<sup>fl/ox/+</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/ox/+</sup>* mice (Figure 5). There was no significant difference in the expression levels of total and active  $\beta$ -catenin between analyzed mice (Figure 5).

### Discussion

Our studies indicate that *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice spontaneously develop hyperplastic intestinal polyps at a mature age. This is in concordance with previous observations that



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**Figure 5.** Immunohistochemical staining of effector of ERK and Wnt signaling pathways. Colonic tissues of *LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>*, *Villin-Cre/Klf5<sup>fl/+</sup>*, *Villin-Cre/LSL-K-Ras<sup>G12D</sup>*, and *Villin-Cre/LSL-K-Ras<sup>G12D</sup>/Klf5<sup>fl/+</sup>* mice after injections with AOM were stained for KLF5 (A, G, M, and S), pMEK (B, H, N, and T), pERK1/2 (C, I, O, and U), cyclin D1 (D, J, P, and V), β-catenin Ser552 (E, K, Q, and W), and whole β-catenin (F, L, R, and X), respectively.



*Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice developed hyper-proliferative regions within small intestinal epithelium and colon [9, 15, 19]. This phenotype is characterized by an increase in the number of mitotic figures, an increase in crypt heights and formation of hyperplastic polyps. Other studies, using different regulatory elements to drive *K-Ras* expression demonstrated no alteration of small intestinal epithelium during homeostasis [20, 21]. Analysis of crypt height, mitotic index and apoptosis levels and the levels of cyclin D1 and  $\beta$ -catenin showed no significant differences [20]. On other hand, Janssen et al demonstrated that activation of *KRAS* in the intestinal epithelium leads formation of intestinal tumors and distress [7]. This murine model was characterized by increased levels of phosphorylated ERK1/2 [7]. In our model, alterations to the morphology of intestinal epithelium are concurrent with the activation of pMEK and pERK signaling, and increased levels of cyclin D1 and MKI67, however these did not induce formation of dysplasia or neoplasia. A similar observation regarding activation of MAPK signaling pathway and higher mitotic index was reported by Haigis et al [9]. Their studies confirmed our observation of a hyper-proliferative state within colonic tissues upon *KRAS* activation [9, 15]. Bennecke et al showed that activation of oncogenic *K-ras* induces serrated hyperplasia and is characterized by p16ink4a overexpression and induction of senescence [22]. They reported increased mitotic index and phosphorylation of ERK in the small intestinal epithelium but not in colon [22]. We showed that the activation of *KRAS* from the endogenous regulatory elements does not affect WNT signaling effector,  $\beta$ -catenin. Thus, the increased levels of KLF5 observed in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice in normal appearing mucosa and in the hyperplastic polyps are the direct results of activation of the *KRAS* pathway. Previous reports showed accelerated tumorigenesis in mice with activation of *KRAS* with simultaneous loss of *Apc* that led to decrease of survival [20]. Additionally, the authors observed dysregulation of *KRAS* pathway manifested by increased levels of pMEK and sporadic nuclear staining of pERK within adenomas [20]. Using a different tumorigenesis model, we showed that *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice are more susceptible to the treatment with AOM in comparison to the controls mice, and they succumbed within one week after the last treatment. This suggest that the hyper-proliferative and hyper-plastic pheno-

type observed during the homeostasis in *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice in the colonic tissue is further exuberated by carcinogen treatment that lead to animal death. In contrast to our data, previous report showed that AOM-treated mice with intestinal activation of *KRAS* developed colonic tumors but did not die due to AOM treatment [22]. However, Bennecke and colleagues did not show any morphological changes to the colonic epithelium during homeostasis, contrary to our data. Importantly, in our model reduction in the expression levels of *Klf5* by fifty percent rescues animals with *KRAS* activation after carcinogen treatment. The reduction in KLF5 contributes to downregulation of pMEK and pERK activity without any impact on  $\beta$ -catenin levels of activity.

In summary, our data indicate that KLF5 is a major mediator of *KRAS* pathway during sporadic carcinogenesis and that the *Villin-Cre/LSL-K-Ras<sup>G12D</sup>* mice can serve as a model for spontaneous intestinal polyposis and inducible colon tumorigenesis driven by oncogenic *K-Ras* from its endogenous locus.

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### Disclosure of conflict of interest

None.

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