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Targeted deletion of *Kif18a* protects from colitis-associated colorectal (CAC) tumors in mice through impairing Akt phosphorylation

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ABSTRACT

Kinesins are a superfamily of molecular motors involved in cell division or intracellular transport. They are becoming important targets for chemotherapeutic intervention of cancer due to their crucial role in mitosis. Here, we demonstrate that the kinesin-8 Kif18a is overexpressed in murine CAC and is a crucial promoter during early CAC carcinogenesis. *Kif18a*-deficient mice are evidently protected from AOM–DSS-induced colon carcinogenesis. Kif18A is responsible for proliferation of colonic tumor cells, while Kif18a ablation in mice promotes cell apoptosis. Mechanistically, Kif18a is responsible for induction of Akt phosphorylation, which is known to be associated with cell survival regulation. In conclusion, Kif18a is critical for colorectal carcinogenesis in the setting of inflammation by mechanisms of increased PI3K-AKT signaling. Inhibition of Kif18A activity may be useful in the prevention or chemotherapeutic intervention of CAC.

1. Introduction

The mitotic spindle is a validated target in cancer chemotherapy and a variety of anti-mitotic drugs, such as taxanes and vinca alkaloids, have been successfully used in the clinic [1,2]. However, these spindle poisons have certain limitations because tumor cells may become resistant to these drugs through tubulin mutations, overexpression of drug efflux pumps, or altered expression of tubulin subtypes [1,3]. Thus, there is a significant effort to find other ways of targeting the mitotic spindle, which could potentially overcome some of the toxicities and mechanisms of resistance. In recent years, the kinesin family of motor proteins has gained significant attention, being crucial for mitosis and thus emerging as a target for chemotherapeutic intervention [4–6]. Kinesins are microtubule-based motor proteins that function at different stages of cell division, intracellular vesicle and organelle transport, and the movement of microtubules [7–9]. Because of their critical cellular functions in mitosis, several kinesins have been implicated in tumorigenesis [10–14], including kinesin-8 Kif18A [15,16].

Kif18A is a member of the kinesin-8 family, has been described to be a molecular motor protein that uses adenosine triphosphate (ATP) hydrolysis to produce force and movement along microtubules [17,18], and plays a crucial role in regulating microtubule dynamics and cell division in eukaryotes [19–26]. During mitosis, Kif18A is concentrated at the plus ends of microtubules, facilitating microtubule depolymerization. This is essential in the accurate alignment of the spindle equator [21]. Kif18A reduces the amplitude of preanaphase oscillations and negatively controls the movement of chromosomes towards the spindle poles during anaphase [23].

Given the fact that Kif18A plays critical roles in modulating microtubule dynamics and mitosis, perturbation of Kif18A function might cause cell transformation and carcinogenesis. In fact, Kif18A was reported to be involved in human breast carcinogenesis and correlated with clinical relevance to colorectal cancer progression [15,16]. In addition, proteomic analysis identified Kif18A as a potential biomarker of cholangiocarcinoma and lung cancer [27,28]. However, there is essentially no information about Kif18A involving carcinogenesis *in vivo*. In this study, we evaluate carcinogenic ability of Kif18A using *Kif18a* knock-out mice. Our results show that *Kif18a* deficiency protects mice from colitis-associated colorectal (CAC) cancers, demonstrating the significance of Kif18A in CAC progression and its functional role *in vivo*.

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2. Materials and methods

2.1. Animals and tumor induction

The *Kif18a*-deficient (*Kif18a*$^{-/-}$) mice had been reported previously [29]. Mice were kept in specific-pathogen free conditions and fed by free access to a standard diet and water. All experiments were approved by the Animal Ethics Committee of Rui-Jin Hospital affiliated to Shanghai Jiao Tong University School of Medicine.

Following previously established methods for inducing colonic neoplasia [30–32], 8- to 10-week-old sex-matched mice were injected intraperitoneally (i.p.) with 10 mg/kg of azoxymethane (AOM; Sigma) at the beginning of the experiment (day 0). After 1 week, mice were treated with 2.5% dextran sodium sulfate (DSS; MP Biomedicals, molecular weight 35,000–50,000 kDa) *ad libitum* in their drinking water for 1 week. After this, mice were maintained on regular water for 2 weeks and subjected to two more DSS treatment cycles. During the DSS treatment and recovery phase the body weights, stool consistency, and stool blood were monitored. At week 10, mice were injected i.p. with 120 mg/kg 5-bromo-2-deoxyuridine (BrdU; Roche) and sacrificed 90 min later. Colons were removed, opened longitudinally, and macroscopic tumors were counted and measured with a caliper. Then, the distal colons were fixed in 10% neutral buffered formalin for 24 h and transferred to 70% ethanol for subsequent paraffin embedding and histological analysis.

2.2. Histological analysis

The sections were stained with hematoxylin-and-eosin. Histologic assessment was performed in a double-blind fashion. The severity scores of mucosal inflammation were determined as follows [33]: 0, normal morphology; 1, focal inflammatory cell infiltrate around the crypt base; 2, diffuse infiltration of inflammatory cells around the crypts or erosion/destruction of the lower one-third of the glands; 3, erosion/destruction of the lower two-thirds of the glands or loss of all the glands but with the surface epithelium remaining; and 4, loss of all the glands and epithelium. Dysplasia was scored as follows [34]: 0 = no dysplasia, 1 = mild dysplasia characterized by aberrant crypt foci (ACF), +.5 for small gastrointestinal neoplasia (GIN) or multiple ACF, 2 = moderate dysplasia with GIN, +.5 for multiple occurences or small adenoma, 3 = severe or high grade dysplasia restricted to the mucosa, 3.5 = adenocarcinoma, invasion through the muscularis mucosa, 4 = adenocarcinoma, full invasion through the submucosa and into or through the muscularis propria.

2.3. Immunofluorescent and immunohistochemical studies

Paraffin-embedded slides were deparaffinized. Antigen unmasking was carried out by incubation in 92–98 °C water bath in 10 mM sodium citrate buffer for 30 min. Slides were incubated with indicated primary antibodies in PBS containing 1% BSA and 10% goat serum overnight at 4 °C. Biotinylated or FITC-conjugated

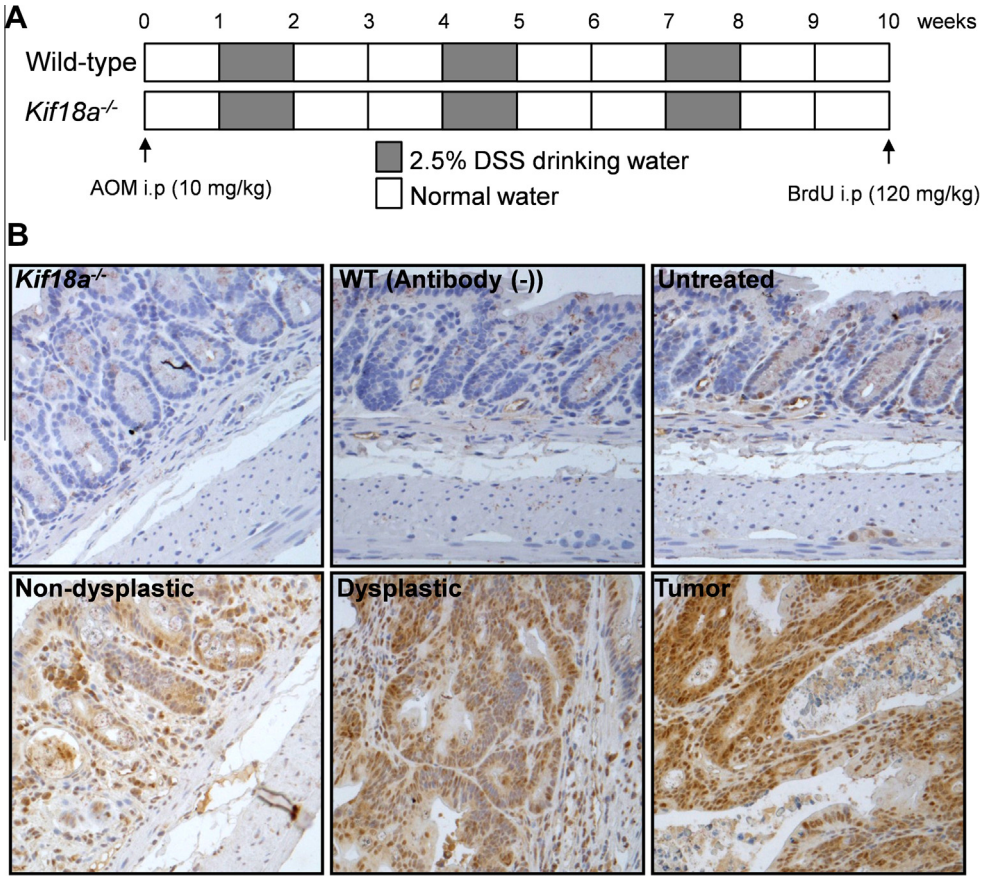

Fig. 1. Kif18a expression is up-regulated in mouse CAC tumors. (A) Study design. Wild-type mice (n = 11) and *Kif18a*$^{-/-}$ littermates (n = 11) (129/Sv background) were treated with AOM and DSS. All mice were sacrificed 10 weeks after the study commenced. (B) Immunohistochemical staining for Kif18a in the WT mouse CAC model. The untreated normal, non-dysplastic, dysplastic and tumor mucosa are shown. Both sections from WT mice without adding primary antibody and *Kif18a*$^{-/-}$ mice with standard IHC staining are used as negative controls. Magnification, 200×.

secondary antibodies (BD Pharmingen) were added and incubated at room temperature for 1 h. For IHC, Streptavidin-HRP (BD Pharmingen) was added, and after 40 min the sections were stained with DAB substrate and counterstained with hematoxylin. For IF, the slides were incubated with DAPI (Sigma). Primary antibodies used were as follows: anti-Kif18a (Proteintech), anti-Akt (Cell Signaling), anti-pAkt (Ser473; Cell Signaling), anti-Caspase 3 (Abnova), anti-BrdU (Thermo) and anti-PCNA (Santa Cruz).

2.4. In situ TUNEL analysis

The *in situ* TUNEL analysis was performed using *In Situ* Cell Death Detection Kit (Fluorescein; Roche) according to the manufacturer's instructions. In brief, slides were dewaxed and rehydrated according to standard protocols, incubated in 10 mM sodium citrate buffer in 92–98 °C water bath for 30 min, and then immersed in 0.1 M Tris–HCl (pH 7.5) containing 3% BSA and 20% normal bovine serum for 30 min. Finally, 50 μl of TUNEL reaction mixture was added to the sections. The sections were then incubated for 60 min at 37 °C in a humidified atmosphere in the dark, and were then evaluated under a fluorescence microscope.

2.5. Statistical analysis

All quantitative data in this study were presented as mean ± SD. A two-tailed Student's *t-test* was used to analyze comparisons between two groups. Analysis of survival was performed using the Kaplan–Meier method and compared using log-rank test. $p < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Kif18a expression is up-regulated in CAC tumors

Kif18A is a molecular motor protein using ATP hydrolysis to produce force and movement along microtubules [17,18], and controls the accurate alignment of the spindle equator, thus playing a crucial role in cell division [21]. To examine the role of Kif18a in colitis-associated tumorigenesis, we treated WT and *Kif18a*$^{-/-}$ mice with AOM and DSS as described in Section 2 (Fig. 1A). We hypothesized that Kif18a expression would be increased in colorectal neoplasia in the induced CAC model. Colon specimens from WT mice with or without AOM–DSS treatment were examined for Kif18a expression by immunohistochemical staining (Fig. 1B). The results showed that untreated normal colon had low expression of Kif18a protein, whereas samples from tumor-load mice showed a higher expression of Kif18a both in epithelial and mesenchymal cells (Fig. 1B). Additionally, the expression level of Kif18a showed a strong correlation with dysplastic severity, which is supported by much higher Kif18a levels in tumors (Fig. 1B), indicating a large demand for Kif18a in the tumor cells which are highly proliferating.

3.2. Kif18a promotes development of colorectal tumors in the setting of chronic colitis

Given the up-regulated expression of Kif18a in CAC tumors (Fig. 1B) and its critical role in mitosis [21], we next addressed

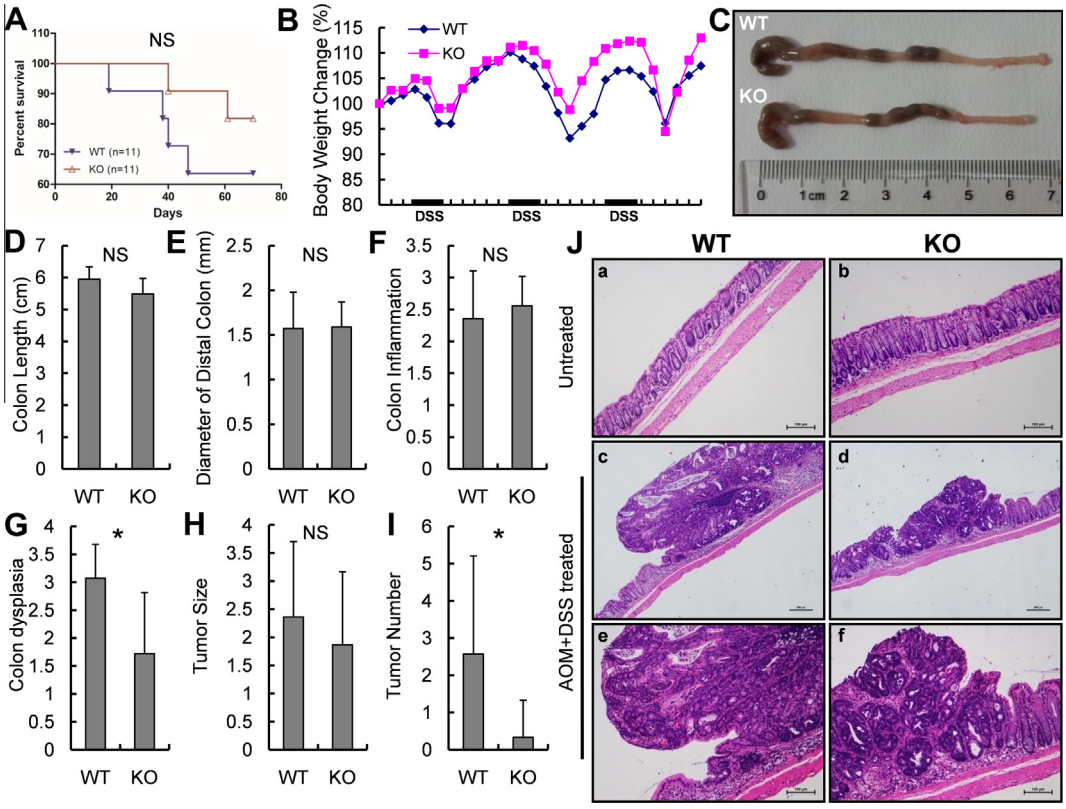

Fig. 2. *Kif18a*$^{-/-}$ mice are protected from CAC tumors in the setting of inflammation. (A) Survival curve of AOM–DSS-treated WT or KO mice during the study period. (B) Body weight changes of all mice are examined during the study period. The values are expressed as a percentage of body weight on day 0. (C–E) Colon length and diameter of WT and KO mice are shown. (F) Colitis severity score is determined by specialist in a double-blind fashion. (G) Dysplastic scores of all mice. (H) Visible tumor sizes are determined using caliper. (I) Incidence of visible dysplasia. The number of dysplastic lesions is counted per mouse. (J) Tumors in the CAC model. Untreated normal mucosal surfaces are shown (a and b). Microscopically, AOM–DSS-treated WT tumors are shown at lower (c) and higher (e) magnifications, and KO dysplasias are shown (d and f), too. Scale bar, (a, b, e and f) 100 μm, (c and d) 200 μm. Data from (D) to (I) are expressed as mean ± SD, WT ($n = 7$), KO ($n = 9$), NS means non significant, ∗ indicate significant differences between groups ($p < 0.05$).

Table 1
Incidence and size of polyps.

	WT ($n = 11$)	*Kif18a*$^{-/-}$ ($n = 11$)	p value
Survival (%)	7 (63.6)	9 (81.8)	0.292
Incidence of survival (macroscopic, %)	5 (71.4)	1 (11.1)	**0.0167**
Incidence of survival (microscopic, %)	7 (100)	4 (44.4)	**0.0213**
Tumors/animal (range)	2.6 ± 2.6 (0–6)	0.3 ± 1 (0–3)	**0.0335**
Tumor size, mm (range)	2.4 ± 1.3 (0.4–5.1)	1.9 ± 1.3 (0.6–3.2)	0.559

Bold, $p < 0.05$, statistically significant.

whether the absence of Kif18a altered the susceptibility to develop CAC in mice. In the experimental procedure, WT mice showed earlier and more mortality than *Kif18a*$^{-/-}$ mice (Fig. 2A), but the difference was not statistically significant ($p = 0.29$). Additionally, *Kif18a*$^{-/-}$ mice showed comparable weight change (Fig. 2B), stool consistency, and stool blood (data not shown) compared with WT mice. At week 10, all mice were sacrificed and the colon samples were removed, measured and fixed in 10% neutral buffered formalin for subsequent paraffin embedding and histological analysis. Consistently, the length and diameter of colons had no significant difference between WT and *Kif18a*$^{-/-}$ mice (Fig. 2C–E). Furthermore, to evaluate the degree of induced colitis and dysplasia, the colonic sections stained with H&E were scored by a specialist in a double-blind fashion according to the criteria reported previously [33,34]. We found that the WT mice load more severe dysplasias because of their higher histological dysplastic scores compared with *Kif18a*$^{-/-}$ mice, while the inflammatory scores were comparable (Fig. 2F and G). Colitis is a critical promoter of CAC progression. Now, in the nearly equal inflammatory microenvironment, we found a striking difference between WT and *Kif18a*$^{-/-}$ mice with respect to development of dysplasia and tumors. 5 of 7 WT mice (71.4%) grossly showed multiple polypoid lesions, but such visible lesions only were seen in 1 *Kif18a*$^{-/-}$ mouse (11.1%). When examined microscopically, all survived WT mice ($n = 7$) had at least 1 dysplastic lesion, and in contrast, only 4 of 9 survived *Kif18a*$^{-/-}$ mice (44.4%) had dysplastic lesions (Table 1). A significant increase in the number of dysplastic lesions was observed between WT and *Kif18a*$^{-/-}$ mice ($p = 0.0335$), whereas the size of dysplasia was comparable ($p = 0.559$) (Fig. 2H and I, Table 1). Furthermore, the histopathological H&E staining showed that most of AOM–DSS treated WT mice bear severe adenocarcinoma compared with untreated mice, but the *Kif18a*$^{-/-}$ mice just developed various degrees of dysplasia (Fig. 2J). We could find that the high differentiated adenocarcinoma with classic cribriform structure invaded to the submucosal layer in the WT mice model. But the dysplastic lesions found in KO mice were small, flat, and low differentiation (Fig. 2Jc–f). These results demonstrate Kif18a is a key promoter in CAC tumor progression.

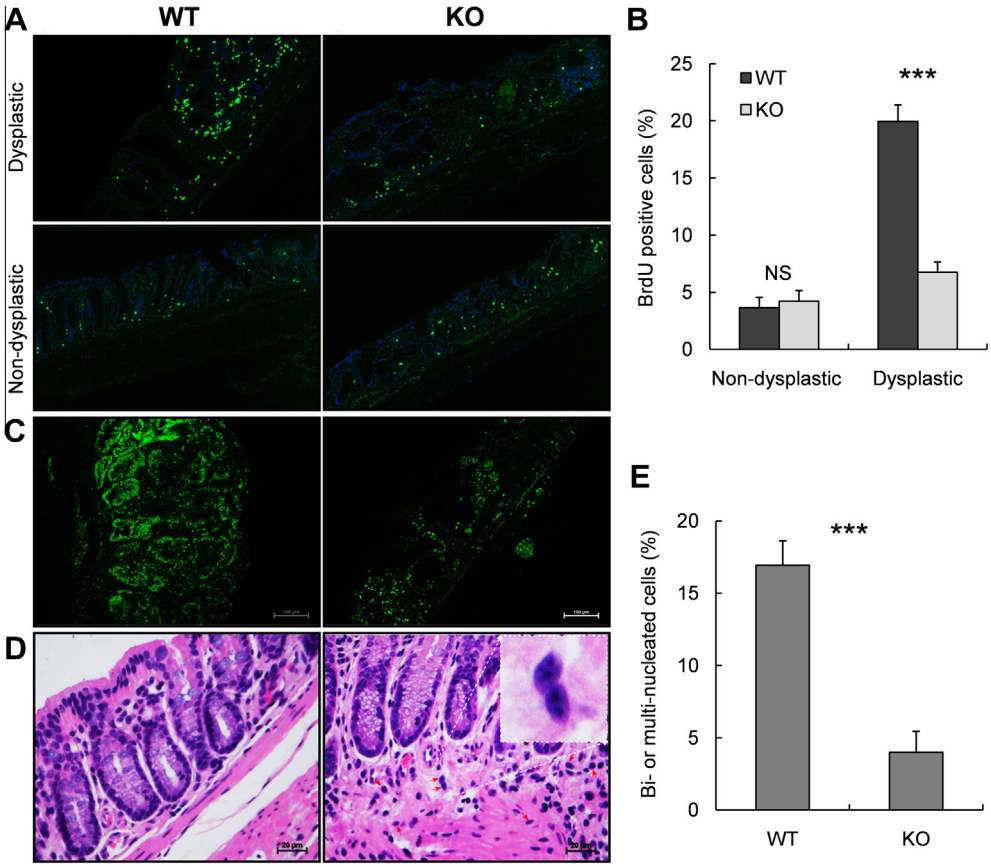

Fig. 3. Kif18a is required for colonic tumor cells proliferation. (A) Proliferation in the CAC model. BrdU incorporation is assessed using anti-BrdU antibody staining (green). DAPI (blue) indicates nucleus. Representative pictures are taken from WT and KO dysplastic lesion and nondysplastic mucosa, respectively. Magnification, 100×. (B) BrdU positive cells quantitation. Three visions of each mouse are calculated. (C) Immunofluorescent staining for PCNA. Representative pictures are taken from WT and KO dysplastic lesion. Magnification, 100×. (D) H&E-stained WT and KO colon mucosa. Red arrows denote cells with multiple- or bi-nuclei. Magnification, 400×. (E) Bi- or multi-nucleated cells quantitation. Three visions of each mouse are calculated. Data from (B) and (E) are expressed as mean ± SD, NS means non significant, *** indicate significant differences between groups ($p < 0.001$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

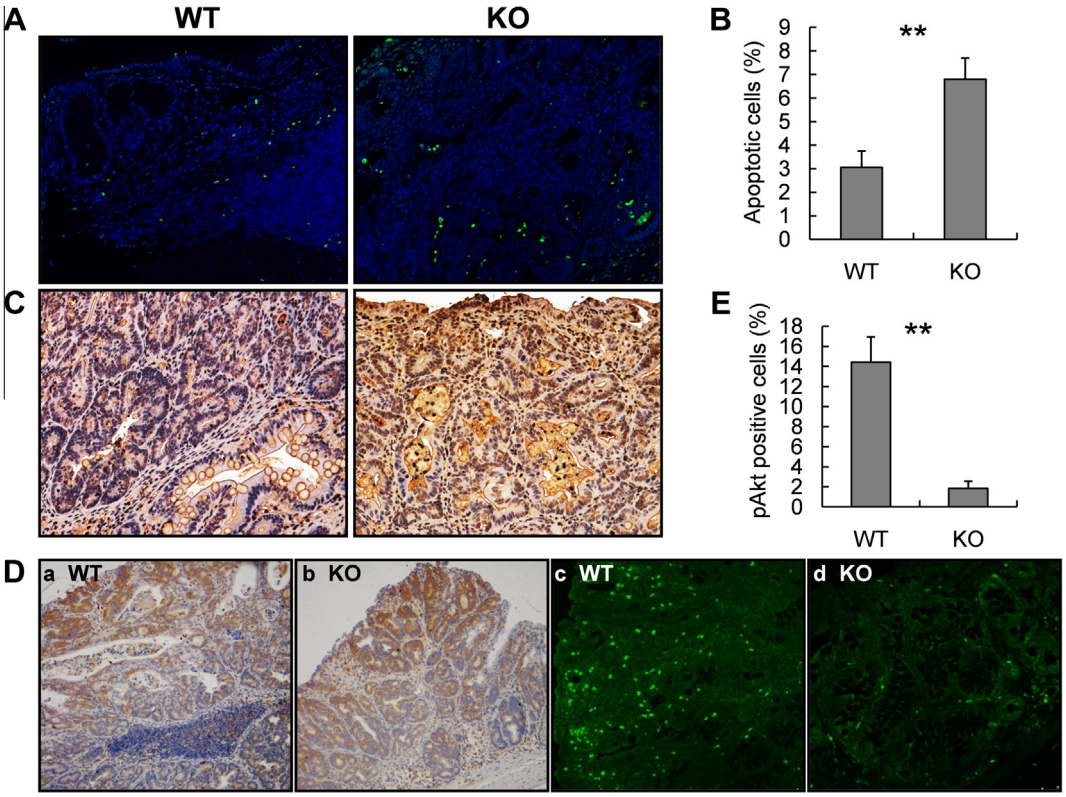

Fig. 4. Kif18a deficiency induces tumor cells apoptosis via inactivation of Akt kinase. (A) Sections of WT and KO colons are subjected to *in situ* TUNEL analysis, and representative images are shown. Green denotes positive spots and clusters. DAPI (blue) indicates nucleus. Magnification, 200×. (B) Percentage of apoptotic (TUNEL positive) cells. Three visions of each mouse are calculated. (C) Sections of WT and KO dysplasias are stained with Caspase 3 antibody. Magnification, 200×. (D) Sections of WT and KO dysplasias are stained with Akt (a and b) or phosphorylated-Akt (c and d) antibodies, respectively. Magnification, 100× for Akt and 200× for pAkt. (E) Percentage of pAkt(Ser473) positive cells. Three visions of each mouse are calculated. Data from (B) and (E) are expressed as mean ± SD, ** indicate significant differences between groups ($p < 0.01$). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

3.3. *Kif18a disruption influences tumor proliferation and apoptosis*

Kif18A is a molecular motor protein, being important in modulating microtubule dynamics and mitosis. We suppose that the oncogenic effect of Kif18a we observed is due to its role in modulating mitosis and proliferation. To this end, in the induced CAC model, WT tumor cells showed more BrdU incorporation than *Kif18a*$^{-/-}$ cells (Fig. 3A and B). This demonstrates that the tumor cells proliferate quickly when Kif18a is present. This result was further confirmed by immunofluorescent staining for proliferating cell nuclear antigen (PCNA), whose levels were generally higher in WT colonic sections (Fig. 3C). Also, we found that there were more multiple- or bi-nuclei in *Kif18a*$^{-/-}$ colonic cells, but this did not occur in WT colons (Fig. 3D and E), suggesting a dysregulated mitotic process in *Kif18a*$^{-/-}$ colons that failed to yield normal daughter cells. This result is consistent with the key role of Kif18a in chromosome congression and segregation.

Additionally, we also found that *Kif18a*$^{-/-}$ tumor cells undergo more apoptosis than WT tumor cells (Fig. 4A and B) evaluated via *in situ* TUNEL assay. It was further certified by increased Caspase 3 amount in *Kif18a*$^{-/-}$ tumor (Fig. 4C). This is considered to contribute partly to the gentle tumor progression in *Kif18a*$^{-/-}$ mice. This increased apoptosis observed in *Kif18a*$^{-/-}$ colons is thought to be a result of disturbed PI3K-AKT signaling [16], which is well known to be associated with cell survival regulation and tumor progression [35]. Thus, we stained the WT and *Kif18a*$^{-/-}$ colons using Akt antibodies, and we found the total Akt protein level was comparable between the WT and *Kif18a*$^{-/-}$ dysplasias (Fig. 4Da and b), but the phosphorylated Akt decreased obviously in the *Kif18a*$^{-/-}$ dysplastic mucosa (Fig. 4Dc, d and E). These data demonstrate that Kif18a promotes cancer cell proliferation via regulating microtubule dynamics and mitosis, and loss of Kif18a induces higher apoptosis through decreased Akt kinase activity.

In recent years, the kinesin family of motor proteins has been emerging as targets for chemotherapeutic intervention of cancer due to their crucial role in mitosis [4–6]. The kinesin-8 Kif18A has been reported to be associated with human breast carcinogenesis and correlated with clinical relevance to colorectal cancer progression [15,16]. Additionally, proteomic analysis has identified Kif18A as potential biomarker of cholangiocarcinoma and lung cancer [27,28]. In this report, we show that Kif18a is up-regulated in induced CAC model, and *Kif18a* deficient mice are protected from CAC carcinogenesis via inactivation of PI3K-AKT pathway, demonstrating the significance of Kif18a in CAC progression and its functional role *in vivo*. Thus, Kif18A could become a target for CAC therapeutic intervention. Fortunately, a small molecule BTB-1 has been reported to inhibit Kif18A activity [36], which is worth further exploration and development. In addition, Kif18A may also be used as a CAC diagnostic biomarker.

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