


# Nuclear factor I/B promotes colorectal cancer cell proliferation, epithelial-mesenchymal transition and 5-fluorouracil resistance

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Nuclear factor I/B (NFIB) is a widely studied transcription factor that participates in tumor progression; nevertheless, studies on NFIB in colorectal cancer (CRC) are limited. In our study, Western blot and RT-PCR analyses showed that NFIB was overexpressed in CRC tissues and cell lines, which was consistent with our bioinformatic analysis results. Furthermore, NFIB expression was closely related to the TNM stage of CRC. NFIB promoted cell proliferation and migration and inhibited cell apoptosis in vitro. Meanwhile, we discovered that NFIB accelerated xenograft tumor growth in vivo. In addition, NFIB weakened the sensitivity of CRC cells to 5-fluorouracil (5-FU). NFIB induced epithelial-mesenchymal transition (EMT) by upregulating snail expression, which was accompanied by decreased E-cadherin and Zo-1 expression and increased Vimentin expression. Because the Akt pathway plays an important role in CRC progression, we examined whether there was a correlation between NFIB and the Akt pathway in cell proliferation and migration. Our results showed that NFIB promoted cell proliferation and increased 5-FU resistance by activating the Akt pathway. In summary, our findings suggested that NFIB induced EMT of CRC cells via upregulating snail expression and promoted cell proliferation and 5-FU resistance by activating the Akt pathway.

## KEYWORDS

Akt, EMT, NFIB, proliferation, Snail

## 1 | INTRODUCTION

Colorectal cancer has become one of the most common types of cancer worldwide; it is the third most common cancer in men and the second most common cancer in women, with more than 1.2 million new cases

and approximately 600 000 deaths each year.<sup>1-3</sup> From stage I to stage IV, the 5-year survival rate sharply decreases from 93.2% to 8.1%, so early diagnosis and standardization of treatment have become the key to improve the 5-year survival rate of CRC.<sup>4</sup> Therefore, more studies are needed to discover new effective diagnostic and prognostic biomarkers.

Liu and Chen contribute equally to this study.

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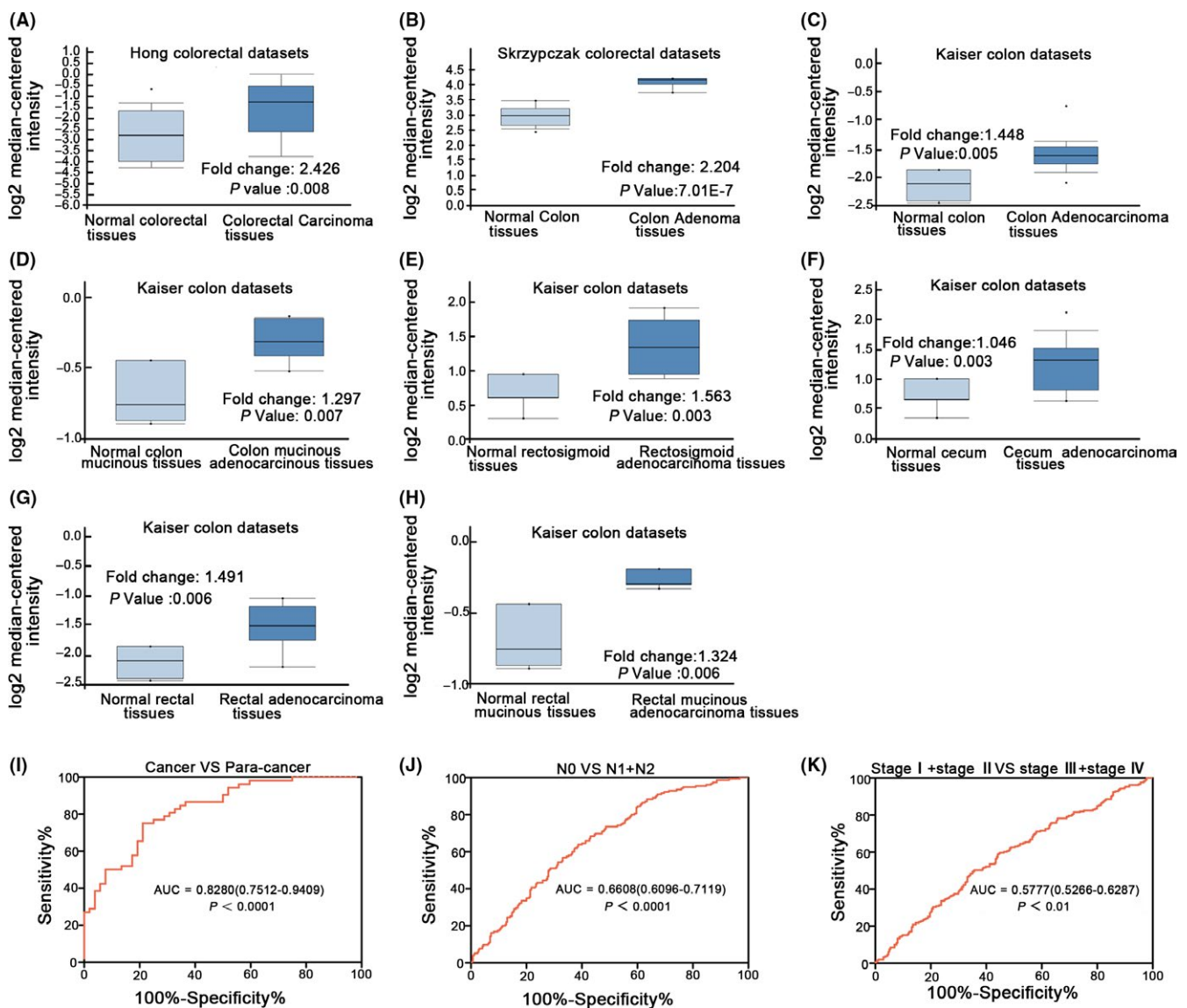
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In recent years, a growing number of studies have reported that NFIB plays an important role in embryonic development, maintenance of stem cell activity, regulation of cell differentiation and tumor-related functions. Wu et al<sup>5</sup> discovered that NFIB could promote cell migration in non-small cell lung cancer. miR-1224 was reported to impair proliferation and enhance apoptosis by repressing NFIB expression in hepatocytes upon ischemia-reperfusion in vivo and in vitro.<sup>6</sup> Another study showed that NFIB could promote dynamic changes in the chromatin state of melanoma cells to facilitate migration, invasion and metastasis. However, other studies report that NFIB serve as an oncogene in small cell lung cancer, melanoma and triple negative breast cancer, while NFIB inhibits tumor development and progression in glioma, glioblastoma and osteosarcoma.<sup>7</sup> Nevertheless, studies

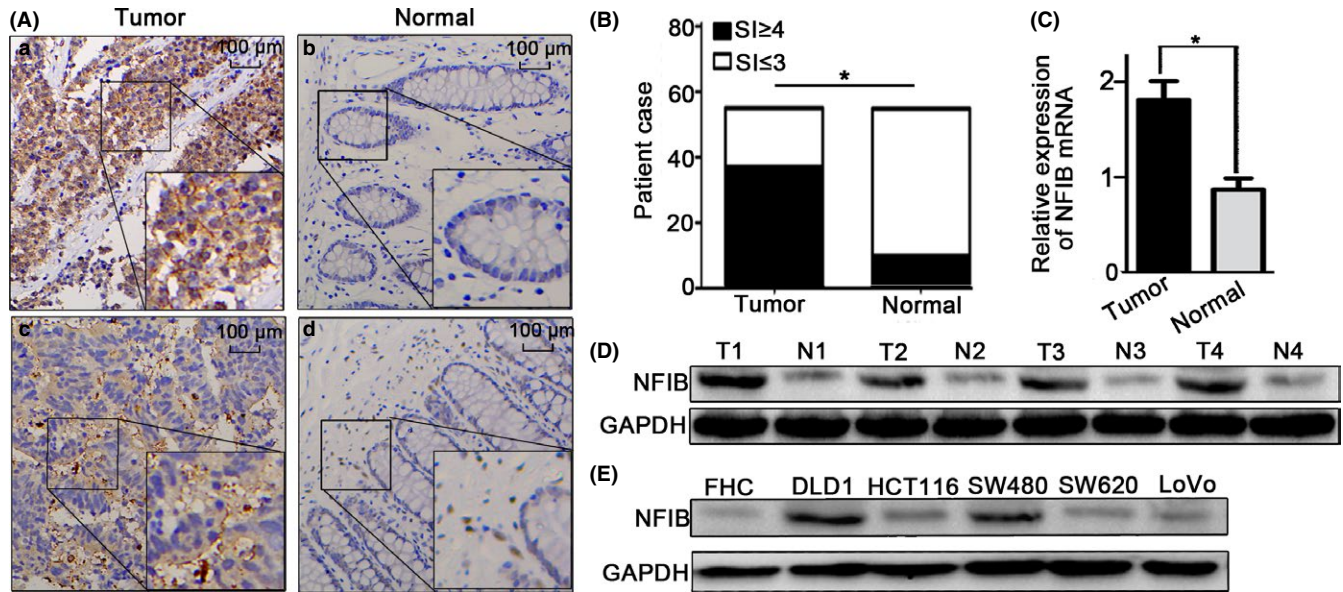
of NFIB in CRC are rare, and the biological function of NFIB in CRC is still unclear.

The Akt pathway plays a key role in cell proliferation, apoptosis, drug resistance and differentiation.<sup>8</sup> NFIB also has been reported to bind to the EZH2 promoter and promote EZH2 expression,<sup>9</sup> which can activate the PI3K/AKT pathway.<sup>10</sup> Wu et al<sup>11</sup> demonstrates that NFIB can activate the Akt/Stat3 signaling pathway. Therefore, we hypothesized that the Akt pathway may be involved in tumor progression caused by NFIB overexpression.

In our study, we discovered that NFIB promoted cell proliferation and 5-FU resistance by activating the Akt signaling pathway and facilitated migration and EMT by upregulating snail expression.



**FIGURE 1** Bioinformatic analysis of nuclear factor I/B (NFIB) expression in colorectal cancer (CRC). A-H, Oncomine databases including 8 data-sets showed that NFIB was overexpressed in various types of CRC tissues. The NFIB expression profile was downloaded from TCGA website and analyzed by ROC curves. I, NFIB could distinguish CRC tissues and noncancerous tissues by ROC curves. NFIB expression in subgroups of CRC patients was analyzed by ROC curves: J, N0 vs N1+N2. K, Stage I+Stage II vs Stage III+Stage IV



**FIGURE 2** Nuclear factor I/B (NFIB) is overexpressed in colorectal cancer (CRC). A, According to the results of immunohistochemical staining, NFIB was highly expressed in the nucleus and/or cytoplasm of CRC tissues but weakly expressed in paracancerous tissues. Magnification,  $\times 200$ . B, In 70.3% (38/54,  $SI \geq 4$ ) of CRC tissues, NFIB showed positive staining, while only 18.5% (10/54,  $SI \geq 4$ ) of adjacent tissues showed positive staining for NFIB. C-D, The NFIB level was higher in CRC tissues than in para-cancerous tissues by RT-PCR and Western blot assays. N, paracancerous tissue; T, CRC tissue. E, NFIB expression was higher in CRC cell lines than in FHC cells.  $*P < 0.05$

**TABLE 1** Clinicopathological characteristics of patients

Clinic pathological factors	n	NFIB expression		P value
		Positive	Negative	
Age (y)				
60	30	18	12	>0.05
$\leq 60$	24	20	4	
Gender				
Male	29	17	12	>0.05
Female	25	20	5	
Pathologic T stage				
T1 + T2	24	11	13	<0.01
T3 + T4	30	27	3	
Pathologic N stage				
N0	15	5	10	<0.01
N1 + N2	39	32	7	
Pathologic M stage				
M0	21	10	11	<0.01
M1	33	28	5	
Tumor differentiation				
Well	9	6	3	>0.05
Moderate	22	18	4	
Poor	23	13	10	

Clinical pathological characteristics of patients in Wuhan Concorde hospital between 2014 and 2017. None of the patients had chemoradiotherapy before surgery. The data was tested by  $\chi^2$  analysis.  $P < 0.05$  was considered to be statistically significant.

## 2 | MATERIALS AND METHODS

### 2.1 | Bioinformatic analysis

The NFIB expression data were downloaded from TCGA website (<https://xenabrowser.net/heatmap/>) and the Oncomine Cancer Microarray database (<https://www.oncomine.org/resource/login.html>); the data included age, gender, and TNM stage. Receiver operating characteristic (ROC) curve analysis was performed using GraphPad Prism 5.0 (GraphPad Software, La Jolla, CA, USA).

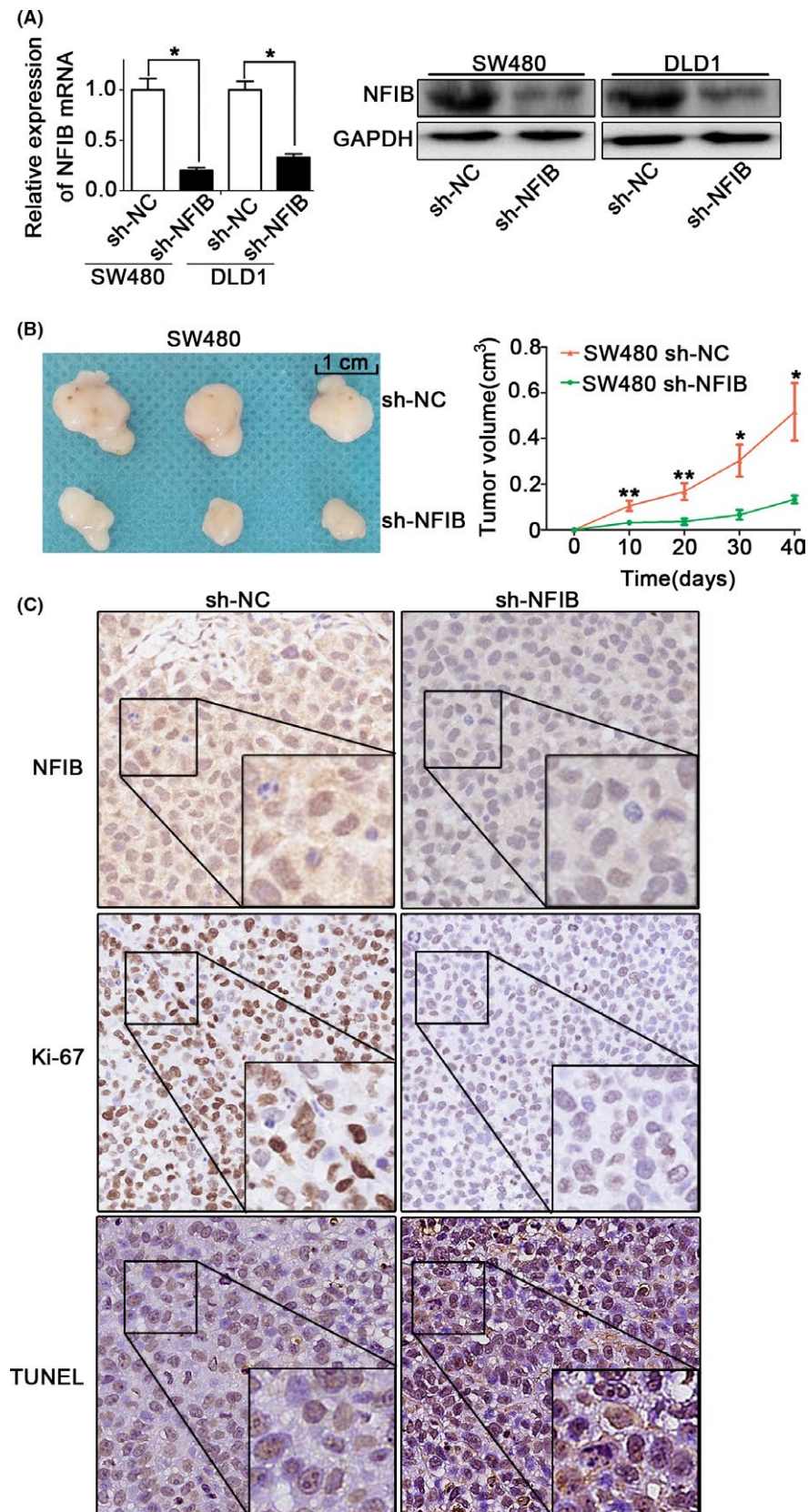
### 2.2 | CRC tissue samples

Fifty four pairs of cancer tissues and adjacent normal tissues were collected from CRC radical surgery at the Wuhan Union Hospital between 2014 and 2017. All samples were from CRC patients without any chemoradiotherapy before surgery. The samples were divided into two parts: one part was snap-frozen in liquid nitrogen for further protein analysis, and the other part was fixed with formalin for immunohistochemistry (IHC) assays. All samples for our study were collected after signed informed consent from the patients. Our study was approved by the Human Research Ethics Committee of Huazhong University of Science and Technology.

### 2.3 | Cell culture and reagents

The normal human colon epithelium cell line FHC was cultured in DMEM/F12 (HyClone, Logan, UT, USA). The CRC cell lines DLD1 and SW480 were maintained with RPMI1640 (HyClone), while HCT116 was grown in McCoy's 5A (HyClone), SW620 in DMEM/L15 (HyClone),

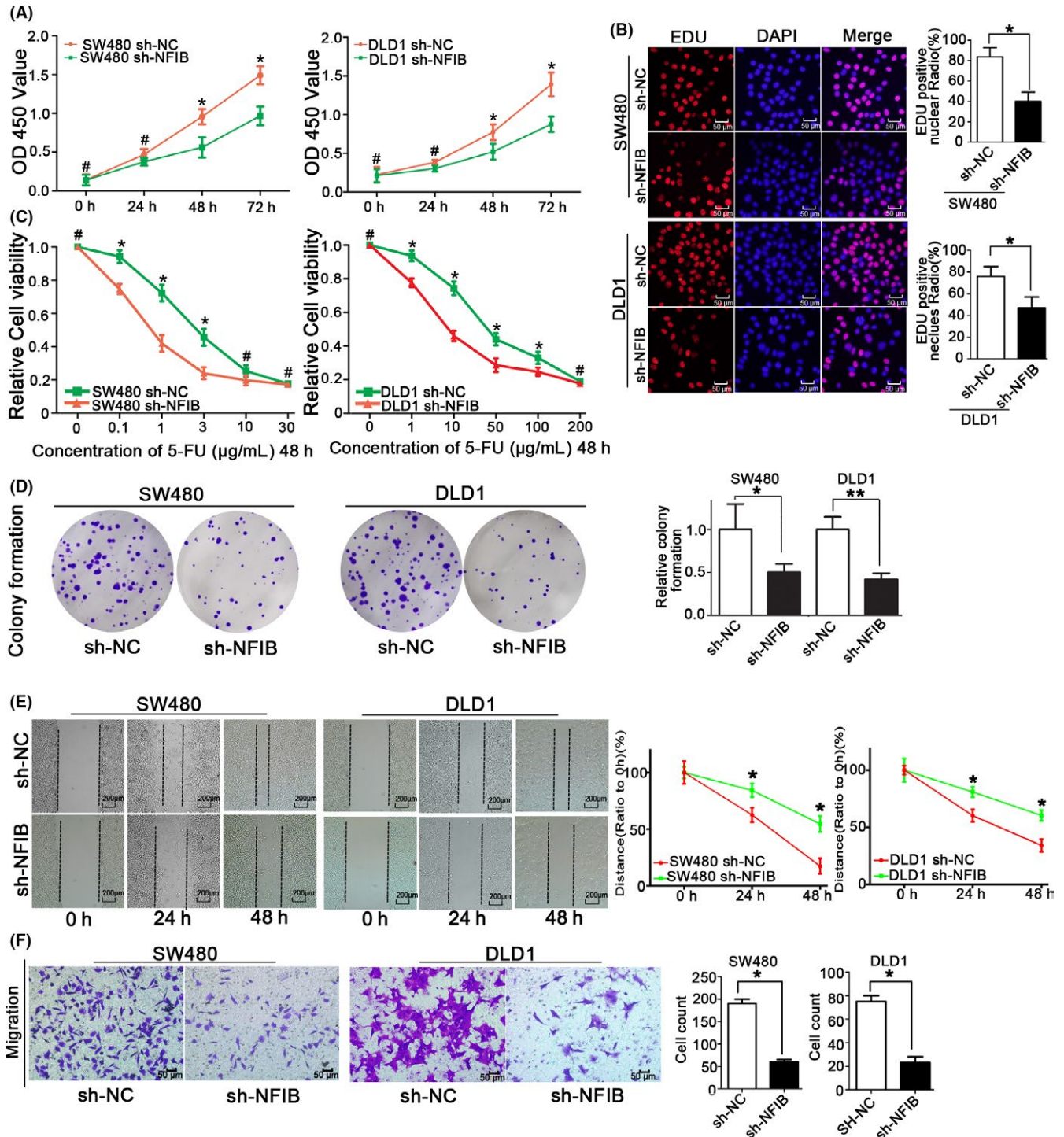




**FIGURE 3** Nuclear factor I/B (NFIB) silencing inhibits tumorigenesis in vivo. A, Western blot and RT-PCR analyses showed that NFIB was knocked down in SW480 and DLD1 cells. B, Compared to sh-NC cells, SW480 sh-NFIB cells resulted in a significantly decreased tumor size. C, The xenograft tumors were analyzed by IHC. Ki-67, proliferation marker; TUNEL, apoptotic marker. Magnification,  $\times 200$ . \* $P < 0.05$ . \*\* $P < 0.01$

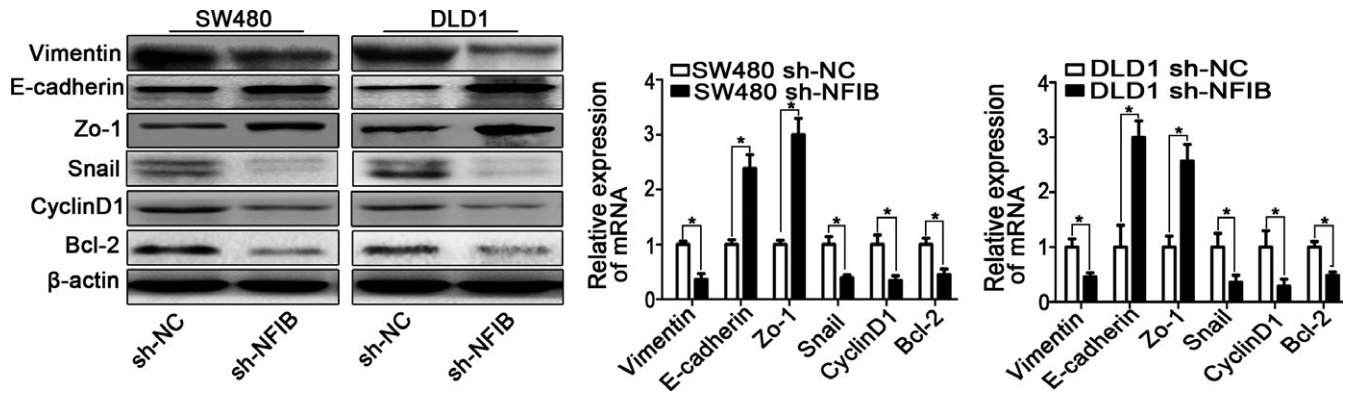
LoVo in DMEM/High glucose (HyClone) and 293T in DMEM F12 (HyClone). All cell lines were maintained in complete medium with 10% fetal bovine serum (FBS, ScienCell, San Diego, CA, USA), streptomycin (100  $\mu\text{g}/\text{mL}$ , Sigma) and penicillin (100 U/mL, Sigma, St. Louis, MO, USA) in a humidified incubator at 37°C and 5% CO<sub>2</sub>. In Western blot

assays, antibodies against CyclinD1 (1:1000, 60186-1-Ig), Vimentin (1:1000, 10366-1-AP), Zo-1 (1:1000, 21773-1-AP) and Snail (1:1000, 13099-1-AP) were purchased from Proteintech (Chicago, IL, USA), while antibodies against Bcl-2 (1:1000, #4223), Akt (1:1000, #4685) and phosphorylated Akt (1:500, #13038) were purchased from CST



**FIGURE 4** Nuclear factor I/B (NFIB) silencing affects cell growth, 5-FU sensitivity, colony formation and migration. A, NFIB silencing inhibited cell growth. B, EDU assays showed that NFIB knockdown decreased the ratio of EDU-positive cells. Magnification,  $\times 200$ . C, The 5-FU sensitivity curve (48 hours) showed that knockdown of NFIB increased the 5-FU sensitivity of colorectal cancer (CRC) cells. D, NFIB silencing inhibited colony formation in CRC cell lines. E-F, The scratch experiments (magnification,  $\times 40$ ) and transwell assays (magnification,  $\times 200$ ) showed that NFIB knockdown inhibited cell migration in CRC. \* $P < 0.05$ , \*\* $P < 0.01$ . #, no significance





**FIGURE 5** NFIB silencing inhibits EMT and CyclinD1 and Bcl-2 expression in colorectal cancer cells. \* $P < 0.05$ . NFIB, nuclear factor I/B

(USA). NFIB (1:1000, ab80835) and E-cadherin (1:1000, sc-71007) antibodies were purchased from Abcam (Cambridge, UK) and Santa Cruz (Dallas, TX, USA), respectively. EDU reagent was purchased from Guangzhou RiboBio Co., Ltd. (Guang Zhou, China).

## 2.4 | Western blotting assays

Cells and tissues were treated with cold RIPA buffer with PMSF (Sigma) and phosphatase inhibitors (Beyotime). Protein samples were separated by SDS-PAGE gels and transferred onto PVDF membranes. The membranes were incubated with primary antibodies. Image J (National Institutes of Health, Bethesda, MD, USA) was used to measure the band density.

## 2.5 | Cell growth assay (CCK8 assay and EDU assay)

Cells (SW480:  $5 \times 10^3/200 \mu\text{L}$ , DLD1:  $4 \times 10^3/200 \mu\text{L}$  and LoVo:  $5 \times 10^3/200 \mu\text{L}$ ) were seeded into a 96-well plate. After 24, 48 and 72 hours, CCK8 assays were carried out to determine cell growth according to the manufacturer's protocol<sup>12</sup>. The EDU assay was carried out using a Cell-Light™ EDU Apollo®488 In Vitro Flow Cytometry Kit according to the manufacturer's protocol. The detailed procedures were described in the Appendix S1.

## 2.6 | Colony formation assay

Five hundred cells were cultured in a 6-well plate for 10 days. The clones were treated with 4% paraformaldehyde and 0.05% crystal violet. The clones were counted via Image J.

## 2.7 | Cell migration and scratch assays

SW480 ( $8 \times 10^4$ ), DLD1 ( $2 \times 10^4$ ) or LoVo ( $5 \times 10^4$ ) cells in 200  $\mu\text{L}$  FBS-free medium were seeded into a transwell chamber (BD, USA). After 22 hours, the transwell chamber was fixed with 4% paraformaldehyde and stained with 0.05% crystal violet. The cells were counted in five random fields. For scratch assays, SW480 cells, DLD1 cells or LoVo cells ( $4 \times 10^5$ ) were seeded in 12-well plates. After the cells reached 85%–95% confluence, 10  $\mu\text{L}$  pipette

tips were used to make a scratch, which was then photographed at 0, 24 and 48 hours.

## 2.8 | Cell apoptosis assay

Cells apoptosis was assessed by Annexin V-APC/PI apoptosis kits (AntGent, China). Cells were collected and then washed with PBS twice. APC and PI were added in the dark room and incubated for 15 minutes. The apoptosis rate was detected by flow cytometry.

## 2.9 | IHC

The detailed procedures for IHC were described in the Appendix S1.

## 2.10 | Transfection assay

The detailed procedures for transfection assay were described in the Appendix S1.

## 2.11 | Real-time PCR

The detailed procedures were described in the Appendix S1. The primer sequences were provided in Table S1.

## 2.12 | Animal experiments

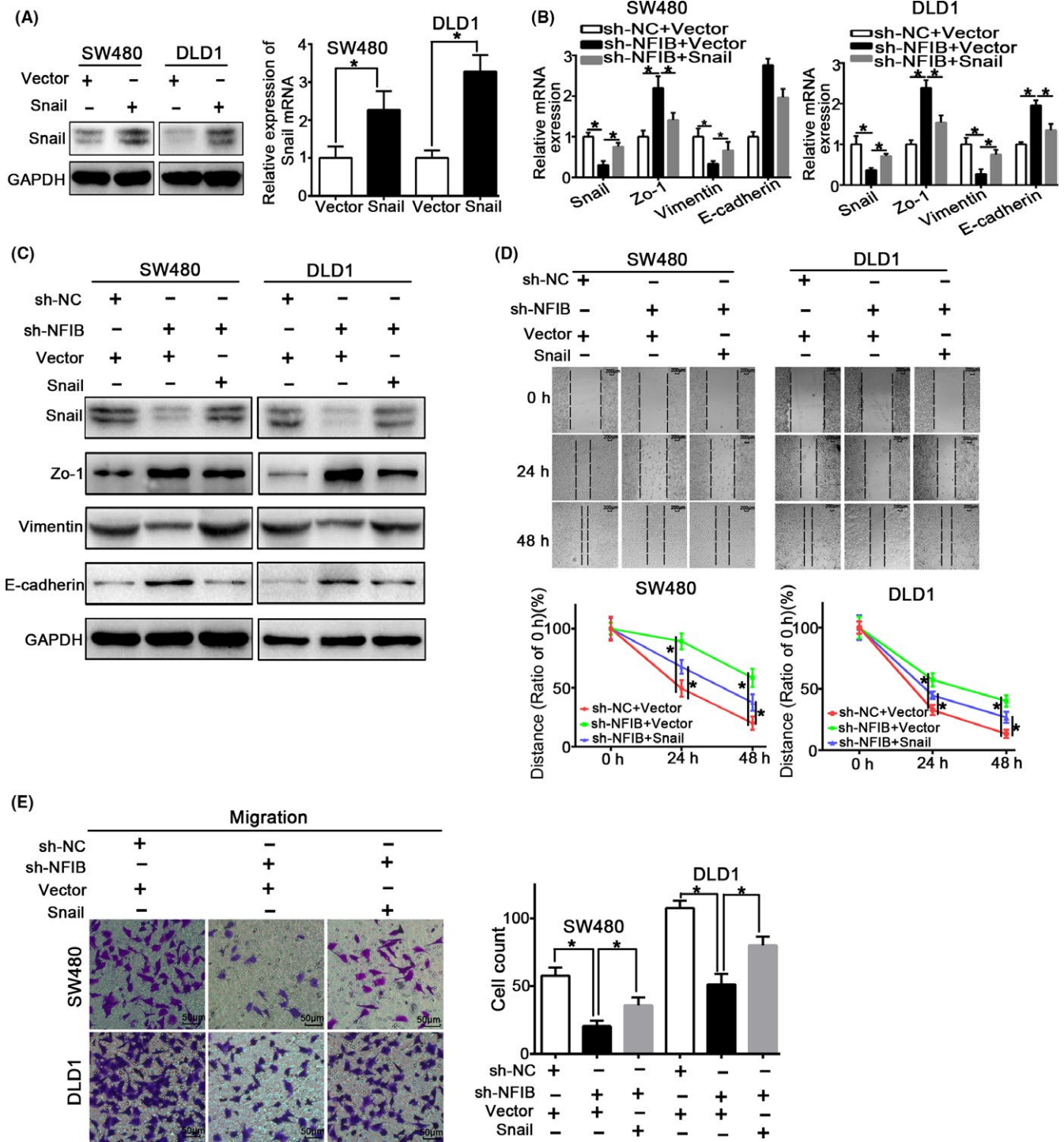
The detailed procedures for Animal experiments were described in the Appendix S1.

## 2.13 | Chromatin immunoprecipitation

The detailed procedures for chromatin immunoprecipitation (ChIP) were described in the Appendix S1.

## 2.14 | Luciferase reporter assays

The detailed procedures for Luciferase reporter assay were described in the Appendix S1.

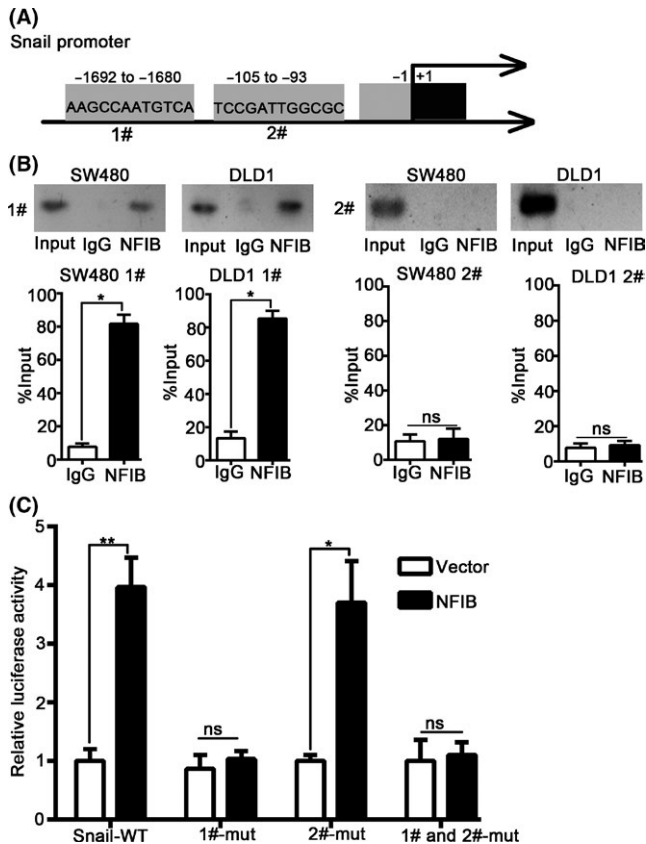


**FIGURE 6** Nuclear factor I/B (NFIB) induces EMT by upregulating Snail in CRC cells. A, Snail protein and mRNA were overexpressed after transfection of Snail cDNA. B-C, Snail overexpression partially restored EMT, which was inhibited by NFIB knockdown. D-E, Scratch assays (magnification,  $\times 40$ ) and transwell assays (magnification,  $\times 200$ ) showed that snail overexpression promoted cell migration, which was impaired by NFIB knockdown.  $*P < 0.05$ . CRC, colorectal cancer

**2.15 | Statistical analysis**

Unpaired t tests were used to test the difference between groups. The expression of NFIB in 54 CRC tissues and paired adjacent noncancerous tissues was analyzed using the paired t test. The relationships between the expression of NFIB and clinical

characteristics of CRC were analyzed by the  $\chi^2$  test.  $P$  value  $< 0.05$  was considered statistically significant, and all statistical tests were two-sided. All assays were repeated at least three times. The statistical analysis was carried out using GraphPad Prism5.0. The data were presented as the mean  $\pm$  standard deviation (SD).



**FIGURE 7** Nuclear factor I/B (NFIB) promotes Snail expression by binding to the Snail promoter. A, Schematic illustration of the human Snail promoter and NFIB potential binding site. B, ChIP assays with NFIB antibody or IgG were performed to verify the binding between NFIB and the Snail promoter in SW480 and DLD1 cells. C, Luciferase activity assays were performed in 293T cells, which were transfected with wild type (Snail-WT) or mutant-type (Snail-mut) Snail promoter-containing pGL3 reporter vectors. These cells were further transfected with pcDNA-NFIB or vector. After 48 hours, firefly luciferase activity was detected and normalized by Renilla luciferase activity; 1#mut: potential binding site 1 mut. 2#mut: potential binding site 2 mut. 1#2#mut: potential binding sites 1 and 2 mut. \* $P < 0.05$ , \*\* $P < 0.01$ . ns, no significance

### 3 | RESULTS

#### 3.1 | NFIB is overexpressed in CRC tissues

We analyzed the expression of NFIB mRNA from the Oncomine database and discovered that the expression of NFIB in tumor tissues was significantly higher than that in paracancerous tissues in some data-sets (data not shown). In most CRC types, NFIB was significantly overexpressed in the cancer tissues (Figure 1A-H). Next, to determine the diagnostic value of NFIB expression in CRC, we plotted the ROC curve according to NFIB expression in cancer tissues and corresponding paracancerous tissues provided by TCGA website. The area under the curve (AUC) was 0.8280 (95% CI: 0.7512-0.9409;  $P < 0.0001$ ), with a sensitivity of 75.00% and a specificity of 78.85% at the cutoff value, which showed that NFIB could clearly distinguish CRC tissues from paracancerous tissues

(Figure 1I). In addition, NFIB mRNA expression in the subgroups of CRC patients was determined by ROC curve analysis, and the results indicated that NFIB overexpression might serve as a diagnostic factor in CRC patients with N0/(N1 + N2) stage (Figure 1J, AUC = 0.6608,  $P < 0.0001$ ) and Stage I+II/Stage III+IV (Figure 1K, AUC = 0.5777,  $P < 0.01$ ). However, NFIB expression did not distinguish between patients in M1 stage and M0 stage by ROC curve analysis (data not shown).

To further clarify the expression of NFIB in CRC, we assessed 54 pairs of CRC tissues and adjacent tissues by IHC staining, Western blot analyses and RT-PCR (Figure 2A-D). IHC staining showed that NFIB was positively stained in 70.3% (38/54, SI  $\geq 4$ ) of CRC tissues, while only 18.5% (10/54, SI  $\geq 4$ ) of adjacent tissues were positively stained for NFIB (Figure 2A-B). Moreover, the sites of staining were different; both the nucleus and cytoplasm were strongly stained in the tumor tissues, but only slight staining of the nucleus was observed in the paracancerous tissue. NFIB was obviously overexpressed in CRC tissues and CRC cell lines (Figure 2C-E). To further explore whether NFIB overexpression was related to the clinicopathological features of CRC, we combined the immunohistochemical scores with clinical data and discovered that high expression of NFIB was positively related to T stage ( $P < 0.01$ ), N stage ( $P < 0.01$ ), and M stage ( $P < 0.01$ ), while the correlations to the gender, age and tumor differentiation were not significant (Table 1).

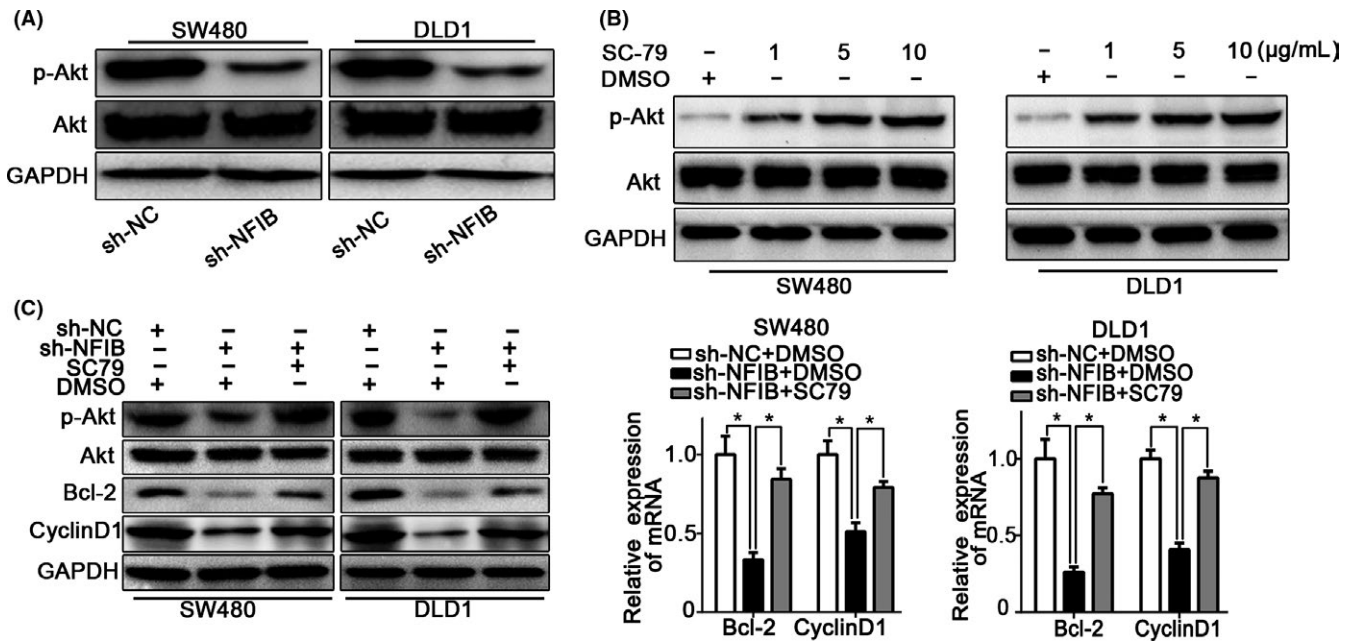
#### 3.2 | NFIB promotes tumorigenesis of CRC cells in vivo

To further explore the effects of NFIB on CRC tumorigenesis, we transfected sh-NFIB into SW480 and DLD1 cells, and NFIB cDNA was transfected into LoVo cells using a lentiviral vector. The expression of NFIB mRNA and protein was knocked down by sh-NFIB and overexpressed by NFIB cDNA (Figure 3A and Figure S1A). We discovered that NFIB silencing significantly reduced the volume of subcutaneous xenograft tumors and that NFIB overexpression promoted subcutaneous xenograft tumor growth (Figure 3B and Figure S1B). Moreover, the IHC results showed that the proliferation marker Ki-67 index was decreased and apoptotic TUNEL staining was increased in the SW480-sh-NFIB groups in vivo (Figure 3C). In contrast, the Ki-67 index was increased, and TUNEL staining was decreased in the LoVo-NFIB groups (Figure S1C). These results indicated that NFIB promoted xenograft tumor growth by enhancing cell proliferation and inhibiting cell apoptosis.

#### 3.3 | NFIB promotes cell growth, colony formation, and migration and decreases sensitivity to 5-FU

In vivo, we found that NFIB played an oncogenic role in CRC cells. To further study the mechanisms of NFIB in promoting tumor progression, we established NFIB knockdown cell lines and NFIB overexpression cell lines in the subsequent experiments. The results showed





**FIGURE 8** Nuclear factor I/B (NFIB) silencing inactivates the Akt signaling pathway in colorectal cancer (CRC) cells. A, Western blot assays were performed to determine the levels of p-Akt and total Akt in sh-NC cells and sh-NFIB cells in CRC. B, The protein levels of p-Akt, Akt, and GAPDH were detected in CRC cells treated with various concentrations of SC-79. C, Western blot and RT-PCR results showed that SC-79 treatment could activate the Akt signaling pathway, which was inactivated by NFIB knockdown, increase the expression of CyclinD1 and Bcl-2. 0.1% DMSO vehicle was selected as the control. \* $P < 0.05$

that NFIB knockdown significantly inhibited cell proliferation, 5-FU resistance, colony formation and cell migration (Figure 4A-F). Conversely, NFIB overexpression promoted cell proliferation, colony formation, 5-FU resistance and cell migration (Figure S2A-F).

Taken together, the results showed that NFIB played a role as an oncogene in CRC by promoting CRC cell proliferation, migration and 5-FU resistance.

### 3.4 | NFIB induces the EMT profile and upregulates the expression of Bcl-2 and CyclinD1 in CRC cells

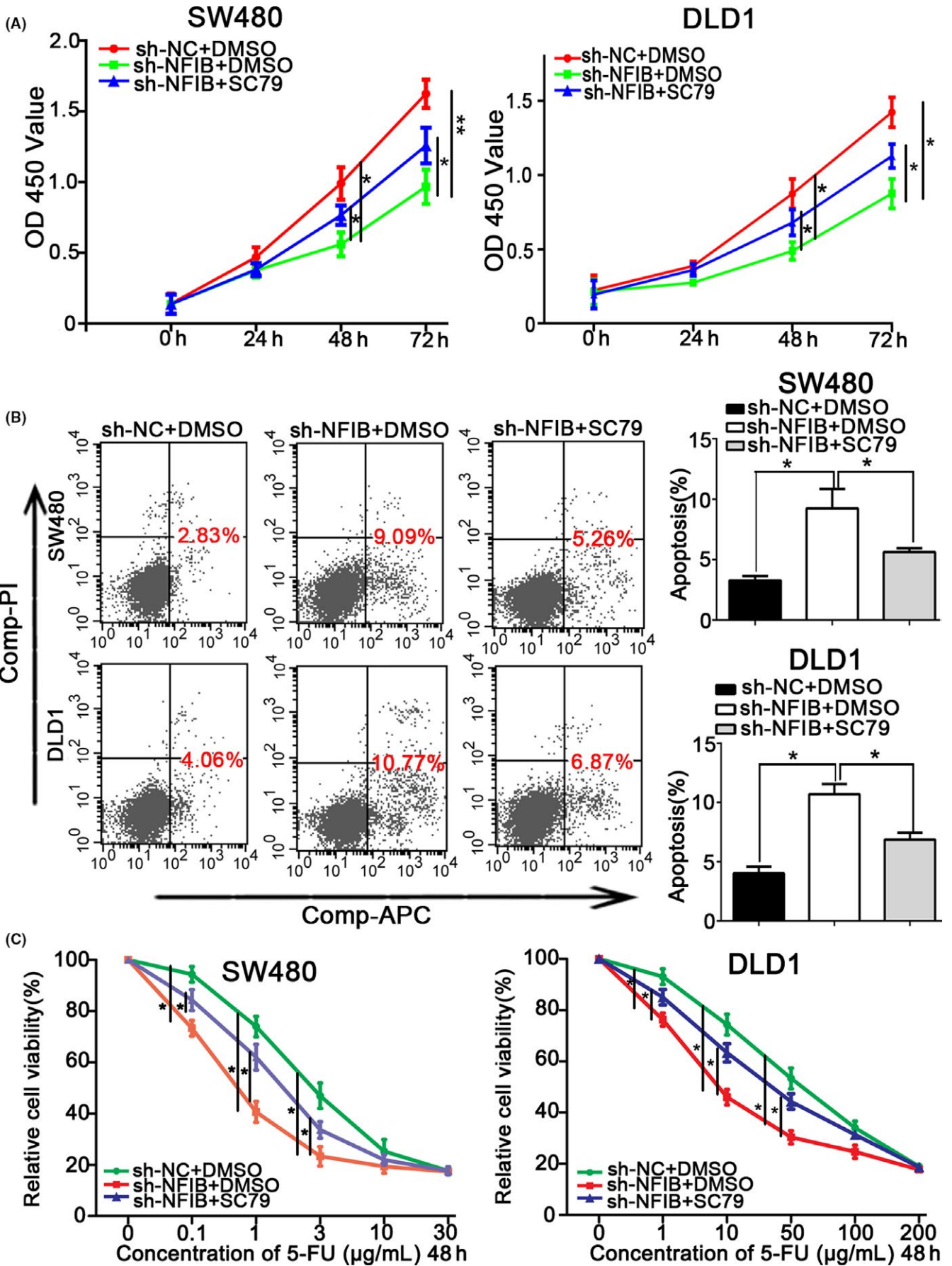
Epithelial-mesenchymal transition is an important mechanism of migration and metastasis. Therefore, the expression levels of the EMT-related transcription factors (EMT-TF) Zeb1, Zeb2, Twist, Slug and Snail were detected by RT-PCR, and the results revealed that NFIB could promote Snail expression (Figure S3 and Figure 5). NFIB knockdown decreased the expression of Snail and Vimentin, whereas E-cadherin and Zo-1 were increased both in SW480 cells and DLD1 cells (Figure 5). Coincidentally, NFIB overexpression increased the expression of Snail and Vimentin and decreased the expression of E-cadherin and Zo-1 (Figure S3).

Bcl-2 and CyclinD1 are involved in tumor cell proliferation by regulating cell apoptosis and the cell cycle. We discovered that the expression of Bcl-2 and CyclinD1 in NFIB knockdown cells was decreased (Figure 5). In contrast, NFIB overexpression significantly increased Bcl-2 and CyclinD1 expression in LoVo cells (Figure S3).

### 3.5 | NFIB induces EMT by upregulating Snail expression

In our study, we found that NFIB induced EMT and upregulated the expression of Snail. As an important EMT-TF, Snail governs EMT.<sup>13</sup> To explore the functions of Snail in NFIB-induced EMT, we transfected Snail cDNA and Snail si-RNAs into NFIB knockdown cells and NFIB overexpression cells, respectively. In SW480 and DLD1 cells, co-transfection of Snail cDNA dramatically alleviated the NFIB-knockdown-induced snail repression, and Zo-1, Vimentin and E-cadherin expression levels were reversed by overexpression of Snail (Figure 6A-C). Snail overexpression partially restored the migratory capacity, which was impaired by NFIB silencing (Figure 6D-E). In addition, Snail siRNA significantly decreased snail expression and reversed NFIB overexpression-induced EMT (Figure S4A-E).

**FIGURE 9** Nuclear factor I/B (NFIB) silencing affects cell proliferation, apoptosis and 5-FU sensitivity via inactivating the Akt signaling pathway. A, CCK8 assays indicated that SC-79 (5 µg/mL) could increase cell proliferation in colorectal cancer (CRC) cells, which was impaired by NFIB knockdown. B, Apoptosis results showed that NFIB regulated apoptosis by the Akt signaling pathway. The concentration of SC-79 was 5 µg/mL, and 0.1% DMSO vehicle was selected as the control. C, The 5-FU sensitivity curve showed that SC-79 pretreatment (5 µg/mL, 2 hours) obviously decreased the 5-FU sensitivity compared with that of NFIB knockdown cells pretreated with 0.1% DMSO vehicle in CRC. \* $P < 0.05$ , \*\* $P < 0.01$



**TABLE 2** The IC50 of 5-fluorouracil in this study

v	IC50 (µg/mL) 48 h
SW480 sh-NC+ DMSO	9.434
SW480 sh-NFIB + DMSO	4.971
SW480 sh-NFIB + SC79	7.176
DLD1 sh-NC+ DMSO	118.711
DLD1 sh-NFIB + DMSO	42.034
DLD1 sh-NFIB + SC79	83.155
LoVo Vector+DMSO	0.935
LoVo NFIB+DMSO	10.3
LoVo NFIB+MK-2206	3.756

The cells were pretreated with SC-79 (5 µg/mL), 0.1% DMSO or MK-2206 (10 µmol/L) vehicle 2 h before adding various concentration of 5-FU. The IC50 was measured after 48 h of 5-FU treatment.

### 3.6 | NFIB promotes Snail transcription by binding to the Snail promoter

The Snail promoter region was searched on the ROMO website (<http://algggen.lsi.upc.es>), and we discovered that there were two potential binding sites for NFIB (Figure 7A). The two potential binding sites were named potential binding site 1# (1#) and potential binding site 2# (2#). Next, ChIP assays were performed in SW480, DLD1 and LoVo cells using NFIB antibodies (NFIB). Compared to the sample bound to IgG, the NFIB-bound complex showed a remarkable enrichment of 1# of the snail promoter (Figure 7B and Figure S5). To further confirm that the binding of NFIB to the Snail promoter is functional, we performed a luciferase reporter assay of 293T cells. The NFIB overexpression group showed substantially increased luciferase activities of the wild-type snail promoter-containing pGL3 reporter vector (Snail-WT) and the 2# mut Snail promoter-containing pGL3 reporter vector (Snail-2#-mut) but not the 1# mut or 1#2# mut Snail promoter-containing pGL3 reporter vectors (Snail-1# mut and 2# mut) when compared with the negative control (vector) group (Figure 7C). These results indicated that NFIB binded to 1# and promoted Snail transcription.

### 3.7 | NFIB increases cell proliferation and decreases cell apoptosis and sensitivity to 5-FU by activating the Akt signaling pathway

The Akt signaling pathway has been proved to be an important mechanism to regulate apoptosis and proliferation in many types of tumors. In our study, we discovered that the p-Akt level was decreased in NFIB knockdown cells and increased in NFIB overexpression cells (Figure 8A and Figure S6A). The Akt activator SC-79 was used to abolish this inhibitory effect of NFIB knockdown. We discovered that 5 µg/mL of SC-79 pretreatment for 2 hours could significantly activate the Akt pathway, so this concentration was used in subsequent studies (Figure 8B). SC-79 treatment for 2 hours reversed NFIB knockdown-induced deactivation of Akt and downregulation

of Bcl-2 and CyclinD 1 in CRC cells (Figure 8C). Furthermore, CCK8 assays, apoptosis assays and 5-FU sensitivity assays revealed that NFIB silencing-mediated inhibition of cell proliferation and enhancement of 5-FU sensitivity were reversed by SC-79 pretreatment (Figure 9A-C).

In contrast, the Akt inhibitor MK-2206 was used in NFIB overexpression cells. We discovered that 10 µmol/L of MK-2206 pretreatment for 2 hours could significantly inhibit the activation of Akt, and this pretreatment time and concentration were used in subsequent experiments (Figure S6B). MK-2206 treatment reversed NFIB overexpression-induced activation of Akt and decreased the expression of Bcl-2 and CyclinD 1 in CRC cells. Furthermore, the proliferation and resistance to 5-FU in NFIB-overexpressing cells were reversed by MK-2206 pretreatment (Figure S6D-E and Table 2). MK-2206 pretreatment also increased cell apoptosis (Figure S6F). In conclusion, NFIB promoted cell proliferation and inhibited cell apoptosis and 5-FU sensitivity by activating the Akt signaling pathway.

## 4 | DISCUSSION

Previous studies reported that NFIB played a controversial role in different cancers. Wu et al<sup>14</sup> show that in Rb/p53 deletion mice, NFIB overexpression can accelerate small cell lung cancer formation. NFIB promotes cell proliferation in estrogen receptor-negative breast cancers by upregulating CDK6 and downregulating p21.<sup>15</sup> Nevertheless, NFIB also plays an anti-oncogenic role in osteosarcoma.<sup>16</sup> NFIB combines with androgen receptor/FOXA1 binding sites in androgen-dependent prostate cancer cells and plays a repressive role in the cell cycle. NFIB also reduces IGFBP5 expression, which leads to weakening IGFBP5-mediated inhibition of IGF-1 and IGF-1/IGF-1R axis-induced osteosarcoma cell proliferation, survival and metastasis.<sup>16</sup> In our study, we discovered that NFIB was overexpressed in CRC cells and played an oncogenic role by promoting EMT, cell proliferation and 5-FU resistance.

Epithelial-mesenchymal transition is an important mechanism of tumor metastasis.<sup>17</sup> In the EMT process, EMT-TFs inhibited the expression of epithelial phenotype proteins, such as E-cadherin and Zo-1, while promoting expression of mesenchymal phenotype proteins, such as Vimentin and N-cadherin. After the EMT process, cells lose polarity and acquire the ability to move freely.<sup>18</sup> Previous studies have reported that NFIB can induce EMT, however, the specific mechanism is still unclear.<sup>11</sup> In our study, we explored the link between NFIB and EMT-TFs and discovered that NFIB could promote snail expression by binding to the Snail promoter. Snail is a zinc finger protein that can mediate EMT through downregulation of cell adhesion molecules, such as E-cadherin, by binding several E-boxes located in the promoter region.<sup>19</sup> Snail can also lead to repression of tight junction proteins, such as claudin and Zo-1.<sup>20</sup> Snail has been reported to upregulate the expression of mesenchymal markers, such as Vimentin, Fibronectin and matrix metalloproteinases



(MMPs).<sup>21-23</sup> Our study demonstrated that NFIB induced EMT by upregulating snail expression.

The Akt signaling pathway plays an important role in cell processes, including cell proliferation, differentiation, apoptosis and drug resistance.<sup>24</sup> Activation of Akt is a “common approach” for many intracellular and extracellular signaling molecules to promote survival and proliferation. Activation of the Akt pathway can alleviate the tumor cell damage caused by chemotherapeutic drugs by enhancing DNA repair and inducing the expression of drug pump proteins.<sup>25-28</sup> NFIB was reported to bind to the EZH2 promoter and promote EZH2 expression,<sup>9</sup> which could activate the PI3K/AKT pathway.<sup>10</sup> Wu et al<sup>11</sup> discovered that NFIB can activate the Akt/Stat3 signaling pathway in gastric cancer. However, the relationship between the effects of NFIB on the biological behavior of CRC and the activation of the Akt pathway has not been reported. In our study, we discovered that NFIB promoted cell proliferation and 5-FU insensitivity by activating the Akt pathway.

We also discovered that NFIB weakened 5-FU sensitivity. The mechanism of chemotherapeutic resistance is very complicated, and three important mechanisms are widely recognized. First, drug efflux proteins, especially the ATP-binding cassette (ABC) transporter family of proteins, play a key role in chemotherapeutic resistance.<sup>29</sup> Second, DNA repair pathways, a defense mechanism, are essential to maintain DNA integrity and resist chemotherapeutic damage.<sup>30</sup> Third, activation of the anti-apoptotic pathway and expression of anti-apoptotic proteins are also an important mechanism of chemotherapeutic resistance.<sup>31</sup> The PI3K/Akt pathway and Bcl-2 protein are important components that can promote cancer cell survival.<sup>31</sup> In our study, we revealed that NFIB promoted Akt pathway activation and upregulated Bcl-2 expression in CRC cells. Furthermore, the link between EMT and cancer cell drug resistance has been known for many decades.<sup>32</sup> In the EMT process, cells show stem cell characteristics and upregulate drug efflux protein.<sup>33</sup> In addition, Saxena et al<sup>34</sup> discover that the activity of EMT-TF binding to the promoter of ABC transporters is increased. In our study, NFIB-induced CRC cell EMT could partially explain how NFIB causes 5-FU insensitivity.

In conclusion, our results revealed that NFIB promoted cell migration and proliferation while inhibited CRC cell apoptosis and 5-FU sensitivity. Subsequently, we demonstrated that NFIB induced EMT by upregulating snail expression and promoted cell proliferation and 5-FU resistance by activating the Akt signaling pathway, which suggested that NFIB might be an important therapeutic target for the treatment of CRC.

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## CONFLICTS OF INTEREST

The authors announced no conflicts of interest.

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### SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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