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Krüppel-like factor 5 promotes mitosis by activating the cyclin B1/Cdc2 complex during oncogenic Ras-mediated transformation

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Abstract **We previously showed that the zinc finger-containing transcription factor Krüppel-like factor 5 (KLF5) is important in mediating transformation by oncogenic H-Ras through induction of *cyclin D1* expression and acceleration of the G_1/S transition of the cell cycle. Here we present evidence of a role for KLF5 in accelerating mitotic entry in H-Ras-transformed NIH3T3 fibroblasts. When compared with non-transformed parental NIH3T3 cells, H-Ras-transformed fibroblasts exhibit an increase in mitotic index, levels of cyclin B1 and Cdc2, and cyclin B1/Cdc2 kinase activity. Inhibition of *KLF5* expression in H-Ras-transformed cells with *KLF5*-specific small interfering RNA (siRNA) results in a decrease in each of the aforementioned parameters, with a concomitant reduction in the transforming potential of the cells. Conversely, over-expression of *KLF5* in NIH3T3 cells leads to an increase in the promoter activity of the genes encoding *cyclin B1* and *Cdc2*. These results indicate that KLF5 accelerates mitotic entry in H-Ras-transformed cells by transcriptionally activating *cyclin B1* and *Cdc2*, which leads to an increase in cyclin B1/Cdc2 kinase activity. Extending our previous observation that KLF5 activates *cyclin D1* transcription to promote G_1/S transition, our current results further support a crucial function for KLF5 in mediating cellular transformation caused by oncogenic H-Ras.**

Keywords: Transformation; Mitotic index; Colony formation; Proliferation; G_2/M transition; Small interfering RNA

1. Introduction

Krüppel-like factors (KLFs) are Sp1-like transcription factors that bear significant homology to the *Drosophila melanogaster* segmentation gene, Krüppel [1–5]. Proteins of this family contain a DNA binding domain consisting of C_2H_2 zinc fingers and exhibit important regulatory functions [2–5]. Two related, yet functionally contrasting, members of the KLF family, KLF4 and KLF5, are differentially expressed in the intestinal epithelium [3,6]. While *KLF4* is predominantly expressed in the post-mitotic, terminally differentiated epithelial cells of the intestinal villi [7,8], *KLF5* is mostly expressed in the undifferentiated, proliferating cells of the crypt compartment [9]. Studies support a role for KLF4 in functioning as a tumor suppressor of the intestinal epithelial cells [10,11]. In contrast, KLF5 has been shown to accelerate cell proliferation in both cultured fibroblasts and intestinal epithelial cells [12–14].

Ras proteins are a family of small GTPases often found to be constitutively activated in tumors. For example, activating K-Ras mutations are found in approximately 90% of pancreatic and 50% of colon carcinomas [15,16]. Oncogenic Ras isoforms cause the constitutive activation of a signaling cascade involving several proteins including Raf and the mitogen-activated protein kinase (MAPK) [17–19]. Expression of these Ras isoforms can lead to a transformed phenotype in various cell lines, including fibroblasts [12,20] and epithelial cells [21]. Previous experiments demonstrate that constitutive activation of the Ras pathway accelerates the G_1/S transition of cell cycle through induction of *cyclin D1* expression [13,22,23]. Consequently, oncogenic Ras-mediated transformation leads to serum- and anchorage-independent growth and increased metastatic potential [12,24,25]. A recent study reveals that oncogenic Ras also accelerates the G_2/M progression of the cell cycle by increasing the level of cyclin B1 protein [26]. Of note is the G_2/M progression is rigorously controlled by the activity of the cyclin B1/Cdc2 kinase complex [27,28].

We recently showed that the level of KLF5 is increased in NIH3T3 fibroblasts transformed by oncogenic H-Ras [13]. This increase is the result of H-Ras-induced activation of MAPK and subsequent induction of the gene encoding early growth response 1 (Egr1), which transcriptionally activates *KLF5* [13]. Activated KLF5 then leads to the transcriptional induction of the gene encoding *cyclin D1* [13]. Importantly, inhibition of *KLF5* expression is accompanied by a reduction in the transforming potential of the cells, including a reduced capacity for anchorage-independent growth [13]. Here we present evidence for an equally important role of KLF5 in accelerating mitotic entry of H-Ras-transformed NIH3T3 fibroblasts through activation of the cyclin B1/Cdc2 kinase complex.

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Abbreviations: DMEM, Dulbecco's modified Eagle's medium; FACS, fluorescence-activated cell sorting; FBS, fetal bovine serum; KLF5, Krüppel-like factor 5; MAPK, mitogen-activated protein kinase; MPM2, mitotic phosphoepitope marker 2; PBS, phosphate-buffered saline; PI, propidium iodide; siRNA, small interfering RNA

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2. Materials and methods

2.1. Cell lines and reagents

Culture media and fetal bovine serum (FBS) were purchased from Mediatech, Inc. (Herndon, VA). The expression construct containing the oncogenic H-Ras was generously provided by Dr. Raul Urrutia [29]. The monoclonal antibody against Cdc2 and Actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA) and EMD Biosciences (San Diego, CA), respectively. A polyclonal antibody against cyclin B1 was acquired from Rockland Immunochemicals (Gilbertsville, PA). Antibodies against phospho-histone H1 and histone H1 were obtained from EMD Biosciences (San Diego, CA) and Upstate USA (Charlottesville, VA), respectively. The mouse monoclonal antibody directed against phospho-Ser/Thr-Pro MPM2 (mitotic phosphoepitope marker-2) was purchased from Upstate USA. FITC-conjugated anti-mouse IgG (Fc-specific) secondary antibodies were purchased from Sigma (St. Louis, MO). The antibody against phosphorylated histone H3 (Serine-10) was purchased as part of Mitotic Index Hitkit from Cellomics, Inc (Pittsburgh, PA). The −287 *cyclin B1* promoter luciferase reporter was generously provided by Dr. Karen Katula [30,31]. The luciferase construct with *Cdc2* promoter was generously provided by Dr. Christopher Glass [32].

2.2. Cell lines

NIH3T3 mouse fibroblasts were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FBS and 1% penicillin–streptomycin at 37 °C in a 5% CO_2 atmosphere. Stable clones of NIH3T3 transformed by oncogenic H-Ras were selected from foci formed in soft agar as described previously [12]. A representative clone, called Ras7, was used in the previous and current studies [13].

2.3. Cell cycle analysis

Fractions of cells at different phases of the cell cycle were analyzed as previously described [33]. In brief, cells were collected and resuspended in 70% ethanol overnight at −20 °C. Before analysis, cells were pelleted again and incubated with a phospho-Ser/Thr-Pro MPM2 monoclonal antibody diluted in Dulbecco's phosphate-buffered saline for 1 h and then with a FITC-conjugated anti-mouse secondary antibody for 30 min. Cells were then resuspended in a propidium iodide (PI) solution consisting of 50 μg/ml PI, 50 μg/ml RNase A, 0.1% Triton X-100 and 0.1 mM EDTA for 15 min. Cells were then sorted using a FACSCalibur flow cytometer (BD Biosciences, San Jose, CA).

2.4. Cdc2 activity assays

Cdc2 kinase activity was measured as previously described [31]. Cells were lysed using a cell lysis buffer purchased from Cell Signaling Tech. (Beverly, MA) supplemented with a protease inhibitor cocktail purchased from Roche (Indianapolis, IN). The cell extracts were then immunoprecipitated using a Cdc2 monoclonal antibody and protein G-conjugated sepharose beads (Sigma, St. Louis, MO). The pellets were then washed thrice with lysis buffer and then incubated for 30 min at 37 °C with 20 mM HEPES, pH 7.9, 5 mM $MgCl_2$, 1 μg histone H1 (Roche), 1 mM EDTA and 100 μM ATP in a total volume of 20 μl. The samples were then examined by Western blotting using antibodies against phospho-histone H1 and histone H1.

2.5. siRNA transfection

Cells were transfected with small interfering RNA (siRNA) using a protocol similar to that described previously [13]. The *KLF5*-specific siRNA was designed to target against sequences corresponding to nucleotides between 875 and 895 of the coding region of mouse *KLF5* (GenBank Accession No. NM_009769). Control siRNA was also described previously [13]. Cells were cultured in 100-mm culture dishes until 40% confluent and then transfected with siRNA in Opti-MEM with reduced serum content (Invitrogen, Carlsbad, CA) using the Oligofectamine transfection reagent (Invitrogen) for 4 h. The cells were then provided with DMEM containing 10% FBS and 1% penicillin–streptomycin.

2.6. Measurement of mitotic indices

Mitotic indices were measured using Hoechst 33258 stain and phospho-histone H3 (Ser-10) antibodies. Cells were first fixed in a 3% formaldehyde solution in phosphate-buffered saline (PBS) for 15 min. For Hoechst staining, cells were then incubated at room temperature for 20 min after the addition of cold 100% methanol. After rinsing with PBS, a Hoechst 33258 solution was added to a final concentration of 0.2 μg/ml and samples were incubated at room temperature for 15 min. Samples are then rinsed again with PBS and nuclei were visualized using an inverted fluorescence microscope (Nikon, Melville, NY). Mitotic index was scored as the number of mitotic cells (with condensed nuclei) among a total population of 500 stained cells. For staining with anti-phospho-histone H3, protocol described in the Mitotic Index Hitkit (Cellomics, Pittsburgh, PA) was applied. In brief, cells were permeabilized using a permeabilization buffer after fixing cells with 3.7% formaldehyde solution, for 15 min. After rinsing with blocking solution, cells were then incubated with the primary antibody followed by the staining solution (containing Alexa 488 labeled secondary antibody and Hoechst dye) for 1 h each. Cells were then washed and nuclei were visualized using a fluorescent microscope. Mitotic index was scored as the number of phospho-histone H3-positive cells among a total population of 1000 cells counted.

3. Results

3.1. H-Ras-transformed NIH3T3 fibroblasts exhibit an increase in mitotic index, which is decreased by inhibition of KLF5

A clonal derivative of H-Ras-transformed NIH3T3 fibroblasts, called Ras7 [13], was transfected with *KLF5*-specific or non-specific siRNA and examined daily for mitotic indices using several independent methods. The non-transformed parental NIH3T3 cells and untransfected Ras7 cells were used as controls. Fig. 1 shows that the mitotic index, as measured by either Hoechst 33258 staining (Fig. 1A) or phospho-histone

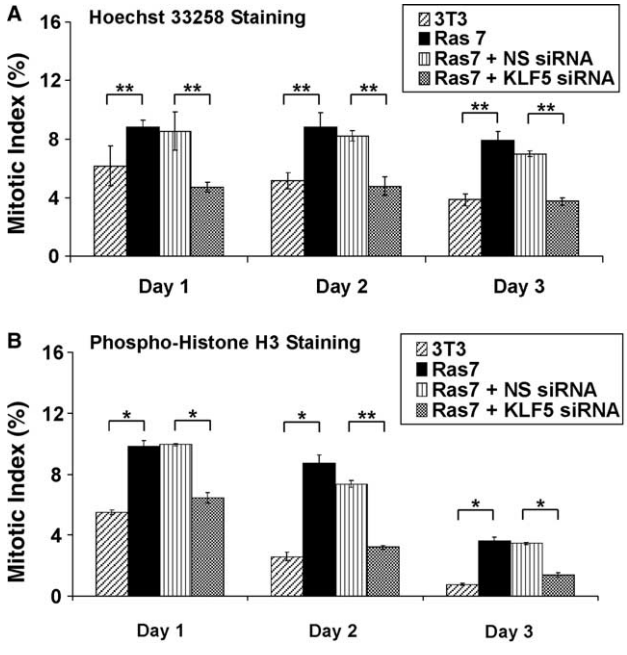

Fig. 1. KLF5 increases mitotic activity in oncogenic H-Ras-transformed NIH3T3 cells. (A) NIH3T3 cells, untransfected Ras7 cells, and Ras 7 cells transfected with non-specific (NS) control siRNA or *KLF5*-specific siRNA from day 1 to day 3 were stained with the Hoechst 33258 dye to label those cells in mitosis. The mitotic index is the percent of cells in mitosis among a total of 500 cells counted. $N = 4$; $^{**}P < 0.01$ by two-tailed Student's t test. (B) Cells were immuno-stained with a phospho-histone H3 antibody to label mitotic cells. Hoechst dye was used to stain nuclei. Mitotic index is represented as percent of phospho-histone H3-positive cells among a total of 1000 cells counted. $N = 3$; $^{*}P < 0.05$; $^{**}P < 0.01$ by two-tailed Student's t test.

H3 staining (Fig. 1B), was significantly higher in Ras7 cells when compared with the non-transformed parental NIH3T3 cells on all 3 days of culture. Transfection of Ras7 cells with *KLF5*-specific siRNA resulted in a significant reduction in mitotic index when compared to untransfected or control siRNA-transfected Ras7 cells.

To provide an additional means of measuring mitosis, we immuno-stained cells with a phospho-MPM2 antibody and sorted cells by FACS following staining with PI [33,34]. As shown in Fig. 2A, the population of MPM2-positive cells was significantly greater in Ras7 cells as compared to non-transformed NIH3T3 cells on days 2 and 3 of culturing. Again, transfection of Ras7 cells with *KLF5*-specific siRNA, but not non-specific siRNA, significantly reduced the proportion of MPM2-positive cells when compared to untransfected Ras7 cells. A similar finding is observed when the ratios between cells in the M and G_2/M phase of the cell cycle were measured (Fig. 2B). These results indicate that KLF5 is responsible for the increase in mitotic index and acceleration in the G_2/M transition of the cell cycle in Ras7 cells.

3.2. *H-Ras-mediated transformation of NIH3T3 fibroblasts is accompanied by an increase in the levels of cyclin B1 and Cdc2, and cylinB1/Cdc2 kinase activity, all of which are decreased by inhibition of KLF5*

As mitosis is controlled by cyclin B1 and Cdc2 [27,28], we measured KLF5, cyclin B1 and Cdc2 protein levels in NIH3T3 and Ras7 cells, as well as Ras7 cells treated with *KLF5*-specific siRNA or control siRNA, by Western blot analysis. As shown in Fig. 3A, Ras7 cells contained a higher level of KLF5, cyclin B1 and Cdc2 than NIH3T3 cells (compare lane 2 to lane 1). Transfection of Ras7 cells with *KLF5*-specific siRNA resulted in a reduction in the level of KLF5 and a concomitant reduction in the levels of cyclin B1 and Cdc2 (Fig. 3A, lane 4). In contrast, transfection of Ras7 cells with control non-specific siRNA did not change the levels of any of the three proteins (Fig. 3A, lane 3). Importantly, Fig. 3B shows that Ras7 cells contained a higher level of cyclin B1/Cdc2 kinase activity than NIH3T3 cells (compare lane 2 to lane 1) and that this increase was reduced in Ras7 cells treated with *KLF5*-speicfic siRNA but not control siRNA (compare lane 4 to lane 3). These results indicate that KLF5 is responsible for the increased cyclin

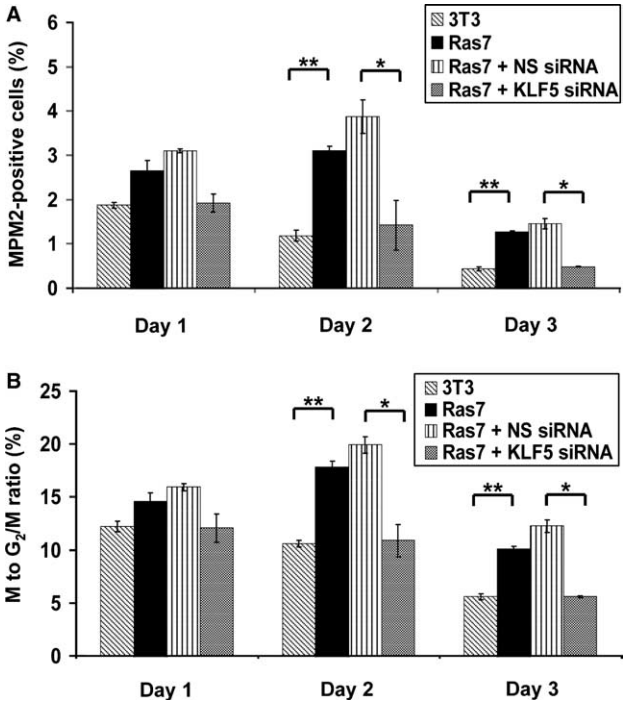

Fig. 2. KLF5 accelerates G_2/M transition of the cell cycle in H-Ras-transformed NIH3T3 cells. (A) Immuno-staining was performed with a phospho-Ser/Thr-Pro MPM2 antibody to identify cells in the M phase of cell cycle. Cells were then stained with PI to measure the DNA content and subjected to FACS analysis. The percentages of MPM2-positive cells among all cells analyzed were plotted at different time points. $N = 3$; $^*P < 0.05$; $^{**}P < 0.01$ by two-tailed Student's t test. (B) The ratio of M-phase population to G_2/M-phase population was calculated by dividing the number of MPM2-positive cells by those in the G_2/M phase (i.e., cells with a DNA content of $4N$). $N = 3$; $^*P < 0.05$; $^{**}P < 0.01$ by two-tailed Student's t test.

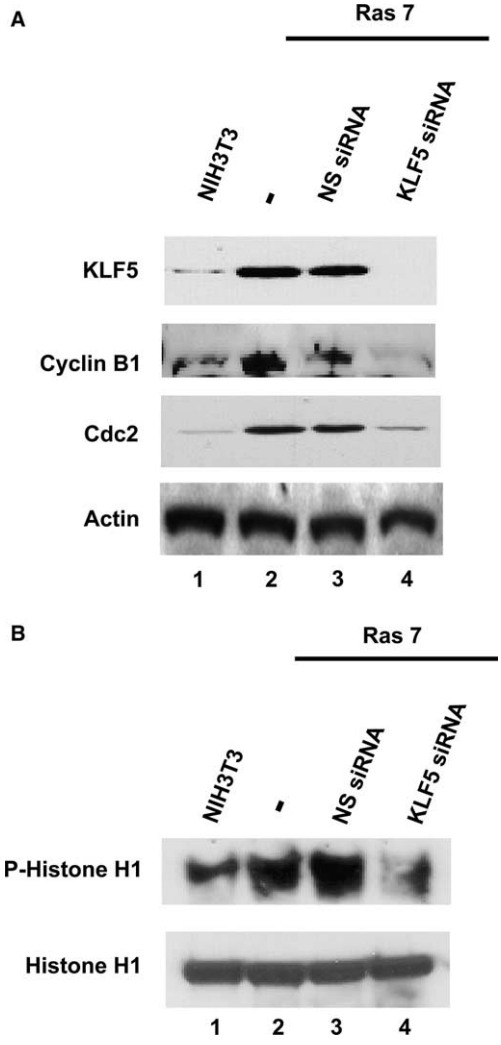

Fig. 3. Effect of KLF5 inhibition by siRNA on cyclin B1, Cdc2, and cyclin B1/Cdc2 kinase activity in H-Ras transformed cells. (A) The levels of KLF5, cyclin B1, and Cdc2, in NIH3T3 cells, untransfected Ras7 cells, and Ras7 cells transfected with non-specific (NS) control siRNA or *KLF5*-specific siRNA, were determined by Western blot analysis. Actin serves as a loading control. (B) The cyclin B1/Cdc2 kinase activity was measured by immunoprecipitation with a Cdc2 antibody followed by in vitro kinase reaction using histone H1 as a substrate. P-histone H1 is the phosphorylated product.

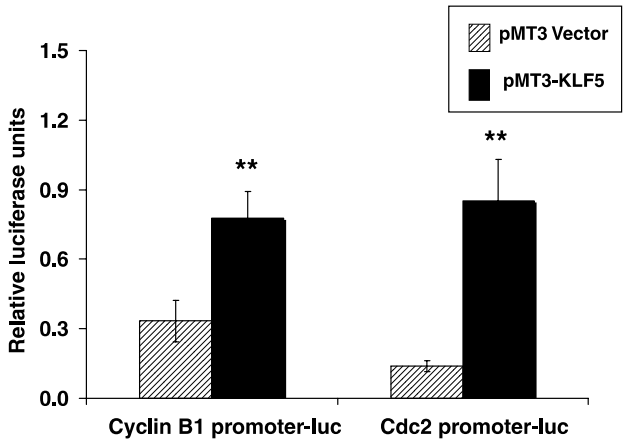

Fig. 4. KLF5 increases cyclin B1 and Cdc2 promoter activity. NIH3T3 cells were co-transfected with a luciferase plasmid containing cyclin B1 promoter (cyclin B1 promoter-luc) [30] or Cdc2 promoter (Cdc2 promoter-luc) [32] and either a control vector (pMT3 vector) or with a *KLF5*-expression vector (pMT3-KLF5). Luciferase activity was determined 2 days following transfection and normalized to the internal control, Renilla luciferase. $N = 4$; $^{**}P < 0.001$ when compared to PMT3 vector-transfected cells using two-tailed Student's t test.

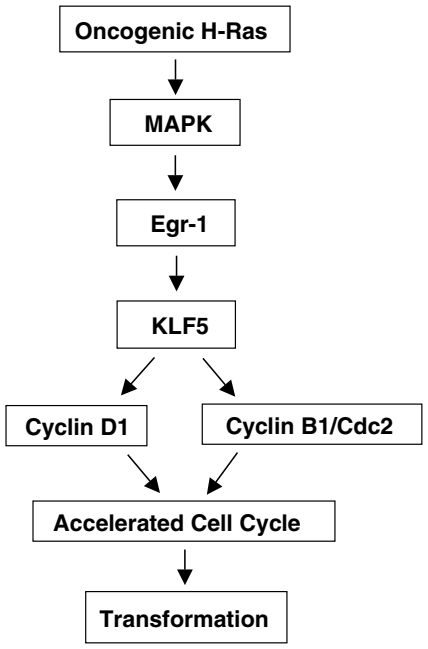

Fig. 5. A model for the role of KLF5 in mediating H-Ras-induced transformation. The induction of *KLF5* by H-Ras is mediated by MAPK and Egr-1 as previously demonstrated [13]. KLF5 then activates the transcription of the genes encoding *cyclin D1* [13] and *cyclin B1*/*Cdc2* (this study). The combined effect is an acceleration of the cell cycle, which eventually results in cellular transformation.

B1/Cdc2 kinase activity and that such an increase leads to the observed increase in mitotic activity in Ras7 cells.

3.3. KLF5 activates cyclin B1 and Cdc2 promoter activity

To determine the mechanism by which KLF5 increases cyclin B1 and Cdc2, we conducted co-transfection experiments using a *KLF5*-expression vector and a luciferase reporter construct containing the cyclin B1 or Cdc2 promoter. Cells co-transfected with an empty vector served as a control. As seen in Fig. 4, cells transfected with the *KLF5*-expression vector exhibited significantly higher activities from both cyclin B1 and Cdc2 promoters when compared to those transfected with the empty vector. These results indicate that KLF5 is a transcriptional activator of the *cyclin B1* and *Cdc2* genes.

4. Discussion

Cellular transformation caused by oncogenic Ras is characterized by increased cell proliferation, loss of contact inhibition and anchorage-independent cell growth [35,36]. Cell proliferation is generally associated with accelerated G_1/S and G_2/M cell cycle transitions [37]. The response of the G_1/S cell cycle machinery to Ras-induced MAPK stimulation is well established, consisting of the upregulation of cyclins D [23,38], E [39], and A, as well as their corresponding cyclin dependent kinases (Cdks) [40]. There is also evidence that the Ras signaling cascade can accelerate the G_2/M transition [41,42].

Ample evidence indicates that cyclin B1 is an important protein regulating proliferation. Depletion of cyclin B1 is found to inhibit proliferation and induce apoptosis in human tumor cells [43]. Previous studies also demonstrate that cyclin B1 is crucial for mediating oncogenic H-Ras-induced transformation [26,41]. The onset of mitosis in the cell is strictly regulated by several factors, among them cyclin B1 and Cdc2 [27,44]. The results of our study, which demonstrate that H-Ras-transformed fibroblasts exhibit increased cyclin B1/Cdc2 protein levels and activity, as well as increased mitotic index, are consistent with these previous findings.

We previously reported that transformation by oncogenic H-Ras results in an increase in the rate of cell proliferation and the S-phase population of cells [13]. In H-Ras-transformed cells, *KLF5* is up-regulated due to H-Ras-activated MAPK and the increased KLF5 is responsible for an increase in *cyclin D1* expression at the transcriptional level [13]. This leads to accelerated proliferation. In the current study, we identified cyclin B1 and Cdc2 as two novel targets of KLF5 upon its induction by H-Ras (Figs. 3A and 4). Consequently, there is an increase in cyclin B1/Cdc2 kinase activity, which becomes inhibited upon treatment of H-Ras-transformed cells with *KLF5*-specific siRNA (Fig. 3B). Importantly, inhibition of *KLF5* expression in H-Ras-transformed cells results in a reduction of mitotic index and the M-to-G_2/M ratio of cells (Figs. 1 and 2). These results demonstrate that KLF5 serves a crucial function in mediating the biological activity of H-Ras in accelerating the G_2/M transition, an event which promotes cellular transformation. A model describing the signal pathway as elicited by oncogenic H-Ras and mediated by KLF5 is illustrated in Fig. 5.

KLF5 has previously been shown to exert a pro-proliferative effect on non-transformed NIH3T3 fibroblasts and intestinal epithelial cells including IEC-6, IEC-18, and IMCE [12,45]. Stable expression of *KLF5* in NIH3T3 cells has also been shown to result in a transformed phenotype including increased proliferation and anchorage-independent growth [12]. In addition, all-*trans* retinoic acid (ATRA) inhibits proliferation of IEC-6 cells by inhibiting *KLF5* gene expression [14]. In contrast, over-expression of *KLF5* in several human cancer

cells has been reported to inhibit their growth in vitro [45,46]. Thus, KLF5 may exhibit different functions at different stages of tumor formation. It is of interest to note that a reason for KLF5's pleiotropic effect may be its differential regulation by the ubiquitin–proteasome pathway in non-transformed and cancer cells [47]. Nonetheless, the exact mechanism by which KLF5 affects growth of cancer cells remain to be established.

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