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Phosphatidylinositol 3-Kinase/Akt Mediates Integrin Signaling To Control RNA Polymerase I Transcriptional Activity

Chuanyue Wu,[a,b,c] Jin You,[a] Jijun Fu,[a] Xingshun Wang,[a] Yandong Zhang[a]

Department of Biology, South University of Science and Technology of China, Shenzhen, China[a]; Shenzhen Key Laboratory of Cell Microenvironment, South University of Science and Technology, Shenzhen, China[b]; Department of Pathology, University of Pittsburgh, Pittsburgh, Pennsylvania, USA[c]

RNA polymerase I-mediated rRNA production is a key determinant of cell growth. Despite extensive studies, the signaling pathways that control RNA polymerase I-mediated rRNA production are not well understood. Here we provide original evidence showing that RNA polymerase I transcriptional activity is tightly controlled by integrin signaling. Furthermore, we show that a signaling axis consisting of focal adhesion kinase (FAK), Src, phosphatidylinositol 3-kinase (PI3K), Akt, and mTOR mediates the effect of integrin signaling on rRNA transcription. Additionally, we show that in kindlin-2 knockout mouse embryonic fibroblasts, overactivation of Ras, Akt, and Src can successfully rescue the defective RNA polymerase I activity induced by the loss of kindlin-2. Finally, through experiments with inhibitors of FAK, Src, and PI3K and rescue experiments in MEFs, we found that the FAK/Src/PI3K/Akt signaling pathway to control rRNA transcription is linear. Collectively, these studies reveal, for the first time, a pivotal role of integrin signaling in regulation of RNA polymerase I transcriptional activity and shed light on the downstream signaling axis that participates in regulation of this key aspect of cell growth.

RNA polymerase I (Pol I) plays a central role in regulating cellular growth and proliferation (1). Eukaryotic cells contain hundreds of ribosomal DNA (rDNA) copies that occupy several different chromosomal locations (2). The production of rRNA can be divided into several steps, i.e., rRNA transcription, modification, and processing, all of which occur in the nucleolus (3, 4). The rate-limiting step is rRNA transcription (1, 5). On sensing of outside stimuli, a preinitiation complex comprised of the transcriptional factors upstream binding factor (UBF), SL1, TBP, Rrn3, and TTF assembles in the promoter region of rDNA. This complex then recruits RNA polymerase I to rDNA loci, and rRNA transcription starts (6–8). In mammalian cells, a single precursor rRNA transcript, 47S rRNA (14.3 kb), is transcribed from rDNA by the RNA polymerase I complex. This large polycistronic transcript encompasses 18S, 5.8S, and 28S rRNAs and includes several spacer regions, which are later processed into distinct rRNA species before assembly into preribosomal subunits (9).

The transcriptional activity of Pol I is a fundamental determinant of cell proliferation capacity (3). In rapidly proliferating cells, rRNA production takes more than 50% of all nuclear transcriptional activity. In yeast cells, this percentage can reach more than 80% (10). As such, the tremendous energy consumption demands tight control.

At the tissue level, cells attach to the extracellular matrix (ECM) through cell surface receptors termed integrins (11). Integrins are heterodimeric transmembrane receptors comprised of α subunits and β subunits that bind to extracellular ligands, such as laminin, collagen, vitronectin, and fibronectin. Different combinations of the 18 α subunits and 8 β subunits confer specificity on the integrin-ECM interactions (12). After binding to a specific ligand, integrins undergo a conformational change (13). Multiple integrins cluster together to trigger intracellular signaling via a concerted interaction between the integrin β subunits and intracellular proteins, such as talin and kindlin (14–17). The signal is then transmitted to catalytic proteins, such as focal adhesion kinase (FAK), which is a key component of the signal transduction pathways downstream of integrins (18). The cytosolic tyrosine kinase FAK undergoes autophosphorylation that leads to its association with other adaptor proteins or kinases. By selectively recruiting adapters, such as growth factor receptor-bound protein 2 (GRB2), integrins play an important role in stimulating the activity of Ras, which in turn activates mitogen-activated protein (MAP) kinase and phosphatidylinositol 3-kinase (PI3K)/Akt signaling cascades (19). Autophosphorylated FAK can also recruit another tyrosine kinase, Src, and leads to its phosphorylation and activation. Src signaling can further activate the Ras pathway and Rho GTPase to control cell proliferation and migration (20). Integrin signaling has been implicated in the regulation of diverse cellular activities. However, whether integrin activation controls RNA polymerase I transcriptional activity is not known.

To investigate whether integrin signaling controls rRNA synthesis, we analyzed the transcriptional activity of RNA polymerase I in response to integrin-mediated ECM adhesion. Through use of a nuclear *in situ* run-on assay, we found that RNA polymerase I activity is tightly regulated by integrin and kindlin-2 signaling. We further unveil the signaling axis underlying this regulation; we identified the FAK/Src/PI3K/Akt/

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Address correspondence to Chuanyue Wu, wucy@sustc.edu.cn, or Yandong Zhang, zhangyd@sustc.edu.cn.

C.W., J.Y., and J.F. contributed equally to this article.

mTOR pathway as the key downstream signaling pathway. Our results provide innovative findings about the importance of integrin and kindlin-2 in controlling a key aspect of cell growth.

MATERIALS AND METHODS

Cell culture. Mouse embryonic fibroblast (MEF) cells were isolated as described previously (21) and maintained in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 2 mM L-glutamine, and penicillin-streptomycin (PS).

Inhibitors. Saracatinib, U0126, PND1186, rapamycin, and wortmannin were purchased from Selleck. All these inhibitors were dissolved in dimethyl sulfoxide (DMSO). A stock solution of U0126 was made up to 100 mM, while stock solutions of the other three inhibitors were made up to 10 mM.

Antibodies. Anti-kindlin-2 antibody was obtained from Cell Signaling Technologies. Anti-pan-Akt, anti-phospho-AKT(308), an anti-Src and anti-pSrc (pSrc-Y416 and pSrc-Y527) sampler kit, anti-FAK, anti-pFAK (pFAK-Y397, pFAK-Y576/577, and pFAK-Y925), anti-extracellular signal-regulated kinase 1/2 (anti-ERK1/2), and anti-pERK1/2 (pERK1/2,T202/Y204) were also obtained from Cell Signaling Technologies. Anti-gamma-tubulin, anti-UBF, and anti-RPA194 were obtained from Santa Cruz Biotechnology. Anti-talin1 was purchased from Santa Cruz Biotechnology. Anti-pS6, anti-p4EBP1, and anti-S6 were purchased from Cell Signaling Technologies.

Plasmids. pLVX-RasV12 and pLKO.1-shARF were kind gifts from Jason Weber's lab at Washington University in St. Louis, MO. A pLKO.1 scrambled short hairpin RNA (shRNA) control plasmid was constructed by cloning a fragment of DNA containing sense and antisense strands of scrambled shRNA into pLKO.1. To clone shTalin1 into pLKO.1, the following validated sequence of murine talin1 (mTalin1) was used: 5′-CGCTCCAAGAGTATTATTAAT-3′. pMSCV-GFP-myr-Akt was purchased from Addgene. pMSCV-GFP was a kind gift from Jason Weber's lab at Washington University. pLNCX-chickenSrc (E378G) and pLNCX-chickenSrc (K295R) were purchased from Addgene.

Adenovirus infection. Adenoviruses were purchased from Vigene; adeno-LacZ or adeno-Cre has a titer of 1.0×10^{11}. MEFs at early passages were trypsinized, approximately 2.0×10^6 cells were resuspended in 3 ml of fresh DMEM containing 10% FBS-PS, and adenovirus was added to a multiplicity of infection (MOI) of 150 and incubated for 4 h at 37°C in a CO_2 incubator. Cells were then supplemented with 6 ml of fresh and complete DMEM and further incubated overnight. Cells were then replenished with fresh complete medium.

Lentivirus/retrovirus production. To produce a Ras overexpression virus, pLVX-Ras was cotransfected with pCMV-VSV-G and pHR8.2ΔR into 293T cells. To produce a lentivirus to knock down p19ARF and talin1 expression in MEFs, 293T cells were cotransfected with pLKO.1-shARF/pLKO.1-shTalin1, pCMV-VSV-G, and pHR8.2ΔR. To produce an Akt-overexpressing virus, 293T cells were cotransfected with ψ2 and pMSCV plasmids. To produce an Src-overexpressing virus, 293T cells were cotransfected with the pLNCX2, pUMVC, and pHR8.2ΔR plasmids. Culture supernatants were harvested 24 h and 48 h after transfection and then centrifuged at 2,000 rpm for 5 min.

Immunoblotting. Whole-cell lysates were prepared by incubation with whole-cell lysis buffer that included 0.5% NP-40 and 1% SDS supplemented with Halt protease and phosphatase inhibitors (Sigma). Lysates were cleared by centrifugation, and the protein concentration was tested by DC assay (Bio-Rad). Lysates were boiled in SDS sample buffer, separated by SDS-PAGE, and transferred to polyvinylidene difluoride membranes (Millipore). Membranes were blocked in 5% nonfat dry milk with TBS-T (10 mmol/liter Tris-HCl [pH 7.4], 150 mmol/liter NaCl, 0.1% Tween 20) buffer and incubated with primary antibodies diluted in blocking buffer at 4°C overnight. Blots were washed with TBS-T buffer and incubated with horseradish peroxidase-conjugated secondary antibodies (1:10,000; GE Healthcare) in blocking buffer at room temperature. Immune complexes were visualized with an enhanced chemiluminescence kit (GE Healthcare).

***In situ* nuclear run-on assay.** Subconfluent cells were plated onto coverslips precoated with fibronectin and then incubated for 30 min, and cells were then pulsed with 2 mM 5-fluorouridine (5-FUrd) for 10 min and fixed with 10% formalin-10% methanol. Cells were then incubated with mouse antibromodeoxyuridine (anti-BrdU; Sigma) at a 1:100 dilution. Goat anti-mouse antibody–rhodamine was applied to facilitate the visualization of newly synthesized RNA.

Chromatin immunoprecipitation (ChIP). Four vials of MEFs at passage 1 were taken from a liquid nitrogen tank, thawed rapidly in a 37°C water bath, and then plated in 100-mm dishes. The next day, cells were replenished with fresh medium and cultured for 2 more days until the cells reached 95% confluence. On the day of the experiment, we first coated 4 100-mm dishes with fibronectin. Cells were then trypsinized and resuspended in 40 ml of fresh DMEM. From this suspension, 5 ml of cells was added to each fibronectin-coated plate (total of 4 plates), and 5 ml of cells was added to regular plates without fibronectin treatment (total of 4 plates). Cells were then gently shaken well to make sure that they were distributed equally across the whole plates. The plates were placed in a 37°C CO_2 incubator and incubated for 30 min. Formaldehyde was added to a final concentration of 1% at room temperature for 10 min with a shaker to cross-link DNA and its interacting proteins. To stop cross-linking, 1.25 M L-glycine was added to a final concentration of 0.125 M, and samples were shaken at room temperature for another 5 min. Cell scrapers were used to gently scrape the cells off the plates. After washing with 1× phosphate-buffered saline (PBS), the cells were resuspended in a lysis buffer containing 1% SDS, 10 mM EDTA, and 50 mM Tris (pH 8.1) with protease and phosphatase inhibitors. To shear chromatin, cell lysates were sonicated extensively, centrifuged to pellet debris, and then diluted in a buffer containing 0.5% NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl at a 1:5 ratio. Cell lysates were precleared by incubation with 2.5 μg of sheared salmon sperm DNA and 50 μl protein A/G beads for 30 min at 4°C. Following incubation with 5 μg of antibody overnight at 4°C, 2.5 μg sheared salmon sperm DNA and 50 μl of protein A/G beads were added and then further incubated for 1 h. The beads were then washed twice in radioimmunoprecipitation assay (RIPA) buffer, twice in RIPA buffer containing 500 mM NaCl, and once with buffer containing 0.5% NP-40, 50 mM Tris (pH 7.5), and 150 mM NaCl. The beads were then extracted three times with a solution containing 1% SDS and 0.1 M $NaHCO_3$. To reverse the cross-linking, 6 M NaCl was added to the pooled extraction samples, to a final concentration of 0.3 M, and samples were heated at 65°C for 5 h. DNA fragments were extracted by use of a Qiagen QuickSpin column and eluted. Quantitative PCR (qPCR) was performed with these purified DNA samples.

Quantitative PCR. The primers were purchased from Life Technologies. Total RNA was extracted by use of a NucleoSpin II (Clontech) RNA isolation kit and was reverse transcribed into cDNA by use of a SuperScript III first-strand synthesis kit (Invitrogen). PCRs were performed with a Step One Plus thermal cycler. For analysis of 47S rRNA transcript levels, SYBR green mix (Bio-Rad universal mix) was used, and transcript quantification was performed by comparison with standard curves generated from dilution series of cDNA for human 47S rRNA. SYBR green mix from Bio-Rad was used for all other quantitative real-time PCR (qRT-PCR) analyses. Transcript quantification was calculated based on the $\Delta\Delta C_T$ value after normalization to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) values. Melting curve analysis confirmed that single products were amplified.

RESULTS

Integrin-mediated ECM adhesion stimulates RNA polymerase I transcriptional activity. To study the effects of integrin-mediated ECM adhesion on rRNA synthesis, we monitored the transcrip-

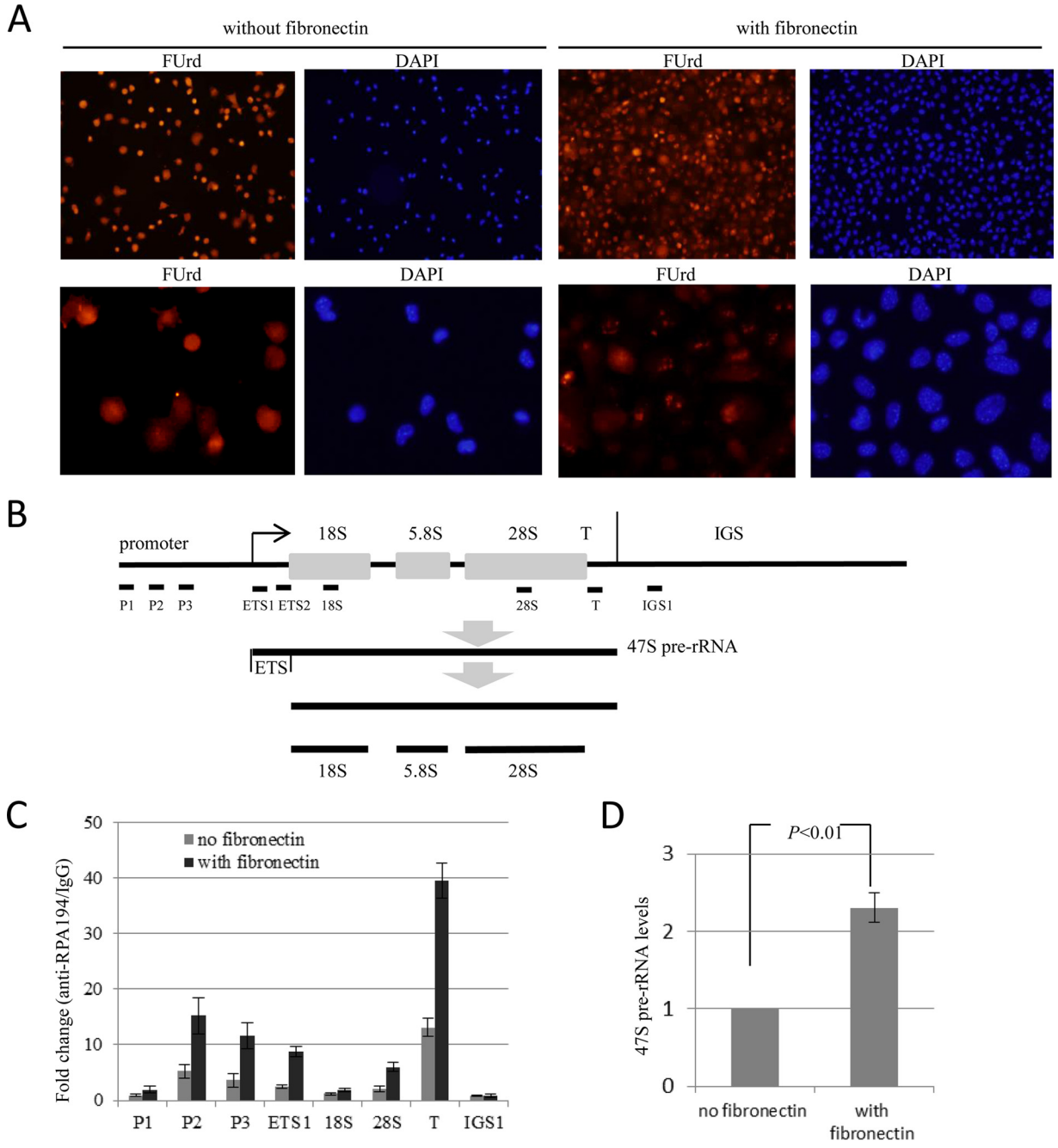


FIG 1 Integrin-mediated cell-ECM adhesion stimulates RNA polymerase I transcriptional activity. (A) A 5-FUrd *in situ* nuclear run-on assay was performed on MEFs cultured on coverslips precoated or not precoated with fibronectin. MEFs were added equally to coverslips precoated or not precoated with fibronectin at 10 μg/ml. Twenty-five minutes later, cells were then pulsed with 2 mM 5-FUrd for 10 min. Cells were then instantly fixed and stained with anti-BrdU antibody to visualize newly synthesized RNA *in situ*. (Top) Representative images. Magnification, ×10. (Bottom) Representative images. Magnification, ×40. Red, 5-FUrd; blue, DAPI (4′,6-diamidino-2-phenylindole). (B) Diagram depicting transcribed, nontranscribed, and promoter regions of rDNA and the locations for primer sets used for chromatin immunoprecipitation (ChIP). IGS, intergenic sequence. (C) MEFs added to coverslips precoated or not precoated with fibronectin were cross-linked by 1% formaldehyde, lysed, and incubated with nonimmune mouse IgG or an antibody recognizing RPA194. PCR analysis using the above-mentioned rDNA primers was used to detect DNA sequences associated with immunoprecipitation. Error bars are for two independent ChIP analyses. (D) MEFs cultured on coverslips precoated or not precoated with fibronectin were analyzed for pre-rRNA levels. Standard deviations were calculated for three separate experiments.

tion of ribosomal genes in mouse embryonic fibroblasts (MEFs). The large copy number and tandem array distribution of ribosomal transcription units allow measurement of the transcription kinetics in real time (4). After we added MEFs to fibronectin-coated coverslips for 25 min, we then exposed MEFs to the uridine analog 5-fluouridine for only 10 min. We detected the incorporation of 5-fluouridine in cells after fixation and staining. As shown in Fig. 1A, on fibronectin-coated coverslips, we found that most

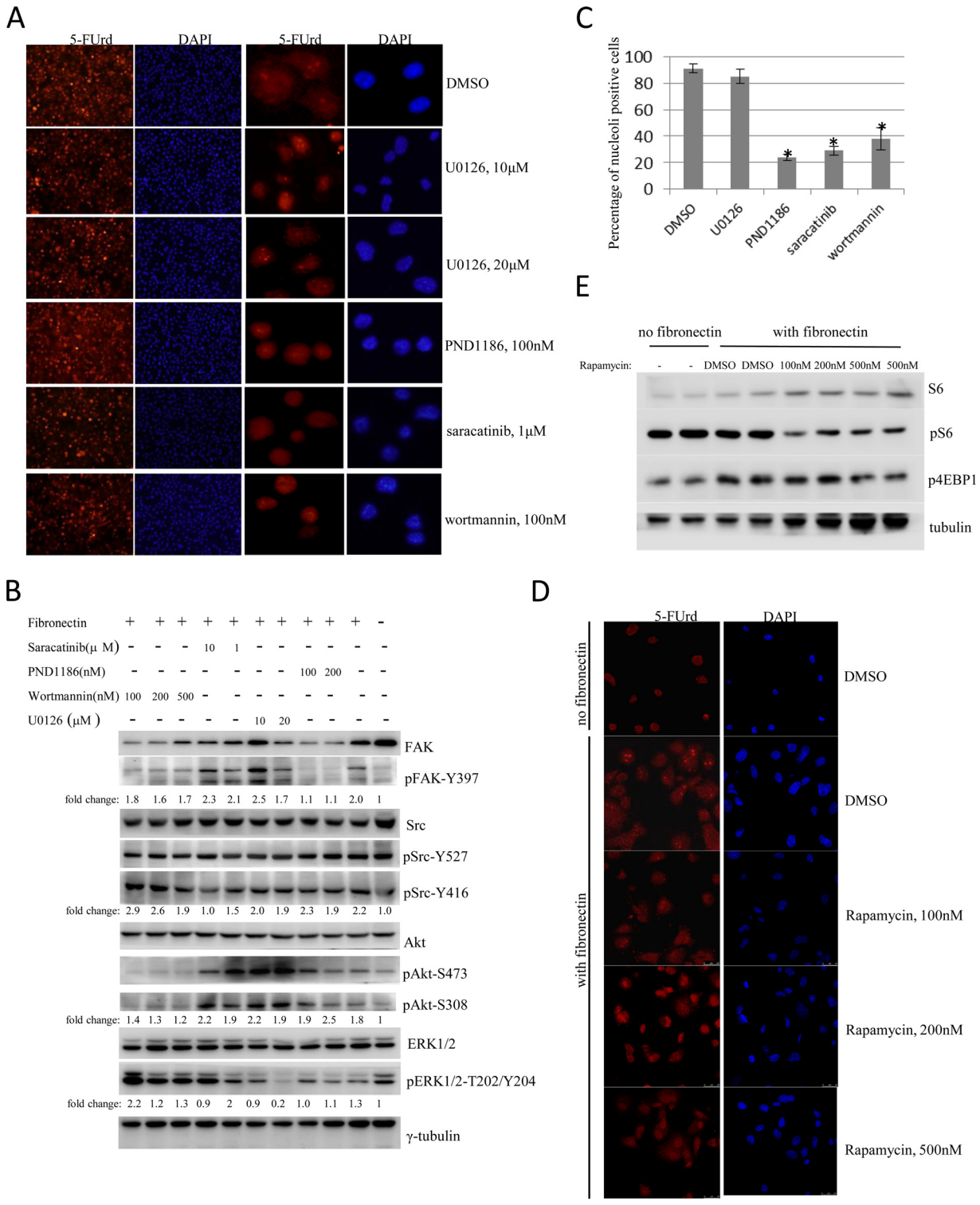


FIG 2 Signaling axis during integrin-mediated RNA polymerase I activation. (A) Cells were added equally to coverslips pretreated with fibronectin at 10 μg/ml. Kinase inhibitors were then applied to these cells at the indicated concentrations. U0126 is a MAP kinase inhibitor, PND1186 is an FAK kinase inhibitor, saracatinib is an Src kinase inhibitor, and wortmannin is a PI3K inhibitor. Twenty-five minutes later, cells were pulsed with 2 mM 5-FUrd for 10 min. Cells were then instantly fixed and stained with anti-BrdU antibody to visualize newly synthesized RNA *in situ*. Representative images are shown. (First column) 5-FUrd staining. Magnification, ×10. (Third column) 5-FUrd staining. Magnification, ×40. (Second and fourth columns) DAPI staining. (B) Western blotting was performed on the above-mentioned cells after treatment with different inhibitors. To verify the activation or inhibition of typical kinases in integrin signaling, FAK, Src, Akt, and ERK1/2 were each analyzed for total kinase and phosphorylated kinase levels. Fold changes were calculated after quantitation of the signal strength and normalization with γ-tubulin levels. (C) Quantitation of 5-FUrd (nucleolus)-positive cells from the above-mentioned samples. Error bars show standard deviations for three separate counts of 5-FUrd (nucleolus)-positive cells from a population of 50 nuclei. *, $P < 0.01$ ($n = 3$). (D) Cells were added equally to coverslips pretreated with fibronectin at 10 μg/ml. Rapamycin was then applied to these cells at the indicated concentrations. Twenty-five minutes later, cells were pulsed with 2 mM 5-FUrd for 10 min. Cells were then instantly fixed and stained with anti-BrdU antibody to visualize newly synthesized RNA *in situ*. Representative images are shown. (Left column) 5-FUrd staining. Magnification, ×63. (Right column) DAPI staining. (E) Western blotting was performed on the above-mentioned cells with the indicated antibodies after treatment with different inhibitors.

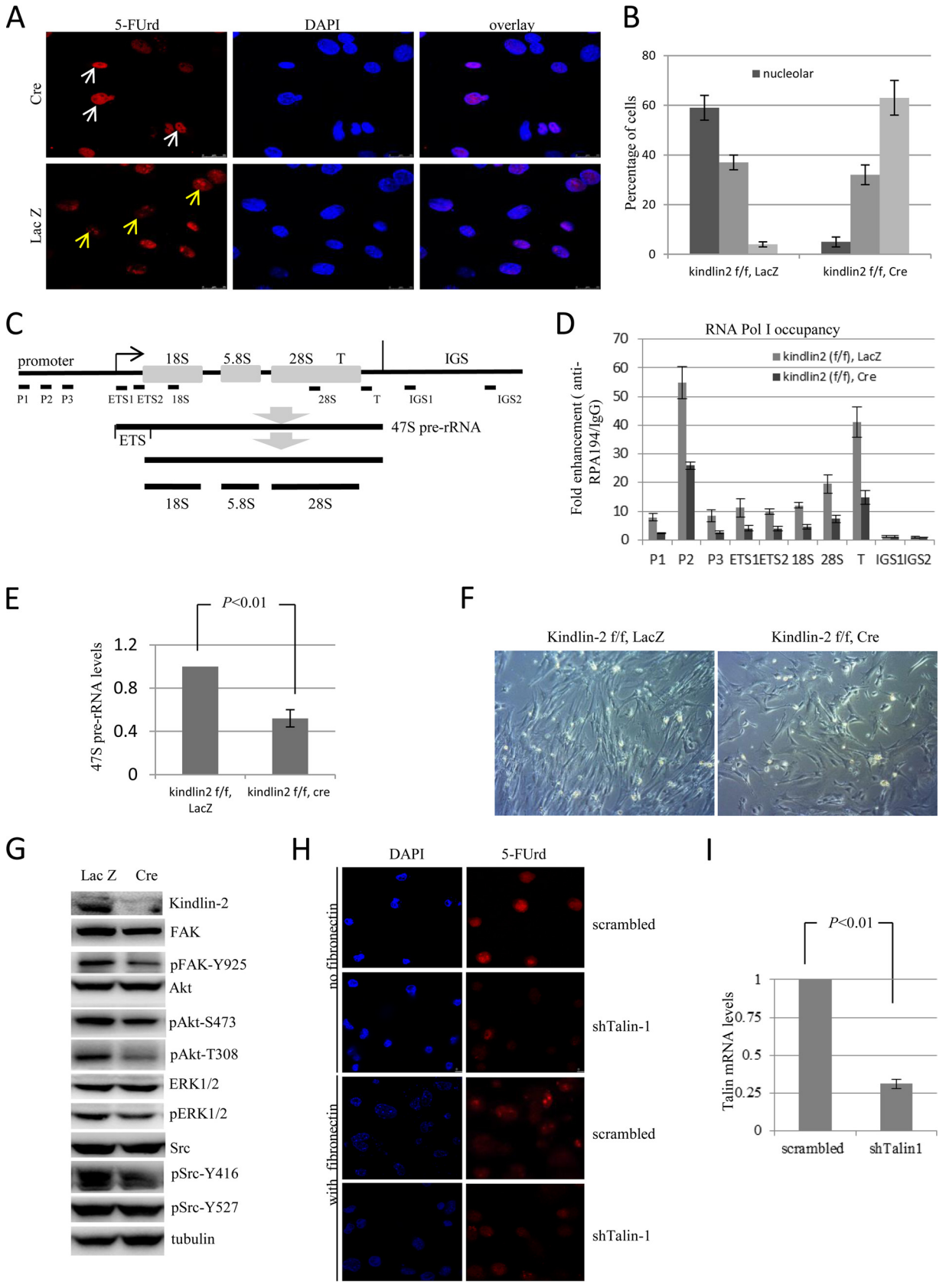


FIG 3 Loss of kindlin-2 causes a reduction of RNA polymerase I transcriptional activity. (A) Kindlin-$2^{flox/flox}$ MEFs were infected with an adenovirus encoding either LacZ or Cre recombinase at a low MOI (MOI = 150). At 3 days postinfection, cells were then analyzed for RNA polymerase I transcriptional activity by the

cells demonstrated strong and distinct nucleolar staining as opposed to nuclear staining, while without fibronectin, cells showed nuclear staining without distinct and punctate nucleolar staining. Because this nuclear *in situ* run-on assay has been regarded as the gold standard for detecting real-time RNA transcription activity in cells (22), we believe that the different RNA transcriptional activity indicated that integrin signaling controls RNA polymerase I activity.

To study the mechanism underlying the increase in RNA polymerase I transcriptional activity, cells with or without fibronectin stimulation were analyzed for RNA polymerase I occupancy on the rDNA gene. As shown in Fig. 1B and C, after fibronectin stimulation, we found that the loading of RNA polymerase I was significantly increased compared to that for cells without fibronectin stimulation. We also analyzed the pre-rRNA levels in MEFs with or without fibronectin stimulation (Fig. 1D), and we found that fibronectin treatment for 30 min stimulated ribosomal transcription up to 2-fold. The level of 47S pre-rRNA has been regarded as a reflection of RNA polymerase I activity (23). These data strongly suggest that integrin activation signals to cells to produce rRNA and that the response is very quick.

The FAK-Src-PI3K signaling axis controls RNA polymerase I activation. To study which signaling axis controls the activity of RNA polymerase I during integrin-mediated ECM adhesion, we first used several specific kinase inhibitors to block typical downstream kinases. These include FAK, Src, PI3K, and MAP kinase. As shown in Fig. 2A, we used PND1186 at various concentrations (from 100 nM to 200 nM) to inhibit FAK kinase activity. The FAK inhibitor PND1186 at 100 nM and 200 nM efficiently reduced the phosphorylation of FAK at Y397 and Y925, as shown in Fig. 2B. At 100 nM, the nucleolar staining of 5-fluorouridine decreased drastically (Fig. 2A), indicating that phosphorylation of focal adhesion kinase is critical for integrin activation-induced rRNA production. We used saracatinib at different concentrations to inhibit Src kinase activity, as shown in Fig. 2B. At 1 μM and 10 μM saracatinib, the phosphorylation of Src was significantly decreased. Accordingly, from Fig. 2A, we found that the transcriptional activity of RNA polymerase I in the nucleoli was abolished. These results clearly showed that both FAK and Src are pivotal kinases downstream of integrin to stimulate RNA polymerase I activity. Next, because the PI3K/Akt pathway and the Ras/Raf/ERK/MAP pathway are two major signaling pathways downstream of Src, we sought to test which one can control rRNA transcription. To do this, we used U0126 at various concentrations to inhibit MAP kinase and wortmannin at various concentrations to inhibit PI3K. As shown in Fig. 2A, U0126 at both 10 μM and 20 μM did not decrease RNA polymerase I transcriptional activity significantly, despite the fact that phosphorylation of ERK1/2 was drastically inhibited. However, wortmannin at 100 nM readily reduced the transcription of rRNA. The Western blot in Fig. 2B shows that at 100 nM wortmannin, the phosphorylation of Akt on Thr308 was reduced. From all the above-described experimental results, we believe that the FAK/Src/PI3K pathway constitutes the major signaling axis to control RNA polymerase I transcriptional activity after integrin activation.

We further quantitated the percentage of nucleolus-positive cells by 5-FUrd analysis as shown in Fig. 2C. The results are consistent with the previous imaging data, demonstrating that inhibition of the FAK/Src/PI3K pathway causes a significant reduction of rRNA transcription.

mTOR plays an important role in mediating integrin signaling to control Pol I transcriptional activity. To further unravel the signaling mechanisms downstream of PI3K during integrin activation to control RNA polymerase I transcriptional activity, cells were further treated with the mTOR inhibitor rapamycin at various concentrations. As shown in Fig. 2E, rapamycin at 100 nM, 200 nM, and 500 nM inhibited the activity of mTOR efficiently, as shown by the reduced phosphorylation of S6 and eIF4E-BP, two prominent substrates for the mTOR Ser/Thr kinase. Decreased activity of mTOR had a dramatic effect on cells after fibronectin stimulation. They demonstrated a drastic reduction in nucleolar staining by 5-FUrd, as shown in Fig. 2D. These data indicate that mTOR plays an important role in mediating integrin signaling to promote RNA polymerase I transcriptional activity.

Loss of kindlin-2 causes inhibition of RNA polymerase I transcriptional activity. Kindlin-2 has been shown to cooperate with talin to mediate integrin activation; multiple lines of evidence suggest that kindlin-2 plays pivotal roles in controlling both the outside-in and inside-out signaling of integrin (14, 24, 25). To study whether kindlin-2 is important for RNA polymerase I transcriptional activity, we analyzed RNA transcription in MEFs in the absence of kindlin-2. We infected kindlin-$2^{flox/flox}$ MEFs with an adenovirus encoding either LacZ or Cre at a low MOI to deplete endogenous kindlin-2. After infection, cells were analyzed by the nuclear *in situ* run-on assay with a 5-fluorouridine pulse for a short time. As shown in Fig. 3A, we found that there was a significant decrease in rRNA transcription, as shown by the unstained nucleoli in a majority of cells. This phenomenon was observed at 3 days post-adenovirus infection, when kindlin-2 was found to be depleted (Fig. 3G), while the nucleus still showed transcriptional activity comparable to that of the wild type. A quantitation of the nucleolus-positive and nucleolus-

5-FUrd *in situ* nuclear run-on assay. Images are shown for 5-FUrd (red) and DAPI (blue). Magnification, ×63. White arrows indicate no nucleolar staining (nucleolus exclusive), and yellow arrows indicate nucleolar staining. (B) Quantitation of the 5-FUrd (nucleolus)-positive, all-nuclear, and nucleolus-exclusive cells from the above-mentioned samples. Error bars show standard deviations for three separate counts for each type of 5-FUrd-positive cells from a population of 50 nuclei. kindlin2 f/f, kindlin-$2^{flox/flox}$. (C) Diagram depicting transcribed, nontranscribed, and promoter regions of rDNA and the locations of primer sets used for ChIP. (D) After adenovirus infection, the above-mentioned kindlin-$2^{flox/flox}$ MEFs were cross-linked by 1% formaldehyde, lysed, and incubated with nonimmune mouse IgG or antibodies recognizing RPA194. PCR analysis using the above-mentioned rDNA primers was used to detect DNA sequences associated with immunoprecipitation. The graph shows ChIP data for two independent ChIP analyses. (E) The above-mentioned cells were harvested for total RNA extraction, and pre-rRNA was analyzed by quantitative real-time PCR. Error bars show standard deviations for 3 separate experiments. (F) Kindlin-$2^{flox/flox}$ MEFs were infected with an adenovirus encoding either LacZ or Cre recombinase at a low MOI (MOI = 150). Typical images are shown for live cultures of the above-mentioned cells at 3 days postinfection. (G) Cells were analyzed by Western blotting with the indicated antibodies. (H) Kindlin-$2^{flox/flox}$ MEFs were infected with a lentivirus encoding either scrambled shRNA or shTalin1. At 3 days postinfection, cells were then analyzed for RNA polymerase I transcriptional activity by the 5-FUrd *in situ* nuclear run-on assay. (Left column) DAPI staining. Magnification, ×63. (Right column) 5-FUrd staining. Magnification, ×63. (I) Cells were analyzed by qPCR to detect mRNA levels of talin1 ($n = 3$).

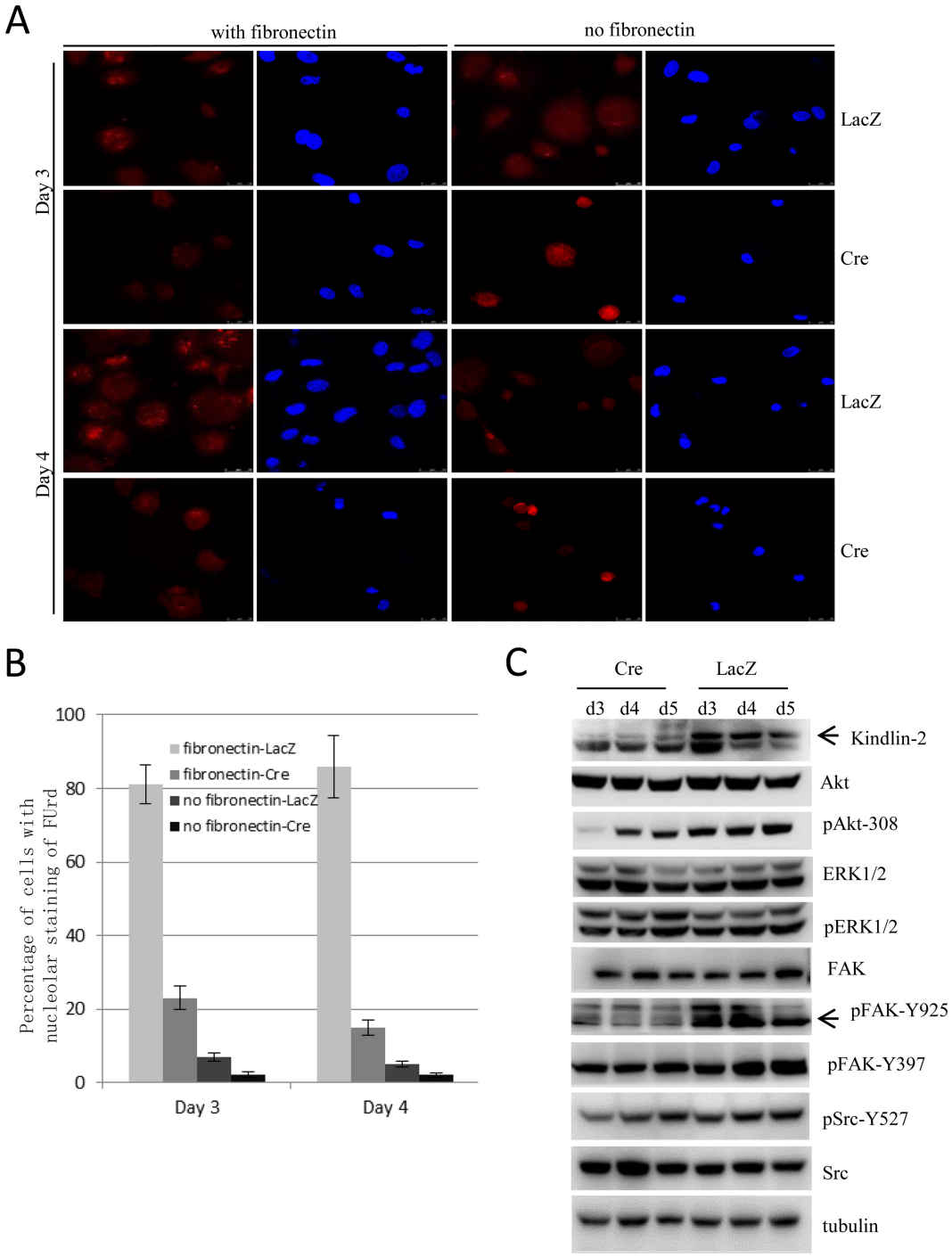


FIG 4 Kindlin-2 mediates integrin activation to control RNA polymerase I transcriptional activity. (A) Kindlin-$2^{flox/flox}$ MEFs were infected with an adenovirus encoding either LacZ or Cre at an MOI of 150 at early passages (P1 and P2). Cells were then trypsinized and plated onto coverslips, with or without fibronectin treatment. At the indicated time points postinfection, cells were further incubated for 25 min in a CO_2 incubator at 37°C and then pulsed with 2 mM 5-FUrd for 10 min. Cells were then instantly fixed with 10% methanol-10% formaldehyde in PBS. A standard protocol was followed to analyze RNA polymerase I activity. Representative images are shown. Magnification, ×63. (B) Quantitation of 5-FUrd (nucleolus)-positive cells from the above-mentioned samples. Error bars show standard deviations for three separate counts of 5-FUrd (nucleolus)-positive cells from a population of 50 nuclei. (C) The above-mentioned cells were harvested for Western blot analysis with anti-kindlin-2 antibody and anti-γ-tubulin.

exclusive populations is shown in Fig. 3B. We further analyzed the RNA polymerase I occupancy of the rDNA region by chromatin immunoprecipitation after adenovirus infections of kindlin-$2^{flox/flox}$ MEFs. As shown in Fig. 3C and D, we found that depletion of kindlin-2 reduced RNA polymerase I occupancy of the rDNA region; this region includes the promoter, the rRNA-encoding region, and the termination region but not the intergenic region. Our results suggest that kindlin-2 plays

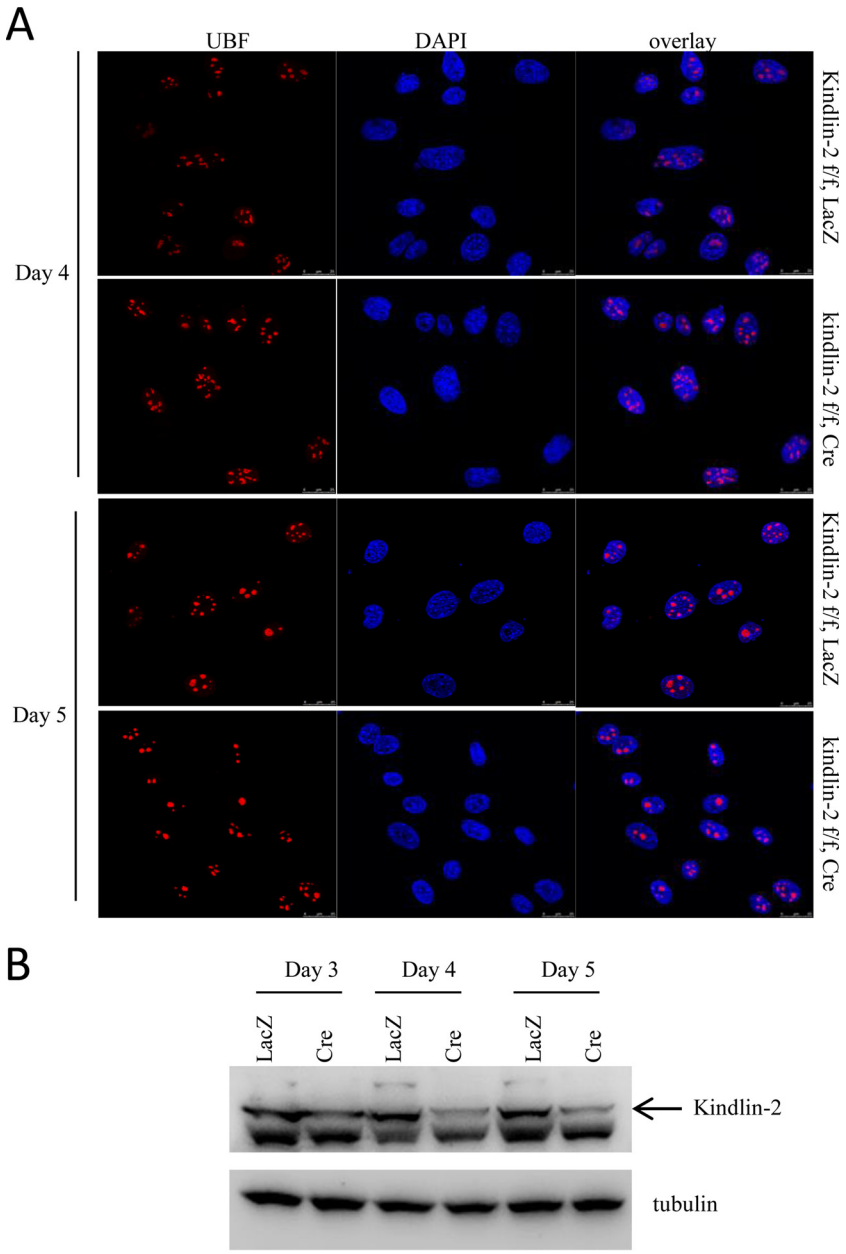


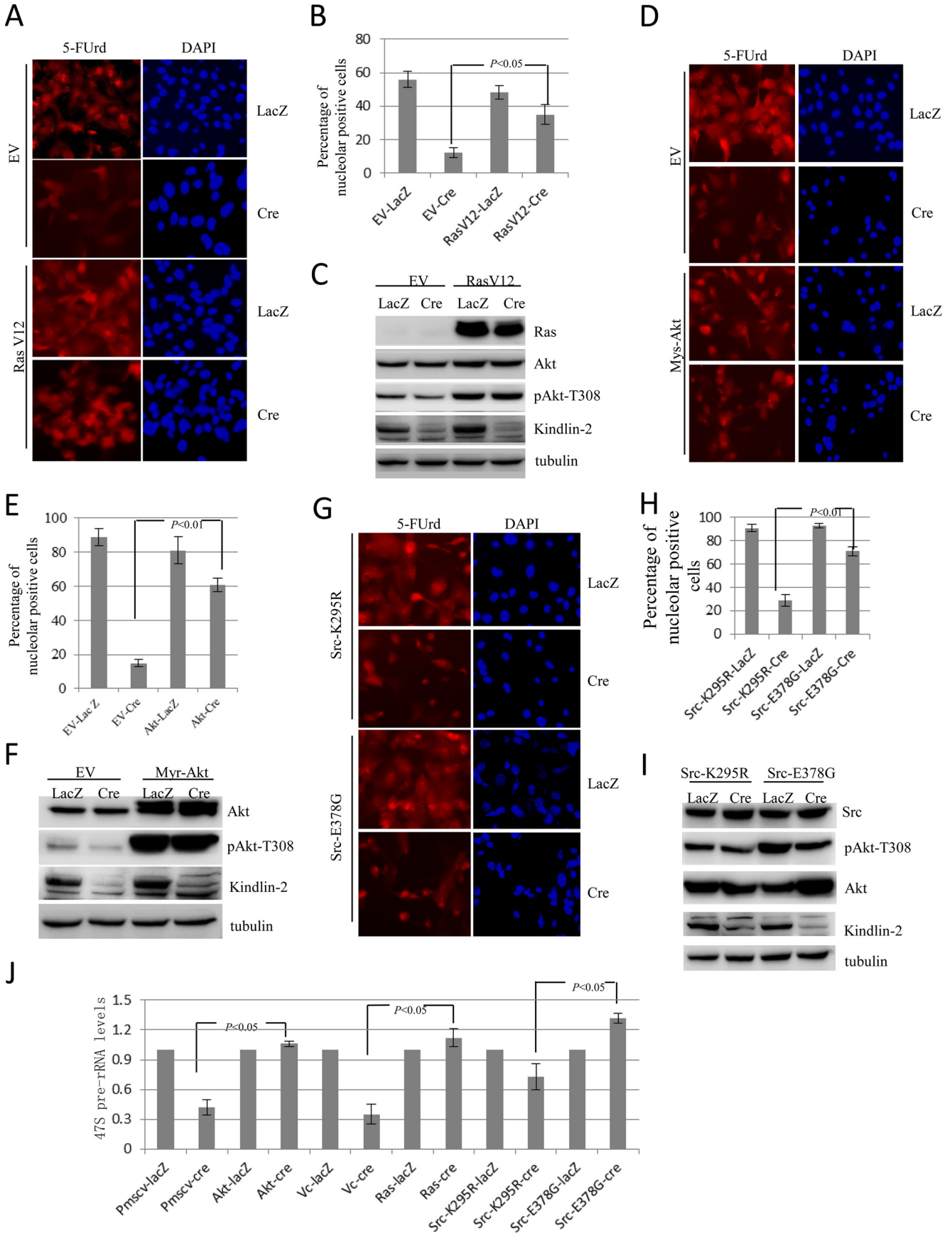
FIG 5 Nucleoli are intact in cells with defective integrin signaling. (A) Kindlin-2$^{flox/flox}$ cells were infected with an adenovirus encoding either LacZ or Cre at an MOI of 150 at early passages (P1 and P2). Immunofluorescence microscopy was performed on these cells at the indicated time points, using antinucleophasmin (anti-NPM) antibody. Nuclei are demarcated by DAPI staining. A typical image is shown for each sample. (B) Western blot analysis at the indicated time points after adenovirus infection for detection of kindlin-2 protein levels, with γ-tubulin as an internal control.

pivotal roles in controlling rRNA transcription. The steady-state level of pre-rRNA per cell was also reduced in kindlin-2-deficient cells (Fig. 3E): the relative amount was decreased to approximately 50% of the control level on a single-cell basis. At 3 days post-adenovirus infection, MEFs still remained attached to the plates, albeit the cell sizes appeared to be smaller (Fig. 3F).

We analyzed the effects of kindlin-2 depletion on the phosphorylation states of key signaling kinases. As shown in Fig. 3G, depletion of kindlin-2 markedly inhibited the activating phosphorylation of Src, FAK, and Akt.

Talin1 depletion impairs RNA polymerase I transcriptional activity. Kindlin-2 has been shown to be a coactivator of talin in mediating integrin signaling. To analyze whether talin itself plays an important role, we further analyzed Pol I activity in talin1-depleted cells. As shown in Fig. 3H and I, we found that depletion of talin1 decreased RNA polymerase I transcriptional activity, as expected by the 5-FUrd assay.

Kindlin-2 mediates integrin signaling to control RNA polymerase I transcriptional activity. Kindlin-2 has been shown to possess functions independent of integrin activation (26). To further test whether kindlin-2 depletion results in



defective integrin activation, we also analyzed the effect of kindlin-2 depletion on RNA polymerase I transcriptional activity in cells that were stimulated with fibronectin. As shown in Fig. 4A, at both 3 days and 4 days post-adenovirus infection, cells were analyzed for rRNA synthesis after stimulation with fibronectin. We found that for the LacZ control group, distinct nucleolar staining indicated strong polymerase I transcription, while in the Cre samples, the majority of cells demonstrated little or no distinct nucleolar staining. The quantitation of nucleolus-positive cells is shown in Fig. 4B. The protein levels of kindlin-2 in MEFs after adeno-Cre and adeno-LacZ infections are shown in Fig. 4C. The above results strongly suggest that defective integrin signaling causes a loss of RNA polymerase I transcriptional activity.

Depletion of kindlin-2 does not alter nucleolar integrity. Because the nucleolus is a sensor for various kinds of cellular stresses (27), stress signals can be transmitted to the nucleolus and sometimes result in a disruption of nucleolar integrity (28). Thus, we sought to analyze whether the reduction of RNA polymerase I transcriptional activity occurred due to nucleolar disruption or disintegration. We analyzed the morphology of nucleoli by fluo rescence microscopy after depletion of kindlin-2. As shown in Fig. 5A, we found by staining of nucleoli with UBF that the nucleoli were not disrupted at any time point post-adenovirus infection. The Western blots in Fig. 5B confirm that kindlin-2 protein levels were reduced from day 3 until day 5 post-adenovirus infection.

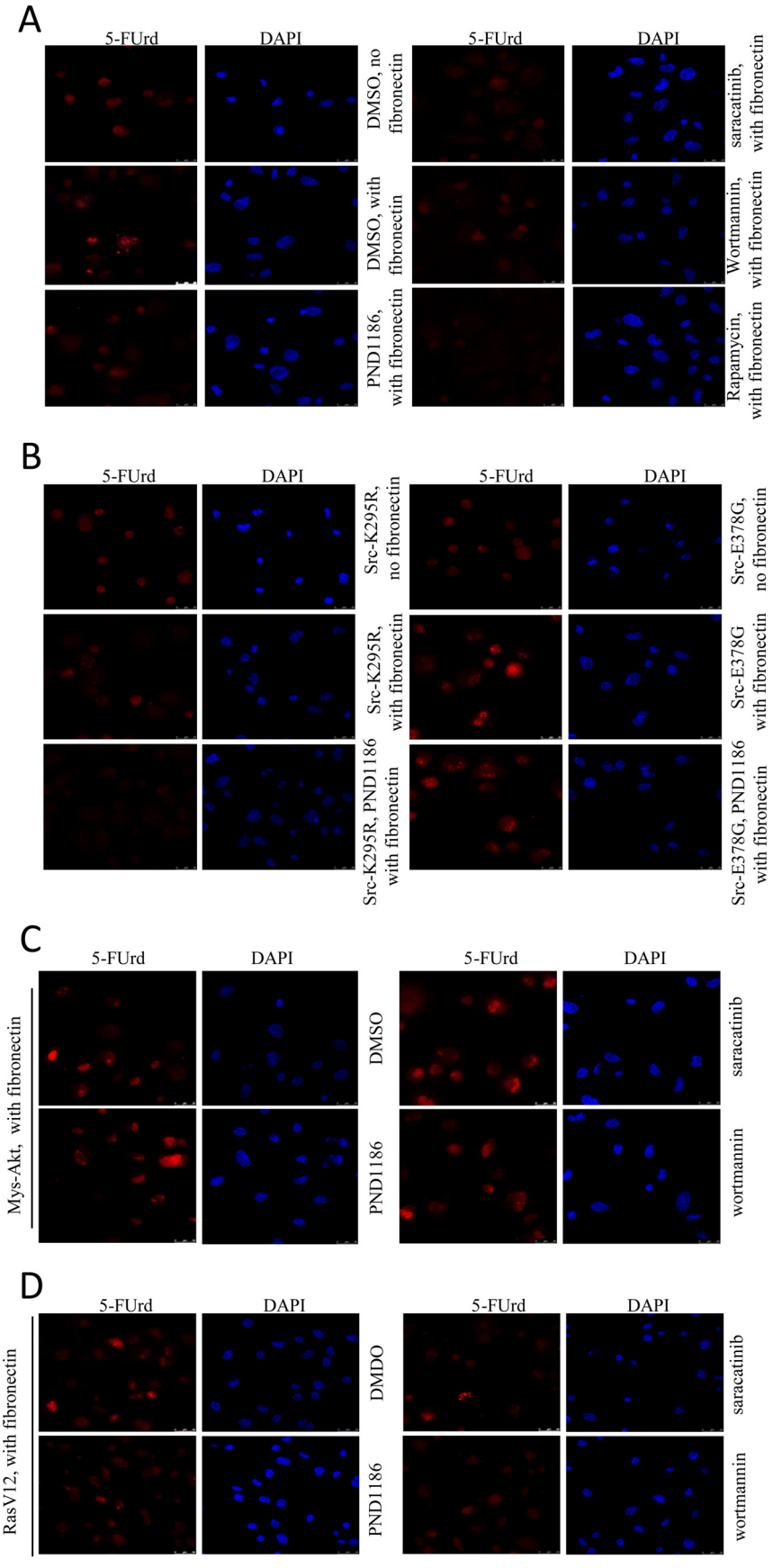
Overactivation of Ras, Akt, and Src can rescue RNA Pol I activity in MEFs with defective integrin signaling. To further investigate the signaling axis downstream of integrin that controls the RNA polymerase I transcriptional activity, we performed a rescue assay in the absence of kindlin-2 in MEFs through overactivation of Ras, Src, and Akt. To do this, we first immortalized MEFs by depleting the tumor suppressor ARF. A deficiency of ARF does not influence the genomic integrity but allows these cells to overcome the Hayflick limit and to bypass the cellular senescence caused by overexpression of oncogenic products, such as oncogenes (29, 30). After these immortalized cells were infected with lentiviruses encoding Ras, Src, and Akt, they were then infected by an adenovirus encoding Cre recombinase to deplete kindlin-2 or the control LacZ protein. The Western blots in Fig. 6C, F, and I confirmed the overexpression of each gene and the deficiency of kindlin-2. We then detected the RNA transcription activity in real time by performing the nuclear *in situ* run-on assay after plating cells on fibronectin-coated coverslips. As shown in Fig. 6A, D, and G, the results showed that overexpression of the activated forms of Ras, Akt, and Src readily rescued the defect induced by kindlin-2 depletion. Quantitation of nucleolus-positive cells is shown in Fig. 6B, E, and H, which demonstrate significant differences between LacZ and Cre in both the control group and the activated Ras, Akt, and Src groups. In addition, we also analyzed the pre-rRNA levels as shown in Fig. 6J and found that overexpression of activated Ras, Akt, and Src can markedly rescue the defective RNA polymerase I activity. Collectively, these results strongly suggest that kindlin-2 mediates integrin-mediated cell-ECM adhesion through the FAK-Src-PI3K/Akt pathway to control RNA polymerase I transcriptional activity.

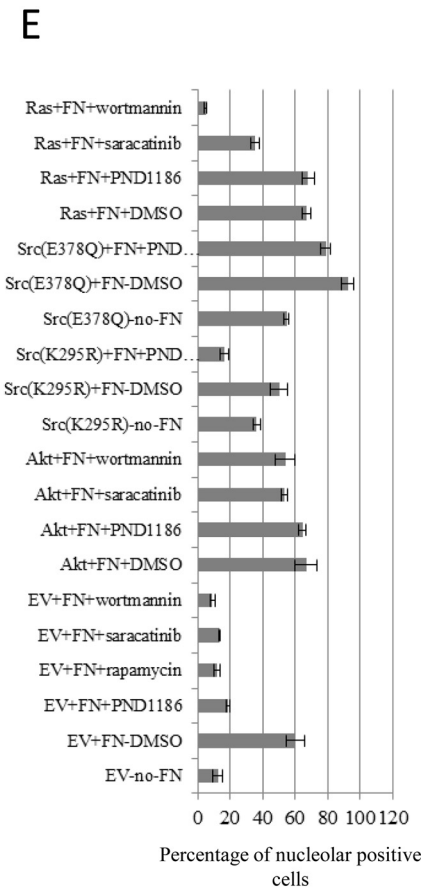
The FAK/Src/PI3K/Akt pathway is a linear signaling pathway to control RNA polymerase I activity. To confirm the signaling pathway for controlling rRNA transcription and to investigate whether it is a linear pathway, we further performed rescue experiments in the presence of the above-mentioned inhibitors. We overexpressed myristoylated Akt in cells and then treated the cells with PND1186 to inhibit FAK activity, with saracatinib to inhibit Src activity, and with wortmannin to inhibit PI3K. Compared to cells with no Akt overexpression (Fig. 7A), cells that overexpressed Akt all demonstrated nucleolar RNA transcriptional activity comparable to that of the cells without inhibitor treatment (Fig. 7C). We also overexpressed active (SrcE378G) and inactive (SrcK295R) Src and then treated the cells with PND1186 (FAK inhibitor). We found that only SrcE378G can successfully rescue RNA polymerase I activity, while SrcK295R cannot (Fig. 7B). Finally, in RasV12-overexpressing cells, we found that RasV12 can rescue RNA Pol I activity in PND1186- and saracatinib-treated cells but not wortmannin-treated cells (Fig. 7D). Taking all the data together, we deduced that the FAK/Src/PI3K/Akt pathway should be linear to control RNA polymerase I transcriptional activity. The quantitation of nucleolus-positive cells in each sample is summarized in Fig. 7E.

DISCUSSION

In this study, we present original findings on the control of RNA polymerase I transcriptional activity by integrin-mediated cell-ECM adhesion. As cell surface receptors, integrins mediate cell-ECM interactions, and they have been shown to control diverse cellular activities, such as proliferation, differentiation, and migration. While rRNA production is an essential aspect and a prerequisite for cell proliferation, it is also the most energy-consuming cellular activity; we therefore deduced that the signaling mediated by integrins might be able to control rRNA synthesis. Previously, it was reported that integrin signaling can mobilize ribosomes to the focal adhesions and control localized protein translation (31). Early studies also indicated that integrin controls general protein synthesis through the Akt/mTOR pathway (32). However, no link has been found to relate integrin signaling to the activity of RNA polymerase I, which is an essential activity for cell growth. In

FIG 6 Overactivation of Ras, Akt, and Src can rescue defective integrin signaling for control of RNA Pol I transcriptional activity. Kindlin-2$^{flox/flox}$ MEFs were infected with a lentivirus encoding an shRNA targeting p19ARF. At 2 days postinfection, cells were then selected by use of 2 μg/ml of puromycin for 2 days. These cells were then infected with either pLVX-RasV12 or empty vector (EV), pMSCV-GFP or pMSCV-GFP-Akt, or pLNCX2-Src(E378G) or pLNCX2-Src(K295R) and then selected by use of hygromycin B at 200 μg/ml or puromycin at 5 μg/ml for 2 to 3 days. Cells were then infected by an adenovirus encoding either LacZ or Cre to delete kindlin-2. (A, D, and G) Cells were analyzed by 5-FUrd assay. Representative images are shown. (B, E, and H) Quantitation of 5-FUrd (nucleolus)-positive cells from the above-mentioned samples. Error bars show standard deviations for three separate counts of 5-FUrd (nucleolus)-positive cells from a population of 50 nuclei. (C, F, and I) Western blots to detect overexpression of Ras and deletion of kindlin-2 in the above-mentioned samples. (J) The above-mentioned cells were harvested for total RNA extraction, and pre-rRNA was analyzed by quantitative real-time PCR. Error bars show standard deviations for 3 separate experiments.





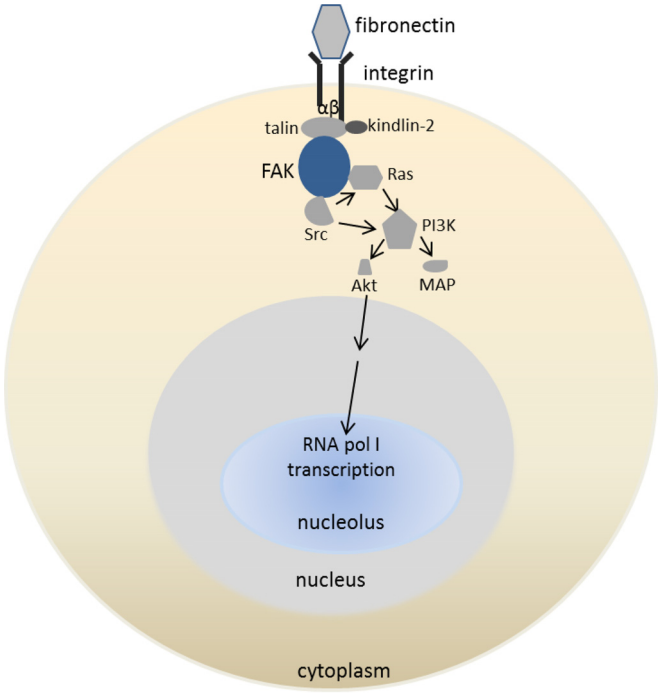


FIG 8 Signaling pathways which control rRNA production in response to fibronectin target RNA polymerase I in the nucleolus. The scheme summarizes the important connections between signaling cascades which target Pol I transcriptional activity. Pol I transcriptional activity is stimulated by integrin, and this is mediated primarily by PI3K and its downstream targets. This leads to stimulation of rRNA gene transcription by activating components of the Pol I transcription machinery during initiation of transcription and by increased occupancy of rDNA promoters.

this report, we demonstrate for the first time that in addition to its other functions, integrin signaling stimulates the assembly of active RNA polymerase I on the rDNA region and controls the production of overall rRNA.

In dissecting the mechanism, we found that the canonical cell growth signaling pathway, PI3K/Akt, is critical for the control of the activity of RNA polymerase I downstream of integrin and FAK/Src, while the ERK/MAP kinase pathway does not participate in this cellular signaling process. There have been reports of the PI3K/Akt pathway being involved in control of the activation of RNA polymerase I through influencing the assembly of the preinitiation complex of RNA polymerase I (33). We believe that integrin signaling promotes the assembly of a functional preinitiation complex to recruit RNA polymerase I to the rDNA gene locus. We proved this through chromatin immunoprecipitation analysis as shown in Fig. 1B and 3C.

Our studies revealed that a linear signaling pathway (FAK/Src/PI3K/Akt/mTOR) controls RNA polymerase I activity during integrin activation. We also proved that activated Ras can markedly rescue RNA Pol I activity during defective integrin signaling, demonstrating the critical role of Ras in the whole process. There is a previous report demonstrating that kindlin-2 promotes Ras activation and its downstream signaling (34). Our study confirms the critical role of Ras in kindlin-2 signaling. As important mediators of integrin signal transduction, three isoforms of kindlin have been detected in the human genome. All isoforms play important roles *in vivo* (35). Kindlin-1 is expressed primarily in epithelial cells; its dysfunction is associated with Kindler's syndrome (36). Kindlin-2 is ubiquitous in all cell types, but mesenchymal cells have larger amounts than other cell types (37). Kindlin-3 is expressed primarily in white blood cells; its loss of function is associated with leukocyte attachment deficiency (LAD) (38). Overexpression of Kindlins has been detected frequently in human cancers (39–41). We focused on the function of kindlin-2 in fibroblasts, as they control the production of many extracellular matrix proteins. Because fibroblasts belong to the primary constituents of connective tissue, it is obvious that kindlin-2 is very important for maintaining the normal function of connective tissues. We recently found that kindlin-2 is pivotal for bone formation in chondrocytes (26). Because serious bone loss has been linked to dysfunction of rRNA production before (42–44), our study provides one of the possible mechanisms for the loss of bone formation in kindlin-2-deficient mice. Loss of kindlin-2 causes decreased ribosome production and thus leads to a dysfunction of fibroblasts. Recent studies showed that kindlin-2 localizes not only to focal adhesions but also to the nucleus, suggesting that kindlin-2 possesses functions in both focal adhesions and the nucleus (26, 45, 46). We recently found that kindlin-2 is critical for integrin outside-in signaling and Src activation (25). Because overexpression of active Ras, Akt, and Src is sufficient to rescue the loss-of-kindlin-2-induced defect of RNA polymerase I transcriptional activity, kindlin-2 likely functions upstream of Src, Ras, and PI3K in the regulation of RNA polymerase I and rRNA production. Based on these findings, we depict a signaling pathway linking integrin and kindlin-2 to RNA polymerase I (Fig. 8). These studies provide novel information on the functions of integrin signaling to control a key aspect of cell growth, rRNA production, and may help in the development of novel therapeutic approaches to control human diseases associated with abnormal cell growth.

FIG 7 The FAK/Src/PI3K/Akt signaling axis is linear in controlling RNA Pol I activity. For the same cells mentioned in the legend to Fig. 6, after selection by hygromycin B and puromycin, cells were plated on coverslips that were precoated with fibronectin. Inhibitors were then added, and cells were incubated for 25 min in a CO_2 incubator. 5-FUrd was then added to a final concentration of 2 mM and incubated for 10 min, and cells were then fixed and stained for detection of 5-FUrd incorporation. Typical images are shown. Magnification, ×63. Red, 5-FUrd; blue, DAPI. (A) Cells infected with empty vector were treated with the indicated inhibitors, and the 5-FUrd *in situ* nuclear run-on assay was performed. Inhibitors of FAK, Src, PI3K, and mTOR can also suppress RNA polymerase I activity. (B) Cells overexpressing Src were treated with FAK inhibitor in the presence of fibronectin stimulation. Overexpression of active Src (SrcE378G) rescued the defective RNA polymerase I activity caused by this inhibitor, while the inactive SrcK295R protein did not. (C) Cells overexpressing myristoylated Akt were treated with FAK, Src, and PI3K inhibitors in the presence of fibronectin stimulation. Overexpression of Akt rescued the defective RNA polymerase I activity caused by these inhibitors. (D) Cells overexpressing RasV12 were treated with FAK, Src, and PI3K inhibitors in the presence of fibronectin stimulation. Overexpression of RasV12 rescued the defective RNA polymerase I activity caused by FAK and Src inhibitors but not that caused by the PI3K inhibitor. (E) Quantitation of 5-FUrd (nucleolus)-positive cells from the above-mentioned samples. Error bars show standard deviations for three separate counts for each type of 5-FUrd-positive cells from a population of 50 nuclei.

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C.W. and Y.Z. independently conceived the study. Y.Z. designed the study. Y.Z., J.Y., J.F., and X.W. performed the experiments. Y.Z. analyzed the data and wrote the manuscript. C.W. performed editorial assistance and provided the conditional kindlin-2 knockout mice.

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