LncRNA Pnky Positively Regulates Neural Stem Cell Migration via Modulating mRNA Splicing and Export of Target Genes

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Abstract

Directed migration of neural stem cells (NSCs) is critical for embryonic neurogenesis and the healing of neurological injuries. LncRNA *Pnky* was reported to regulate neuronal differentiation of NSCs. However, its regulatory effect on NSC migration has never been explored. Herein, we identified that *Pnky* was also a key regulator of NSC migration, as underscored by the fact that *Pnky* silencing restrained the migration of both NSC lines C17.2 and NE4C, whereas *Pnky* over-expression promoted their migration. Meanwhile, *Pnky* regulated the expression of a core set of critical regulators that directing NSC migration, such as MMP2, MMP9, AKT and p38MAPK, etc. Through preliminary bioinformatics, we surprisingly noticed that splicing factors U2AF1 and U2AF1L4, as well as mRNA export adaptors SARNP, Aly/Ref and THOC7, were predicted to strongly interact with *Pnky*. Mechanically, *Pnky* could co-localize and directly bind to U2AF1, SARNP, Aly/Ref and THOC7, indicating the involvement of *Pnky* in modulating mRNA splicing and export processes. Collectively, our data delineated that, through interacting with U2AF1, SARNP, Aly/Ref and THOC7, *Pnky* coupled and modulated the mRNA splicing and export of downstream factors, which consequently affected NSC migration. These findings provide a possible theoretical basis of NSC migration for brain development and damage repair.

1. Introduction

Neural stem cells (NSCs) are multipotent cells that have the capacity to self-renew, migration and differentiate along multiple lineages, such as neurons, astrocytes, and oligodendrocytes (Li et al. 2021). In the embryonic brain, NSC migration is necessary for neurogenesis (Borrell 2019; Bertipaglia et al. 2018), and a lack of NSC migration causes severe brain damage and lethality (Jung et al. 2019; Kaneko et al. 2017). Moreover, NSC migration is also an endogenous regeneration response that, in the acutely injured brain, NSCs can migrate to the sites of injury through blood vessels or neuronal fibers, which makes NSCs attractive candidates for cell replacement therapy for treating neurodegenerative diseases or repairing brain injuries. Numerous studies have revealed that NSCs could proliferate and migrate towards the sites of injury, such as in animal models of traumatic brain injury (Rolfe and Sun 2015), ischaemic stroke (Wang et al. 2020a) and spinal cord injury (Gao et al. 2020), where they contributed to the functional recovery. However, the exact mechanism underlying NSC migration remains unclear.

LncRNAs represent a group of heterogeneous transcripts that range from 200 nt up to ~ 100 kb in length. The mammalian genome encodes many thousands of lncRNAs that have no apparent protein coding potential, but emerging data indicate that such lncRNAs can have critical biological functions in development and disease (Chi et al. 2019; Kopp 2019; Wang et al. 2020b), through controlling gene transcription, pre-mRNA processing, mature mRNA exporting, mRNA degeneration, translation, and protein kinetics, etc (Ali and Grote 2020; Jathar et al. 2017; Zhao et al. 2021). To date, various lncRNAs have been identified to control NSC behaviors. For instance, lncRNA *Neat1* was reported to regulate neuronal differentiation and apoptosis *via* modulating Wnt/β-catenin signaling that was activated by miR-124 (Cui et al. 2019). lncRNA *RMST*, specifically expressed in brain, was identified to participate neurogenesis through interacting with Sox2 (Ng et al. 2013). Recently, another lncRNA *LncKdm2b* was found to control

cortical neuronal differentiation by *cis*-activating *Kdm2b* (Li et al. 2020a). Moreover, Zhang et al. proposed that lncRNA *EPS*, which was highly expressed in biomimetic vesicles, contributed directly to inflammatory resolution and neurogenesis after stroke (Zhang et al. 2020). Nevertheless, few lncRNAs have been investigated to exert possible biological functions in NSC migration till present.

LncRNA *Pnky*, identified by Ramos et al. recently, is a 825 nt, evolutionarily-conserved, nuclear-enriched lncRNA that is expressed in NSCs both *in vitro* and *in vivo* (Ramos et al. 2015; Cho and Hsieh 2015). LncRNA *Pnky* regulates neuronal differentiation of embryonic and postnatal NSCs *via* interacting with PTBP1 (Ramos et al. 2015; Grammatikakis and Gorospe 2016). Further investigation found that lncRNA *Pnky* is a trans-acting regulator of cortical development *in vivo* (Andersen et al. 2019). However, the functional significance of lncRNA *Pnky* in NSC migration is rarely studied.

In this paper, we aim to investigate the possible role of lncRNA *Pnky* in NSC migration, as well as their underlying modulation mechanisms. Remarkably, we discovered that *Pnky* could positively regulate the migration of both C17.2 and NE4C through regulating the expression of some critical regulators and signaling pathways involved in NSC migration, such as MMP2, MMP9, PI3K/Akt and p38MAPK, et al. Regarding the precise internal mechanism, we noticed that, on one hand, lncRNA *Pnky* can directly bind to splicing factor U2AF1 thereby modulating mRNA splicing process. On the other hand, as RNA scaffold, *Pnky* directly recruits SARNP, Aly/Ref and THOC7 to Transcription and Export (TREX) complex, and subsequently regulates mRNA export of downstream factors. These findings not only provide evidence of a novel regulatory role for *Pnky* in NSC migration *via* modulating mRNA splicing and export of target genes, but also shed light on the migration mechanism of NSCs in brain development and damage repair. Over-expression of lncRNA *Pnky* in NSCs may serve as a promising approach to improve functional recovery of neurological injuries and diseases.

2. Materials And Methods

2.1. Plasmids, gene cloning, and reagents

Two different mouse *Pnky* shRNA cDNA fragments were cloned downstream of the U6 promoter in the pLVshRNA-Puro plasmid (#VL3102), which was purchased from Inovogen Tech. Co. (China). A mouse *Pnky* gene was cloned downstream of the CMV promoter in the dual expression lentiviral vector pLV-U6-CMV-Puro that was constructed by Deng's lab (Huang et al. 2019). The lentiviral particles expressing control shRNA (sc-108080) were purchased from Santa Cruz Biotech (USA). Restriction enzymes for gene cloning, such as BamHI (#R0136S), NotI (#R0189S) and HindIII (#R0104S), were purchased from New England Biolab (England). RNA miniprep kits (#AP-MN-MS-RNA-50) were obtained from Axygen (USA). RT-qPCR reagents (#4913914001) were purchased from Roche (Germany). Cy3-*Pnky* FISH probes and Ribo^{TM} Fluorescent In Situ Hybridization Kit (#C10910) were purchased from RiboBio Co., Ltd (China). All general chemicals were obtained from the SinoPharm Chemical Reagent Co. (China). Details about the key resource were listed in Table S1.

2.2. Cell culture and generation of stable cell lines

Murine immortalized NSC line C17.2 was maintained in DMEM (#SH30022.01) with the supplement of 10% fetal bovine serum (FBS, #SV30087), 5% horse serum (HS, #SH30074.02) and 1% penicillin/streptomycin (#SV30010) from Hyclone, Logan, UT, while NE4C cells were cultured in MEM medium (#SH30024.01, Hyclone) supplemented with 10% FBS, 2 mM L-glutamine and 1% penicillin/streptomycin.

C17.2 and NE4C cell lines that stably express *Pnky* shRNA were generated by transduction with the lentiviral particles expressing *Pnky* shRNA. In brief, when HEK 293T cells reached 70~80% confluence, they were co-transfected with the lentiviral packaging plasmids (PH1, PH2, and *Pnky* shRNA) using Lipofectamine 3000 (#L3000001, Invitrogen, MA, USA), and the lentiviral particles (expressing *Pnky* shRNA) were collected and concentrated 48 h later. C17.2 and NE4C cells were infected with the lentiviral particles, and cell lines stably expressing *Pnky* shRNA were screened from single-cell colonies using 96-well plates as described previously (Peng et al. 2020). Using the same strategy, we generated C17.2 and NE4C cell lines stably expressing *Pnky* gene as well as the cell lines expressing control shRNA.

2.3. RNA isolation and RT-qPCR

Total RNA was extracted with the RNA miniprep kit (Axygen), followed by cDNA synthesis using HiScript III 1st Strand cDNA Synthesis Kit (#R312-01, Vazyme, China) for detecting *Pnky* expression, or RevertAid first-strand cDNA synthesis kit (#K1622, Thermo Scientific) for determining the expression of related coding genes, according to the manufacturer's instructions. Quantitative PCR reactions were carried out using FastStart Universal SYBR Green Master (#4913914001, Roche, Germany) and gene-specific primers on a Bio-Rad CFX-96 detection system (Bio-Rad, CA, USA). qPCR data were analyzed with CFX Manager 3.1 software (Bio-Rad), and relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method. The housekeeping gene (GAPDH) was used for normalization.

2.4. Immunofluorescence staining

Immunofluorescence staining for Nestin in the above stable cell lines were performed with a standard protocol. Briefly, cells grown on the 14 mm (diameter) coverslips were fixed with 4% paraformaldehyde (PFA, Beyotime, China) for 20 min, followed by permeabilization with 0.2% TritonX-100 for 10 min at room temperature. Samples were then incubated with 10% goat serum for 1 h to block any non-specific interactions. Antibody against Nestin (#ab6142, Abcam, Cambrige, CA, USA) was added to the samples for incubation at 4 °C overnight. After washing with PBS, cells were further incubated with Cy3-conjuated secondary antibody (#A0521, Beyotime) for 2 h at room temperature. Nuclei were counterstained with mounting medium (#ab104139, containing DAPI, Abcam). Finally, the samples were imaged using an Olympus fluorescence microscopy (CKX53, Olympus, Japan).

2.5. Cell proliferation assays

Cell counting and 5-Ethynyl-2'-deoxyuridine (EdU) incorporation assay were performed to determine the cell proliferation rates of the stable cell lines. For cell counting assays, an equal number of cells (~$3*10^4$) were seeded onto 12-well plates where each cell type was grown in triplicate. Cell samples were collected every 24 hours and differential cell counts were obtained using a Countstar IC1000 cell counter (Countstar, China).

EdU is a thymidine analogue that can be incorporated into cellular DNA during DNA replication, and is usually adopted in the study of cell proliferation (Sun et al. 2016). As for EdU incorporation assay, the BeyoClick™ EdU cell proliferation kit with Alexa Fluor 555 (# C0075S, Beyotime) was used, according to the manufacturer's instructions. Briefly, cells in the logarithmic phase were seeded onto 12-well plates at a concentration of $3*10^5$ and incubated overnight. The nest day, 10 μM EdU solution was added for 2 h incubation. Subsequently, cells were fixed with 4% PFA for 15 min and permeabilized with 0.3% Triton X-100 for 10 min at room temperature. After washing with PBS, 250 μl click reaction solution was added, followed by incubation for 30 min in dark. Finally, nuclei were stained with mounting medium (containing DAPI). At least 10 fields were selected randomly in each group and photos were taken under the Olympus fluorescence microscopy. The percentage of EdU-positive cells was compared between groups.

2.6. Wound healing assay

The effect of lncRNA *Pnky* in NSC migration was explored using wound healing assay. Corresponding cells were seeded onto 6-well plates and allowed to grow to 80% confluences in complete medium. Three parallel scratches were made using sterile pipette tips, and cell debris were washed off using PBS. Then, cells were further incubated in medium with 2% FBS for 48 h. The magnitude of wound healing was captured randomly using the Olympus fluorescence microscopy under bright field at the designated time points of 0 h, 24 h and 48 h, respectively. Widths of the wounds were measured and analyzed using ImageJ software.

2.7. Transwell migration assay

Transwell assay was also used to evaluate the migration of the obtained stable cell lines. In brief, $1*10^5$ cells were suspended in 200 μl of serum-free medium and transferred to the upper chambers (# 3422, 8.0 μm pores, Corning, USA) that placed into 24-well plates. The bottom chambers were filled with 600 μl medium containing 10% FBS. After 24 h of incubation, the chambers were washed gently with cold PBS and replaced to new 24-well plates. Cells that migrated to the lower surface of the membrane were fixed with 1 ml methanol for 10 min, followed by staining with 1% crystal violet for 20 min. Whereas, cells that on the upper membrane surface were wiped off using fluffy swabs that were soaked with PBS. The numbers of migrated cells were photographed and calculated from 5-8 randomly selected fields under microscope. Experiment was performed six times with triplicate samples within each individual experiment.

2.8. Western blot analysis

Western blot analysis was performed as reported previously (Li et al. 2020b). 30 μg of cell lysates per lane were separated by 12% SDS-PAGE and transferred onto nitrocellulose membrane (Millipore, USA). After blocking with 5% nonfat dried milk, the membranes were incubated with the corresponding antibodies as follows. The antibodies against Paxillin (sc-365379), MMP2 (sc-13595), MMP9 (sc-393859), Connexin 43 (sc-13558) and GAPDH (sc-32233) were obtained from Santa Cruz, CA, USA. The antibodies recognizing ERK1/2 (#9102S), p-ERK (Thr202/Tyr204) (#9106S), AKT (#9272S), p-AKT (Ser473) (#4060S), p38 MAPK (#8690S) and p-p38 MAPK (#9216S) were obtained from Cell Signaling (MA, USA). The antibodies against SARNP (#ab225694), Aly/Ref (#ab202894), U2AF1 (#ab172614), THOC7 (#ab155218) and U2AF1L4 (#ab188582) were ordered from Abcam. HRP-conjugated goat anti-mouse or goat anti-rabbit IgG (Santa Cruz) were used to detect the primary antibodies above. Detection of the protein bands was performed with an enhanced chemiluminescence solution (Millipore, USA) using ChemiDocXRS + (Bio-Rad, CA, USA).

2.9. Colocalization of lncRNA Pnky and protein

As described elsewhere (Liu et al. 2015), cells on coverslips were first hybridized with *Pnky* probes conjugated with Cy3 (RiboBio), followed by immunofluorescence staining using antibodies of interest. Briefly, cells were gently rinsed in PBS and fixed in 4% PFA for 10 min at room temperature. Subsequently, cells were permeabilized in PBS containing 0.5% Triton X-100 for 10 min at 4℃, washed with PBS 3 times for 5 min, and pre-hybridizated in pre-hybridization buffer for 30 min at 37℃. Then, using anti-*Pnky* oligodeoxynucleotide probes, hybridization was performed in hybridization solution overnight at 37°C in dark moist chamber. On the second day, after sequential washing with 4ÎSSC□2ÎSSC and 1ÎSSC at 42°C in dark, cells were further fixed in 4% PFA for 5 min and incubated with SARNP (#ab225694), Aly/Ref (#ab202894), U2AF1 (#ab172614), THOC7 (#ab155218) and U2AF1L4 (#ab188582) antibodies at 4°C overnight, respectively. On the next day, cells were incubated with specific Alexa Fluor 488 conjugated secondary antibody (#A0428, #A0423, Beyotime) and counterstained with DAPI. A minimum of 12 fields were selected randomly in each group to analyze the colocalization of *Pnky* and the corresponding proteins. Images were taken using an Olympus confocal laser-scanning microscope (FV3000, Olympus, Japan).

2.10. RNA Immunoprecipitation (RIP)

RIP experiments were performed using a Magna RIP^{TM} RNA-Binding Protein Immunoprecipitation Kit (#17-704, Millipore) according to the manufacturer's instructions. Cells were scraped off from plates, collected, and lysed with complete RIP lysis buffer for 30 min on ice. Magnetic beads were incubated with 5 μg of antibodies, as SARNP (#ab225694), Aly/Ref (#ab202894), U2AF1 (#ab172614), THOC7 (#ab155218), U2AF1L4 (#ab188582) or normal Mouse/ Rabbit IgG (obtained from the above kit), respectively, with rotation for 40 min at room temperature. Then, beads-antibody complexes were washed adequately with RIP washing buffer, and cell lysates were added into the complexes and incubated at 4°C overnight. 10% of cell lysates (input) were stored at -80°C until starting RNA purification. The next day, with the help of magnetic separator, the RNAs associated with the corresponding proteins were pulled

down. Immunoprecipitates and input were then subjected to protease K and heated at 55°C for 30 minutes to digest the proteins. Subsequently, RNAs were purified with phenol:chloroform:isoamyl alcohol, followed by precipitation using absolute ethanol. cDNAs were synthesized using HiScript III 1st Strand cDNA Synthesis Kit and the enrichment of *Pnky* was detected using RT-qPCR method as described above. Fold enrichment of *Pnky* was calculated relative to the percentage of input. The RNA and RNA-binding proteins complexes were also treated with SDS lysis buffer for further Western blot analysis to test the efficiency of immunoprecipitation.

2.11. Bioinformatics analysis

*cat*RAPID (http://s.tartaglialab.com/catrapid/omics) was used to analyze the RNA-protein interactions (Agostini et al. 2013).

2.12. Statistical analysis

Throughout our research, appropriate sample size was determined based on the effect size, α error (0.05) and the Power (0.95), using the program GPower3.1 as reported previously (Serdar et al. 2021). Statistical analysis was carried out using IBM SPSS Statistics (version 20.0, IBM Corp., Armonk, NY, USA). Accordingly, normality and variance homogeneity were firstly assessed using Kolmogorov-Smirnov Test and Levene's Test. As for parametric data, Student's *t*-test or one-way ANOVA were used to assess the statistical significance, as appropriate, and results were expressed as mean ± SD of six independent experiments. If the data were not normally distributed or variance homogeneity was not met, nonparametric tests (Mann-Whitney U tests) were performed and data were displayed as median and interquartile range. Values of $P < 0.05$ were considered to indicate a statistically significant difference (*$p < 0.05$, **$p < 0.01$). GraphPad Prism 5 software (GraphPad Software, Inc., CA, USA) were used to make the statistical charts. As for the statistical analysis of wound healing assay, repeated measures tests were carried out using IBM SPSS Statistics. In brief, Mauchly's Tests of sphericity were firstly performed. If P > 0.05, sphericity was met and sphericity assumed tests were used to assess the variances between the pairs. In case $P < 0.05$, sphericity was violated and Greenhouse-Geisser correction would further come into play.

2.13. Blind study statement

In all our experimental research, study participants, data collectors and data analysts are kept unaware of group assignment (control vs intervention).

3. Results

3.1. LncRNA *Pnky* negatively regulates the proliferation of NSCs

To investigate the biological functions of *Pnky* in NSCs, we generated C17.2 and NE4C cell lines stably expressing *Pnky* shRNA or *Pnky*, as well as their corresponding control cell lines, using *Pnky* shRNA-expressing or *Pnky*-expressing lentiviral vectors. The mRNA levels of *Pnky* were confirmed using RT-qPCR.

As shown in Fig. 1a and Table S2, the expression of *Pnky* shRNA in C17.2 and NE4C cells substantially inhibited the expression of *Pnky*, with the knockdown efficiency of 79.5±8.6% ($F(1,34)=496.17$, $P < 0.001$) and 62.5±20.5% ($F(1,34)=129.00$, $P < 0.001$), respectively. In contrast, in cells stably transfected with *Pnky*-expressing lentiviral vectors, *Pnky* expression was significantly up-regulated (Fig. 1e and Table S3, $P < 0.001$). These data indicate that we generate *Pnky*-knockdown and over-expression stable cell lines successfully.

Next, the effect of *Pnky* on stem cell maintenance was assessed using C17.2 and NE4C stable cell lines. Immunofluorescence staining showed that the differential expression of *Pnky* did not affect the stem fate of C17.2 and NE4C, with the percentage of Nestin-positive cells up to 99% in each stable cell lines (Fig. 1b and f), which indicating the undifferentiated state of NSCs (Engert et al. 2021). Further investigations of *Pnky* on cell proliferation were also carried out using cell counting and EdU assays. Results revealed that *Pnky* silencing promoted cell proliferation for both C17.2 and NE4C cells, with faster cell proliferation rates and more EdU-positive cells in both C17.2 and NE4C *Pnky* shRNA groups, compared with control (Fig. 1c and d). In contrast, *Pnky* over-expression depressed their proliferation (Fig. 1g and h).

3.2. Knockdown of *Pnky* attenuates the migration of NSCs

To preliminarily explore the possible role of *Pnky* in NSC migration, wound healing and transwell assay were performed using C17.2 and NE4C stable cell lines. Transwell migration assay showed that lncRNA *Pnky* inhibition remarkably impaired the vertical migration both in C17.2 and NE4C cells, with the migration rate of 27.97±11.52% ($t(64)=19.68$, $P < 0.001$) and 38.99±10.50% ($t(70)=20.34$, $P < 0.001$), respectively, compared to those of control (Fig. 2a-d and Table S4-5). Meanwhile, both C17.2 and NE4C *Pnky* shRNA groups exhibited slower closing of scratch wounds, as could be seen with the higher percentages of wound widths, in comparison with the controls at 24 h and 48 h (Fig. 2e-h and Table S6-7, $P < 0.001$). Collectively, these data suggest that knockdown of *Pnky* inhibits the migration of NSCs, both in vertical and lateral directions.

3.3. *Pnky* over-expression promotes NSC migration

To further validate the role of *Pnky* in NSC migration, we also tested the differences in migration abilities between *Pnky* over-expression groups and control counterparts. The transwell data illustrated that the number of cells that migrated to the lower surface of the chamber membrane increased significantly in *Pnky* overexpressing C17.2 and NE4C cells, with the migration rates up to 158.22±21.42% ($t(74)=-13.19$, $P < 0.001$) and 203.20±25.70% ($t(64)=-18.55$, $P < 0.001$), respectively, compared to controls (Fig. 3a-d and Table S8-9). The wound widths were also evidently decreased in *Pnky* over-expression groups than those in controls (Fig. 3e-h and Table S10-11, $P < 0.001$). Taken together, these data indicate that *Pnky* plays an evident role in facilitating NSC migration.

3.4. *Pnky* regulates the expression of some critical regulators and signaling pathways that directing NSC migration

NSC migration is a multistep process that includes the extension of lamellar pseudopodia, the formation of new focal contact, the forward contraction of cell body and the dissociation of cell tail (Seetharaman and Etienne-Manneville 2020), and many critical regulators are involved in such process. For example, MMP2 and MMP9 are two key metalloproteinases that participate in the decomposition of extracellular matrix thus facilitating cell migration (Wang et al. 2018). Paxillin, the focal adhesion adaptor protein, functions to recruit structural and signaling molecules involved in cell movement and migration (Rajah et al. 2019). Connexin 43, which forms gap junction channels, exerts crucial cellular functions as proliferation and migration (Zhang et al. 2018).

To investigate whether *Pnky* affects the above key regulators, Western blot and RT-qPCR analysis were adopted to test their expression in C17.2 and NE4C stable cell lines. As expected, *Pnky* depletion depressed the expression of MMP2, MMP9, Connexin 43 and Paxillin, both in mRNA and protein levels (Fig. 4a-b and Table S12-13, $P < 0.001$). In contrast, as shown in Fig. 4c-d and Table S14-15, *Pnky* over-expression enhanced their expression in C17.2 and NE4C cells ($P < 0.001$). These data indicate that *Pnky* positively regulates NSC migration by influencing the expression of some critical regulators.

PI3K/Akt, p38MAPK and Raf/MEK/ERK signaling pathway have always been a focus of interest due to their roles in cell proliferation, migration, and metabolism (Han et al. 2016; Hirata and Kiyokawa 2019; Shahcheraghi et al. 2020). They are involved in the modulation of several downstream effectors, such as MMP2 and MMP9 et al. and thereby affecting cell movement (Cui et al. 2016; Sánchez-Martín et al. 2019). Herein, Western blot was used to detect the pathways engaged in *Pnky* manipulating NSC migration, and data showed that *Pnky* knockdown inhibited the expression and phosphorylation of p38MAPK, ERK and AKT both in C17.2 and NE4C cells, whereas *Pnky* over-expression activated the above pathways (Fig. 4e and f). Overall, these evidences delineate that *Pnky* regulates signaling pathways (PI3K/Akt et al.) and the downstream key effectors (MMP2 and MMP9 et al.), which in turn modulate NSC migration.

3.5. *Pnky* could potentially bind to several factors involved in mRNA splicing and export processes

LncRNAs generally associate with specific sets of RNA-binding proteins (RBPs) to form functional ribonucleoprotein (RNP) complexes. To uncover the possible mechanism by which *Pnky* regulates NSC migration, we screened potential proteins that may interact with *Pnky* using an online tool *cat*RAPID (http://service.tartaglialab.com/email_redir/191801/3e2b203d12), and the top hits were listed in Table 1. It has been reported that *Pnky* was a nuclear-enriched lncRNA that functioned as a trans-acting regulator (Ramos et al. 2015). On the basis of interaction strength, protein location and Z-scores, we identified SARNP, Aly/Ref, U2AF1, U2AF1L4 and THOC7, had high probabilities to directly interact with *Pnky*. Interestingly, among of these, SARNP, Aly/Ref, and THOC7 are core components of TREX complex, which specifically associates with spliced mRNA and functions in mRNA export to the cytoplasm (Heath et al. 2016). U2AF1 is one component of the pre-mRNA splicing machinery, and mainly functions to recruit U2 snRNP to the 3'-splice site, thus playing a critical role in both constitutive and enhancer-dependent splicing (Palangat et al. 2019). U2AF1L4, also known as U2AF26, is another splicing factor that can

functionally substitute for U2AF1 (Preußner et al. 2014). Accordingly, we hypothesized that *Pnky* might positively regulate NSC migration *via* modulating mRNA splicing and export of target genes.

3.6. *Pnky* directly binds to splicing factor U2AF1 to modulate mRNA splicing process

To substantiate our assumption, we first performed primary antibodies specificity determination (anti-U2AF1 and anti-U2AF1L4) using knockdown validation (Fig. S9). Subsequently, co-localization analysis of *Pnky* and U2AF1 (or U2AF1L4) were carried out using FISH and immunofluorescence co-staining technique. Confocal micrographs showed that lncRNA *Pnky* partially co-localized with U2AF1 or U2AF1L4 in the nucleus of NE4C cells (Fig. 5a and b), inferring the direct interactions between them. Furthermore, RIP assay was carried out using antibodies against U2AF1 or U2AF1L4. As expected, *Pnky* was preferentially enriched in U2AF1 antibody-containing beads compared to those harboring control IgG, with *Pnky* enrichment of 28.64±6.03% vs. 1.42±0.59% (Fig. 5c and Table S16, $P < 0.001$), suggesting that *Pnky* could directly bind to splicing factor U2AF1. Contrary to expectations, little *Pnky* RNA was enriched in U2AF1L4 immunoprecipitates (Fig. 5d and Table S17, $P=0.097$), indicating the lower affinity of *Pnky* to U2AF1L4. This may be due to the substitute function of U2AF1L4 for U2AF1, and *Pnky* tends to interact with U2AF1 preferentially. Moreover, the efficient immunoprecipitation of U2AF1 or U2AF1L4 proteins from NE4C cell extracts was confirmed using Western blot (Fig. 5c and d). Overall, these scientific evidences point to the fact that lncRNA *Pnky* could preferentially interact with splicing factor U2AF1 to modulate the pre-mRNA splicing of downstream target genes such as MMP2 et al. which consequently affects NSC migration.

3.7. *Pnky* directly binds to SARNP, Aly/Ref and THOC7, and subsequently facilitates mRNA export of target genes

From the above bioinformatics prediction, lncRNA *Pnky* could also potentially bind to SARNP, Aly/Ref and THOC7. It was reported that SARNP is a participant in mRNA splicing and export that binds to UAP56, an RNA helicase component of the TREX complex which is thought to couple mRNA transcription, processing, and nuclear export (Kang et al. 2020). Aly/Ref is an export adapter that mediates the recruitment of TREX complex, and is involved in nuclear export of spliced mRNA (Shi et al. 2017a). THOC7 usually acts as one component of the THO subcomplex which constitutes the TREX complex, and is required for efficient export of polyadenylated RNA (He et al. 2019). To verify the bioinformatics predictions, we further studied the interaction between *Pnky* and SARNP (or Aly/Ref, THOC7). Likewise, we first determined the specificity of corresponding primary antibodies (anti-SARNP, anti-Aly/Ref and anti-THOC7) using knockdown validation (Fig. S9), followed by co-localization analysis of *Pnky* and SARNP (or Aly/Ref, THOC7). Notably, lncRNA *Pnky* strongly co-localized with SARNP, Aly/Ref or THOC7 in the nucleus of NE4C cells, as manifested clearly in the enlarged micrographs in Fig. 6a-c. Besides, immunoprecipitation of *Pnky* further presented that SARNP, Aly/Ref and THOC7 antibodies could retrieve significantly amounts of endogenous *Pnky* RNA, with ~28.10, 7.92 and 5.04-fold enrichment vs. control IgG (Fig. 6d-f and Table S18-20, $P < 0.001$), confirming the direct binding of *Pnky* to SARNP, Aly/Ref or

THOC7. Besides, the successful immunoprecipitation of SARNP, Aly/Ref and THOC7 was also confirmed using Western blot (Fig. 6d-f).

SARNP, Aly/Ref and THOC7 are key components of TREX complex that plays a vital role in the effective export of mRNAs. It was reported that TREX complex is a dynamic structure that at least composed of Aly/Ref, UAP56, SARNP, CHTOP and the THO subcomplex (including THOC1, THOC2, THOC5, THOC6 and THOC7) (Kumar et al. 2020). During splicing, the TREX complex is recruited in a splicing- and cap-dependent manner to a region near the 5' end of the mRNA where it functions in mRNA export to the cytoplasm *via* the TAP/NFX1 pathway (Katahira 2012). Accordingly, it is reasonable to draw the conclusion that *Pnky* may act as an RNA scaffold to recruit SARNP, Aly/Ref and THOC7 to TREX complex, thus facilitating mRNA export of target genes, such as MMP2 et al., which subsequently modulates NSC migration.

Based on these results, we propose an integrated regulatory model in which lncRNA *Pnky* affect NSC migration. As shown in Fig. 7, in the nucleus of NSCs, lncRNA *Pnky* could directly bind to splicing factor U2AF1 and consequently modulate pre-mRNA splicing of target genes. Meanwhile, *Pnky* could also act as RNA scaffold to recruit SARNP, Aly/Ref and THOC7 to TREX complex, thus boosting mRNA export to the cytoplasm. Through coordinating mRNA splicing and export process, the expression of downstream target genes, such as MMP2, Paxillin, p38MAPK, and Akt et al., was enhanced which subsequently facilitating NSC migration. Moreover, the phosphorylation of p38MAPK, ERK and Akt was also positively regulated in the process of migration, resulting activated transcription of target genes. Consequently, a positive feedback loop is formed in *Pnky*-directed NSC migration process.

4. Discussion

Migration is one of the most crucial characteristics of NSCs and revealing the regulatory mechanism of NSC migration is the key to uncover the process of neurogenesis, and provides new sights for the treatment of neurodevelopmental or neurodegenerative diseases and nerve injuries. In recent years, the fundamental roles of lncRNAs in central nervous system (CNS) is becoming increasingly evident, and more and more lncRNAs have emerged as critical regulators in neurodevelopmental process and diseases, such as self-renewal and proliferation (Fan et al. 2020; Zhao et al. 2020), neural differentiation (Biscarini et al. 2018; Rea et al. 2020; Winzi et al. 2018; Zhao et al. 2020), gliogenesis (Bian et al. 2019; Li et al. 2018; Xia et al. 2020), among others. However, lncRNAs that modulating NSC migration are less well understood.

Previously, lncRNA *Pnky* was known for its important role in regulating neuronal differentiation of embryonic and postnatal NSCs (Ramos et al. 2015). Through interacting with PTBP1, lncRNA *Pnky* regulated the expression and alternative splicing of a core set of transcripts that related to NSCs-neurons transition (Grammatikakis and Gorospe 2016; Ramos et al. 2015). Herein, we identified that lncRNA *Pnky* positively regulates NSC migration, as underscored by the fact that *Pnky* silencing restrained the migration of both C17.2 and NE4C, while *Pnky* over-expression promoted their migration (Fig. 2 and 3).

Moreover, we discovered that *Pnky* negatively regulated the proliferation of NSCs (Fig. 1), which excluded the possibility that the visible changes in NSC migration were due to cell proliferation and further confirmed the regulatory effect of *Pnky* on NSC migration.

It is well established that complicated genes interactions and molecular networks participate in NSC migration process (De Gioia et al. 2020). Metalloproteinases and cell adhesion molecules, which facilitate the degradation of old focal adherens and the formation of new ones, are reported to play pivotal roles in migration process (Alaseem et al. 2019). Moreover, PI3K/Akt, p38MAPK and Raf/MEK/ERK signaling pathways were found to control the migration of many kinds of cells, through regulating the expression of MMP2 and MMP9 et al. (Sánchez-Martín et al. 2019; Zhang et al. 2015). Under our conditions, metalloproteinases MMP2 and MMP9, as well as the focal adhesion adaptor protein Paxillin, were strikingly downregulated both in mRNA and protein levels, as observed in *Pnky* silencing C17.2 and NE4C cells (Fig. 4a and b). Meanwhile, acute knockdown of *Pnky* also led to a significant downregulation of p38MAPK, ERK and AKT phosphorylation both in C17.2 and NE4C cells, while *Pnky* over-expression mainly promoted their phosphorylation (Fig. 4e and f). Interestingly, however, the differential expression of *Pnky* also showed impact on the expression of total p38MAPK, ERK and AKT, suggesting that *Pnky* did regulate the expression of a core set of transcripts that related to NSC migration.

The biological functions of lncRNAs are usually closely related to their intracellular localization (Chen 2016). *Pnky* is reported to be a predominantly nuclear and evolutionarily conserved lncRNA that expresses only in neural tissues (Cho et al. 2015; Ramos et al. 2015). Consistently, our results also signified the nucleus localization of *Pnky* (Fig. 5 and 6). Models suggest that nuclear lncRNAs are not limited to a defined set of functions but can regulate varied activities ranging from structural functions such as chromatin organization or structural scaffolds of nuclear domains, to regulatory functions such as transcriptional regulation or pre-mRNA splicing, among others (Sun et al. 2018; Statello et al. 2021). Moreover, accumulating evidence indicates that nuclear localized lncRNAs often interact with various nuclear RBPs to exert their regulatory functions. Using *cat*RAPID tool, strikingly, we noticed that SARNP, Aly/Ref, U2AF1, U2AF1L4 and THOC7, which commonly participate in mRNA splicing and export processes, were predicted to directly interact with *Pnky* (Table 1), implying that *Pnky* may play an active role in modulating mRNA splicing and export processes.

Regarding pre-mRNA splicing, which is thought to be a post-transcriptional regulation of gene expression, splicesome is the executioner that is responsible for removing introns from pre-mRNAs in all eukaryotes (Yan et al. 2019). Spliceosome is identified to be composed of at least five small nuclear ribonucleoprotein particles (snRNPs, U1, U2, U4/U6, and U5) that recognize intronic splice sites by base pairing, organize the assembly of protein splicing factors, and catalyze the cleavage and ligation reactions (Shi et al. 2017b; van der Feltz and Hoskins 2019). U2AF1 is the small subunit of the U2 auxiliary factor (U2AF) that constitutes the U2 snRNP which is primarily responsible for 3' splice site selection during splicing (Kováčová et al. 2020). Accumulating evidence showed that U2AF1 mutation caused altered pre-mRNA binding and splicing kinetics in a variety of cell types, such as hematological malignancies and lung adenocarcinomas, etc (Carruale et al. 2019; Esfahani et al. 2019; Palangat et al.

2019). In our study, colocalization of *Pnky* and U2AF1, as well as RIP assay, provide evidence in support of the direct interaction between *Pnky* and U2AF1 (Fig. 5a and c). Moreover, the mRNA levels of downstream target genes, such as MMP2 and Paxillin et al., were evidently altered in *Pnky* silencing or over-expression cells (Fig. 4a-d), which further dropping a hint that *Pnky* may regulate the splicing of downstream target genes through interacting with U2AF1. Surprisingly, it seems unlikely that *Pnky* could directly bind to U2AF1L4, another splicing factor that functionally substitute for U2AF1, as evidenced in Fig. 5b and d. Probably, the low affinity of *Pnky* to U2AF1L4 may be attributed to the low expression and substitute function of U2AF1L4.

Mature mRNA export from the nucleus to the cytoplasm couples the process of mRNA splicing and translation. A large proportion of mRNA export is specifically controlled by the dynamic TREX complex, which contains multiple proteins including Aly/Ref, UAP56, SARNP, CHTOP and THO subcomplex (THOC1, THOC2, THOC7 et al.) (Williams et al. 2018). During splicing, UAP56 is firstly recruited to the messenger ribonucleoprotein (mRNP) where it is involved in the ATP-dependent recruitment of Aly/Ref, SARNP and THO subcomplex to the mRNP, thus forming TREX complex (Williams et al. 2018). CHTOP binds to UAP56 in a mutually exclusive manner with Aly/ref (Chang et al. 2013). Abnormal expressions of Aly/Ref, SARNP and THO are found in multiple tumor cells, such as prostate cancer, hepatoma, pancreatic carcinoma and myeloid leukemia cells, which can directly affect their migration and invasion activities through regulating mRNA export (Borden 2020; Tran et al. 2016). Furthermore, many intracellular factors are reported to affect migration behavior of cells *via* modulating mRNA export. For instance, NSun2 promotes cell migration through mediating methylation of ATX mRNA which can facilitate its export in an Aly/Ref-dependent manner (Xu et al. 2020). LncRNA *MALAT1* controls chimeric mRNA export process and regulates myeloid progenitor cell differentiation and migration in malignant hematopoiesis (Chen et al. 2020).

Strikingly, in our present study, we confirmed the direct binding of *Pnky* to SARNP, Aly/Ref and THOC7, manifested as high enrichment of *Pnky* by RIP assay, as well as the colocalization of *Pnky* with the corresponding proteins (Fig. 6). Given the important roles of SARNP, Aly/Ref and THOC7 in mediating mRNA export, it is not surprising to draw the conclusion that *Pnky* modulates mRNA export process. Moreover, effective mRNA export is an essential prerequisite of cytoplasmic protein expression, and blocking of mRNA export results in differentially altered protein expression and cell defect (Okamura et al. 2018). Consistently, protein expressions of MMP2 and MMP9 et al. were also found to be altered in *Pnky* silencing or over-expression NSCs (Fig. 4). Such evidence indirectly supports the assumption that *Pnky* may regulate mRNA export through interacting with SARNP, Aly/Ref and THOC7.

Furthermore, emerging studies showed that TREX complex was recruited to mRNA predominantly by the splicing machinery (Williams et al. 2018). More interestingly, our results showed that lncRNA *Pnky* can directly bind to both splicing factor (U2AF1) and mRNA export adaptors (SARNP, Aly/Ref and THOC7), thus coupling mRNA splicing and export processes of target genes (MMP2 and Paxillin et al.). In view of this, it is reasonable to speculate that, initially, as a splicing machinery regulator, lncRNA *Pnky* modulates pre-mRNA splicing of target genes through interacting with U2AF1. Subsequently, *Pnky* is more likely to

act as RNA scaffold that recruits SARNP, Aly/Ref and THOC7 to TREX complex, thus facilitating mRNA export of target genes.

Taken together, this study adds a new piece to the puzzle of lncRNA *Pnky* contribution to NSC behaviors. *Pnky* actions in NSC migration appear to be mediated by coordinating mRNA splicing and export processes of target genes. Further study is needed to address the details. Once a greater understanding of how these scaffolding complexes is assembled and regulated is achieved, it would then be possible to design strategies to selectively utilize specific signaling components to redirect cellular behavior.

Declarations

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Conflict of Interest

The authors confirm that there are no conflicts of interest.

Availability of data and material

The datasets generated during and/or analyzed during the current study are available online.

https://www.jianguoyun.com/p/DaJknWUQytvbCRiEpIQE

Code availability

Not applicable

Author contributions

JND and **YL** designed the study, performed the research and analyzed the data. **YTS, WQZ and JLZ** helped finish some part of the study. **CZ and JW** helped in material preparation and data analysis. **WSD** participated in study design and modified the manuscript. **SSZ** contributed to study design, data collection and analysis, funding acquisition and preparation of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Prediction of lncRNA *Pnky*-Pretein interactions using *cat*RAPID program

ProteinID	RNAID	Z-score	Interaction Strength (%)	Location	Functions
SARNP (CIP29)	mPnky	1.41	99	nucleus	mRNA export from nucleus, Pre-mRNA splicing, Transcription, Transcription regulation
Aly/Ref (THOC4)	mPnky	1.40	99	nucleus	mRNA export from nucleus, mRNA processing, mRNA splicing
U2AF1	mPnky	1.34	99	nucleus	mRNA processing, mRNA splicing
Nudt16	mPnky	1.34	99	Cytoplasm (predominantly);nucleolus	U8 snoRNA-decapping enzyme, mRNAs degradation, rRNA processing
RPL10A	mPnky	1.27	99	Cytoplasm	cytosolic large ribosomal subunit
PARK7	mPnky	1.24	99	Cytoplasm (predominantly); nucleus ER mitochondrion	Autophagy, DNA damage, DNA repair, Fertilization, Inflammatory response, Stress response
U2AF1L4	mPnky	1.22	99	Nucleus	mRNA processing, mRNA splicing
THOC7	mPnky	1.20	99	Nucleus	mRNA export from nucleus
ZCRB1	mPnky	1.15	99	Nucleus	mRNA processing, mRNA splicing
NOL12	mPnky	1.14	99	nucleolus	rRNA binding

Figures

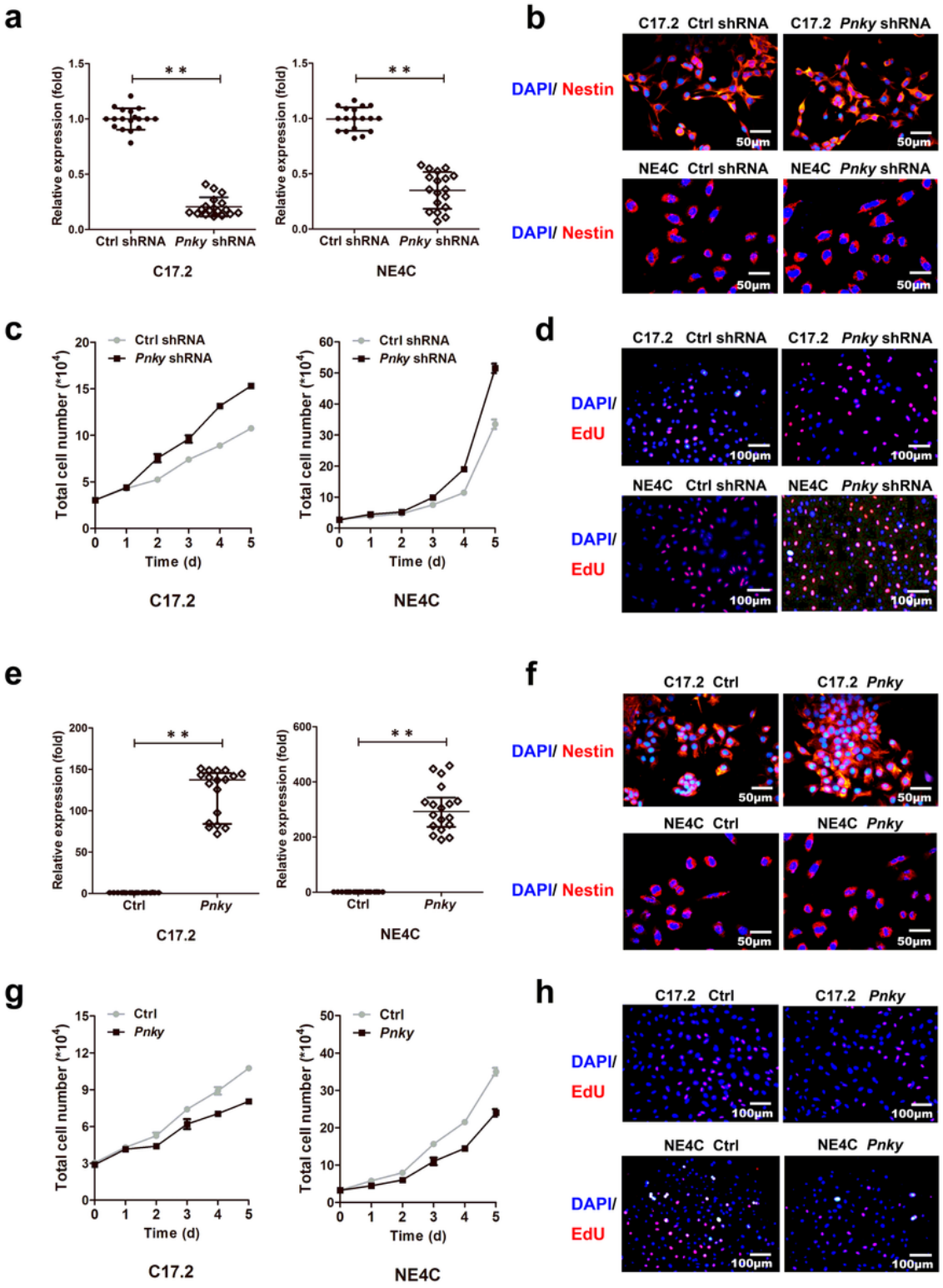

Figure 1

Roles of lncRNA Pnky on stem cell maintenance and proliferation of NSCs. a Pnky expression analysis in C17.2 and NE4C stable cell lines that expressing Pnky shRNA or control shRNA. b Immunofluorescence detection for Nestin (+, red) in the above Pnky silencing stable cell lines. Nuclei were labeled blue with DAPI. c, d Pnky knockdown promoted the proliferation of C17.2 and NE4C. Cell proliferation assays were performed using cell counting methods (c) or EdU incorporation assay (d). e Pnky expression analysis in

the C17.2 and NE4C cell lines that stably expressing Pnky gene or their corresponding controls. f Immunofluorescence detection for Nestin (+, red) in the above Pnky over-expression stable lines. Nuclei were labeled blue with DAPI. g, h Pnky over-expression repressed the proliferation of C17.2 and NE4C. Cell proliferation assays were performed using cell counting method (g) or EdU incorporation assay (h). Accordingly, data were displayed as mean ± SD or median and interquartile range of six independent experiments (three technical replicates each). **, P≤0.01. P values were obtained by one-way ANOVA or Mann-Whitney U test

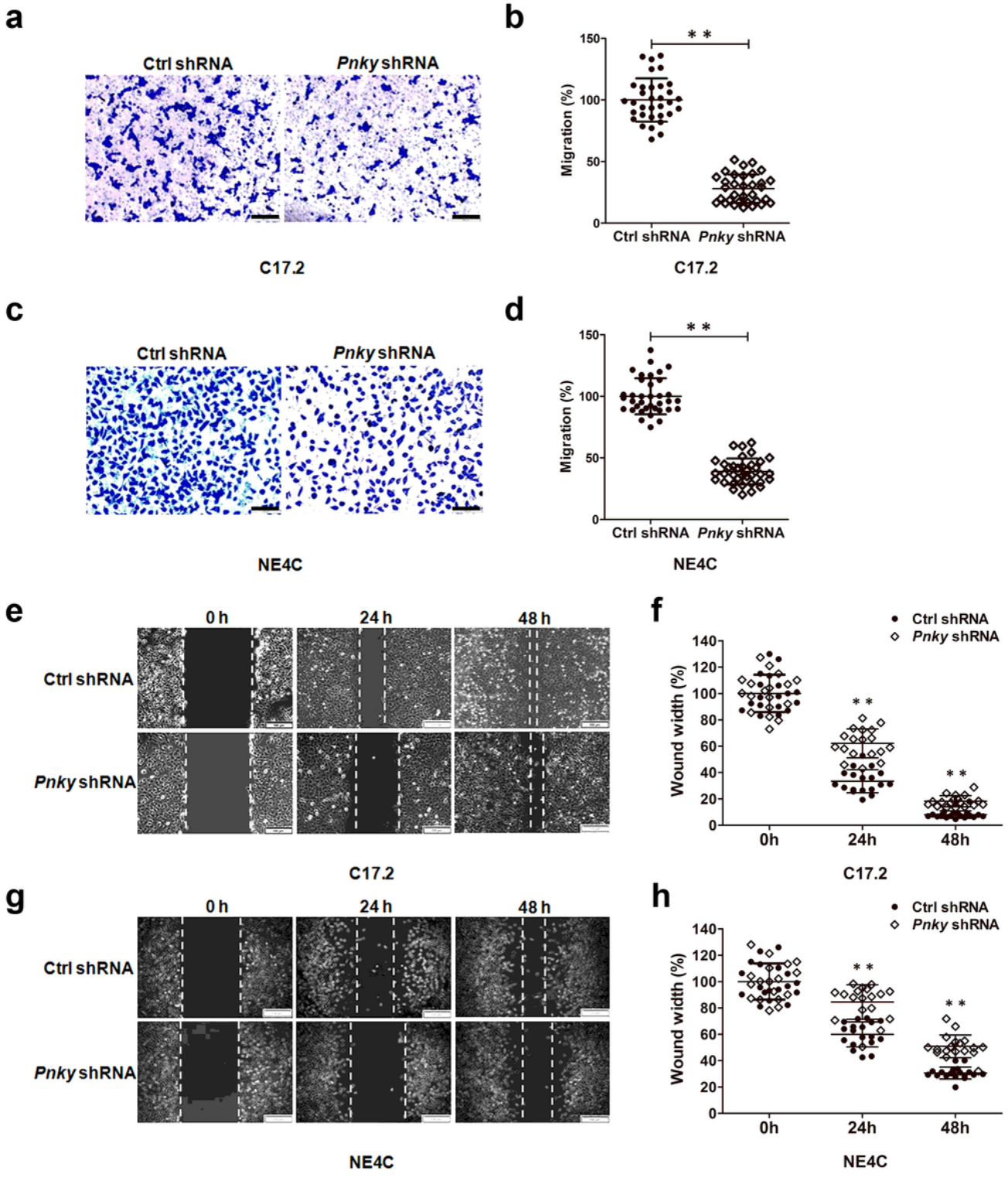

Figure 2

Knockdown of Pnky attenuates the migration of NSCs. a, b Transwell migration assay of C17.2 cell lines stably expressing Pnky shRNA or control shRNA (a), as well as the statistical analysis of the cell numbers passing through the basement membrane of transwell chambers in each group (b). c, d Transwell migration assay of NE4C cell lines stably expressing Pnky shRNA or control shRNA (c), as well as the statistical analysis of the cell numbers passing through the basement membrane of transwell chambers in each group (d). The scale bars in a and c represent 100 μm. Results in b and d were expressed as mean ± SD of six independent experiments. **, $P \leq 0.01$. P values were obtained by Student's t-test. e, f Wound healing assay (e) and the quantification of scratch wound closing (f) using C17.2 Pnky shRNA and control cell lines. g, h Wound healing assay (g) and the quantification of scratch wound closing (h) using NE4C Pnky shRNA and control cell lines. Scale bars in e and g represent 200 μm. Results in f and h were expressed as mean ± SD of six independent experiments. **, $P \leq 0.01$. P values were obtained by repeated measures tests. The Mauchly's test in f suggested the sphericity assumption had been violated ($P = 0.02$), and a Greenhouse-Geisser correction was further performed to assess the variances between the pairs. In case of h, sphericity assumption was met ($P = 0.32$), sphericity assumed test was used

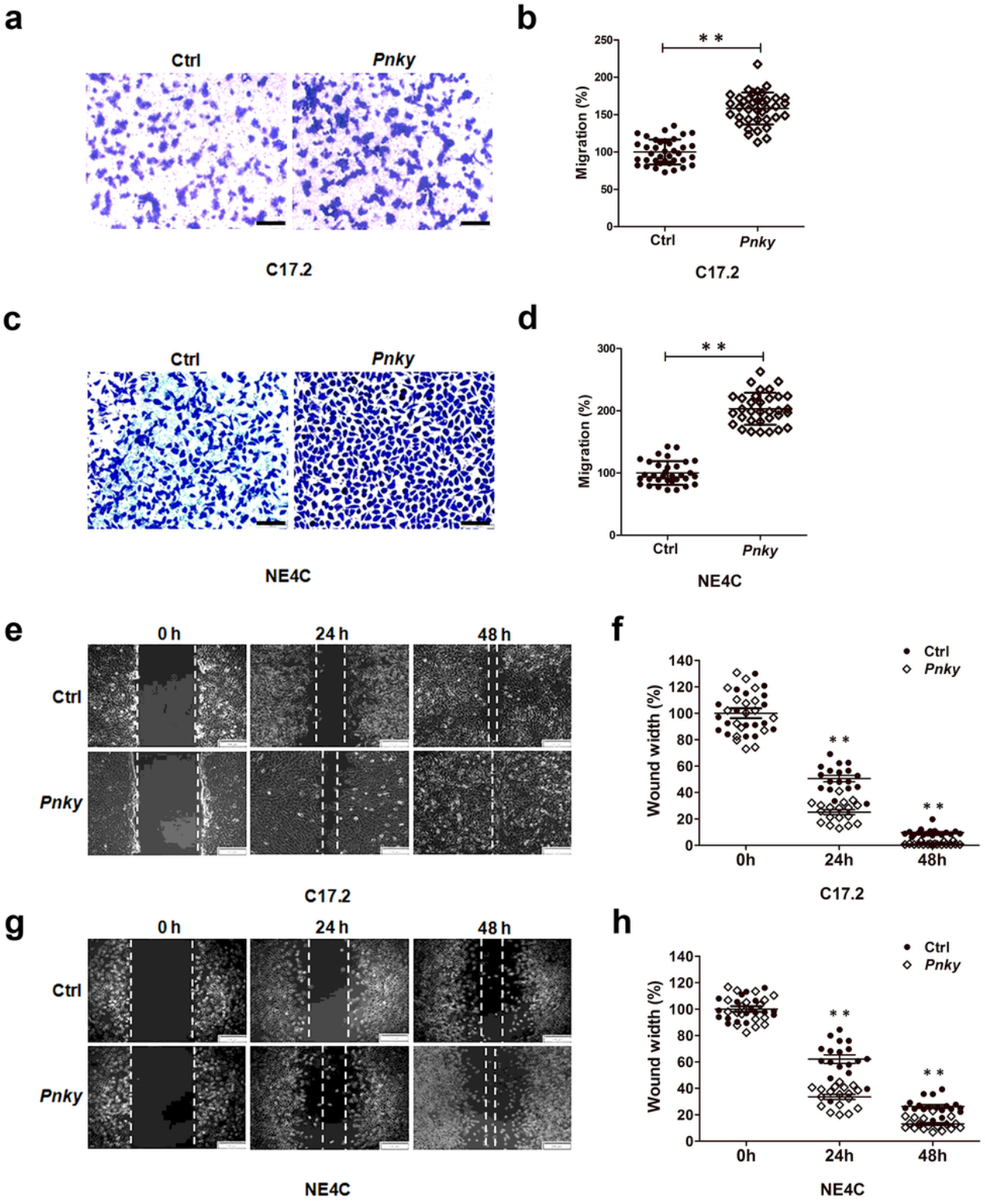

Figure 3

Pnky over-expression promotes NSC migration. a-d Transwell migration assay and statistical analysis of cell numbers that migrated to the underside of the transwell membrane using Pnky over-expression or control stable cell lines (a-b: C17.2 cells; c-d: NE4C cells). The scale bars in a and c represent 100 μm. Results in b and d were expressed as mean ± SD of six independent experiments. **, P≤0.01. P values were obtained by Student's t-test. e-h Wound healing assay and the quantification of scratch wound

closing using the corresponding Pnky over-expression and control cell lines (e-f: C17.2 cells; g-h: NE4C cells). Scale bars in e and g represent 200 μm. Results in f and h were expressed as mean ± SD of six independent experiments. **, P≦0.01. P values were obtained by repeated measures tests. The Mauchly's test in f suggested the sphericity assumption was not met (p□0.01), a Greenhouse-Geisser correction was further performed to assess the variances between the pairs. In case of h, sphericity assumption could be held (P = 0.10), sphericity assumed test was used

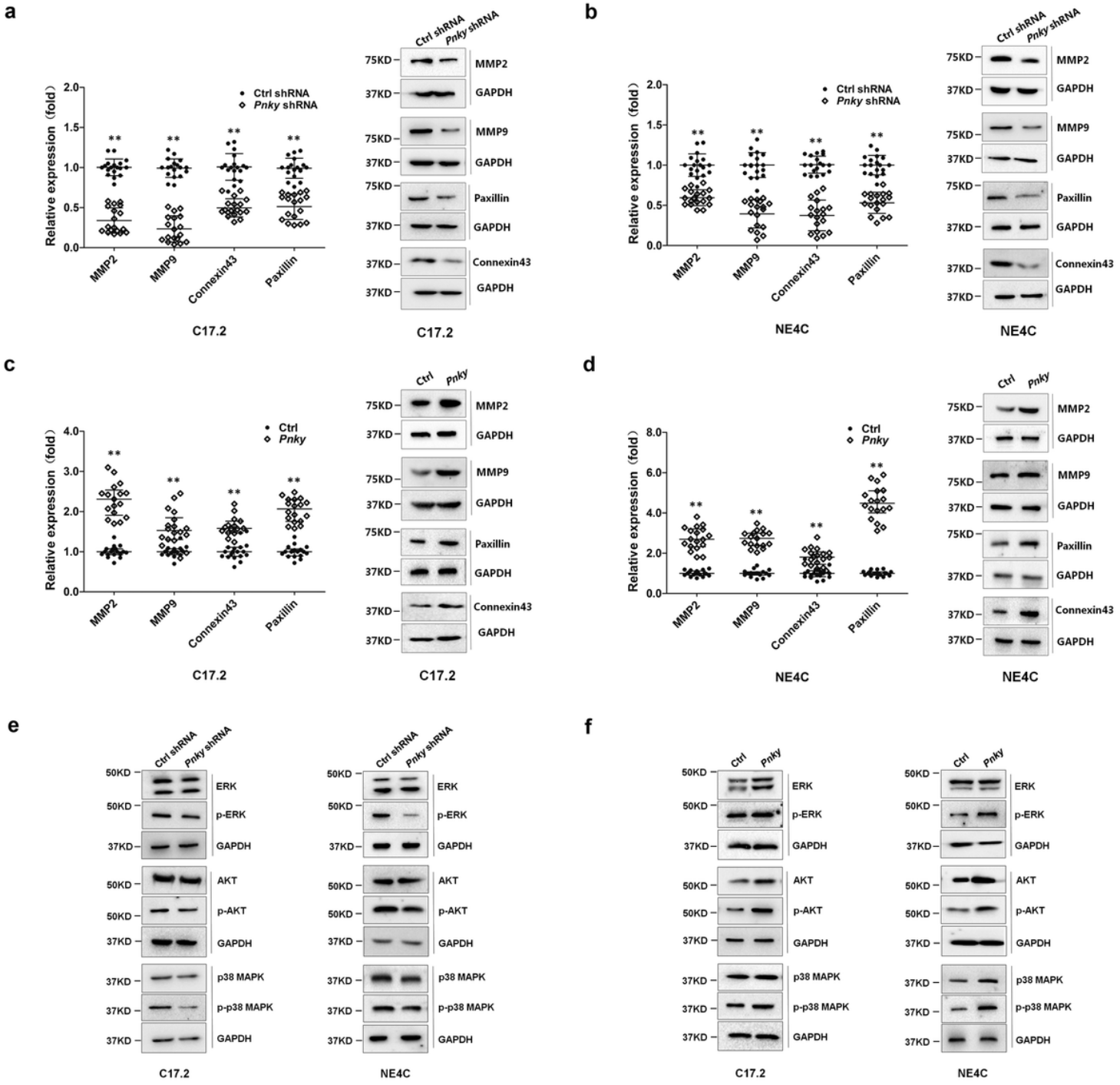

Figure 4

Pnky regulates the expression of some critical regulators and signaling pathways involved in NSC migration. a, b Pnky silencing decreased the expression of MMP2, MMP9, Connexin43 and Paxillin both

in C17.2 (a) and NE4C cells (b). Left panel: RT-qPCR analysis; Right panel: Western blot analysis. c, d Pnky over-expression promoted the expression of MMP2, MMP9, Connexin43 and Paxillin both in C17.2 (c) and NE4C cells (d). Left panel: RT-qPCR analysis; Right panel: Western blot analysis. e Pnky knockdown depressed the expression and phosphorylation of ERK, AKT and p38MAPK both in C17.2 (left panel) and NE4C cells (right panel). f Pnky over-expression increased the expression and phosphorylation of ERK, AKT and p38MAPK both in C17.2 (left panel) and NE4C cells (right panel). Accordingly, data were displayed as mean ± SD or median and interquartile range of six independent experiments (three technical replicates each). **, P≤0.01. P values were obtained by one-way ANOVA or Mann-Whitney U test. Full Western blots with markers and their corresponding loading controls could be found in Fig. S1-S8. ERK and p-ERK, AKT and p-AKT, as well as p38MAPK and p-p38MAPK, were assessed on the same membranes, respectively

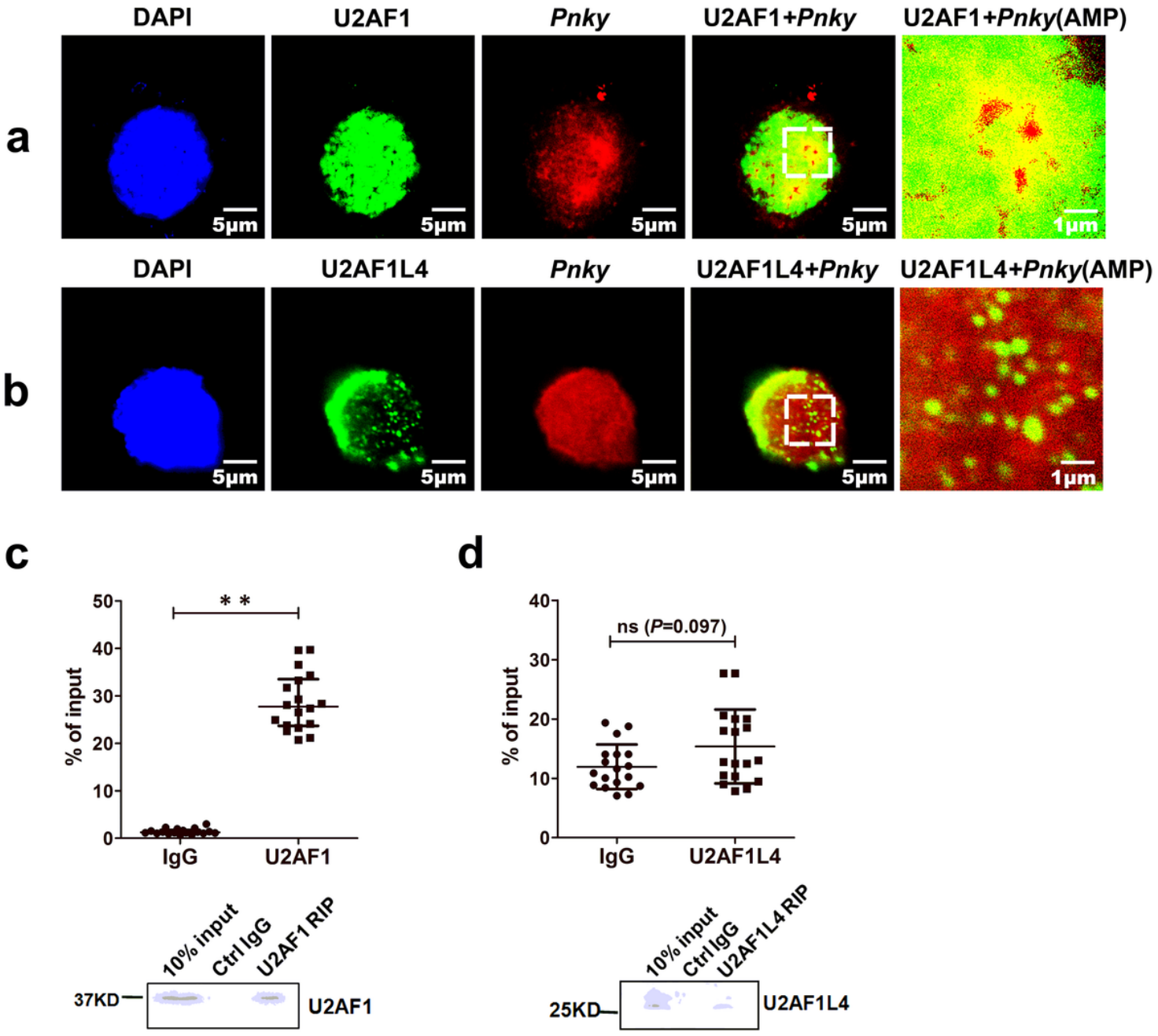

Figure 5

lncRNA Pnky directly binds to splicing factor U2AF1. a, b Confocal images showing the colocalization of lncRNA Pnky with splicing factor U2AF1 (a) or U2AF1L4 (b). c Upper panel: RIP analysis for the binding of U2AF1 to Pnky in NE4C cells. Data were displayed as median and interquartile range of six independent experiments (three technical replicates each), which represent fold-enrichment of Pnky immunoprecipitated by specific U2AF1 antibody or anti-IgG (**P<0.01 vs. IgG, Mann-Whitney U test). Lower panel: Representative immuno-blotting of U2AF1 pulled down using U2AF1 antibody. d Upper panel: RIP analysis for the binding of U2AF1L4 to Pnky in NE4C cells. Results were expressed as median and interquartile range of six independent experiments (three technical replicates each). Individual data points represent fold-enrichment of Pnky immunoprecipitated by specific U2AF1L4 antibody or anti-IgG (P=0.097 vs. IgG, Mann-Whitney U test). Lower panel: Representative immuno-blotting of U2AF1L4 pulled down using U2AF1L4 antibody. Full Western blots with markers could be found in Fig. S10

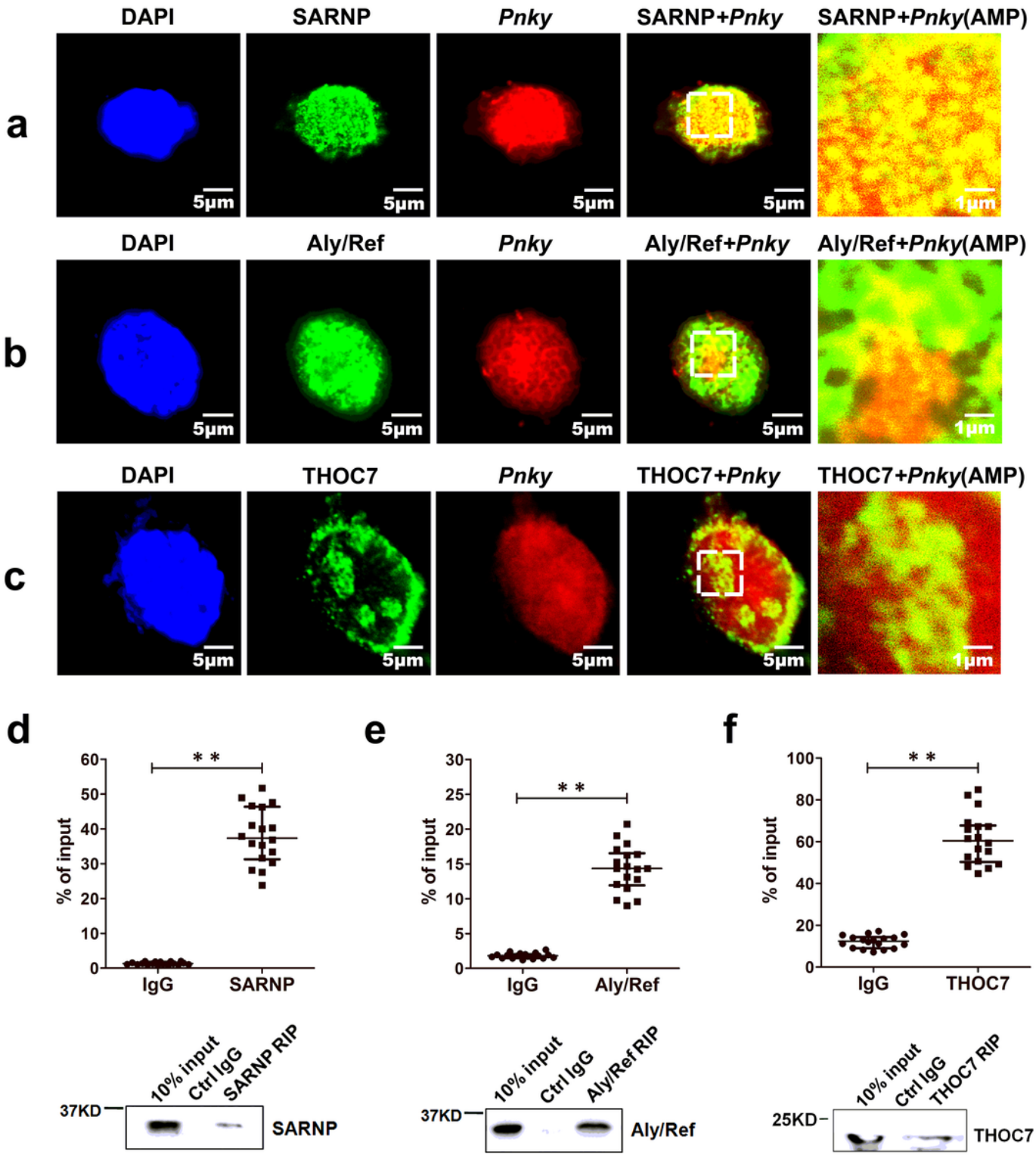

Figure 6

lncRNA Pnky directly binds to SARNP, Aly/Ref and THOC7. a-c Confocal images showing the colocalization of lncRNA Pnky with SARNP (a), Aly/Ref (b) and THOC7 (c). d-f Upper panel: RIP analysis for the binding of Pnky with SARNP (d), Aly/Ref (e) or THOC7 (f) in NE4C cells. Data were displayed as median and interquartile range of six independent experiments (three technical replicates each). Individual data points represent fold-enrichment of Pnky immunoprecipitated by corresponding SARNP,

Aly/Ref or THOC7 antibodies, as well as anti-IgG (**P<0.01 vs. IgG, Mann-Whitney U test). Lower panel: Representative immuno-blotting of SARNP (d), Aly/Ref (e) or THOC7 (f) pulled down using corresponding antibodies. Full Western blots with markers could be found in Fig. S11

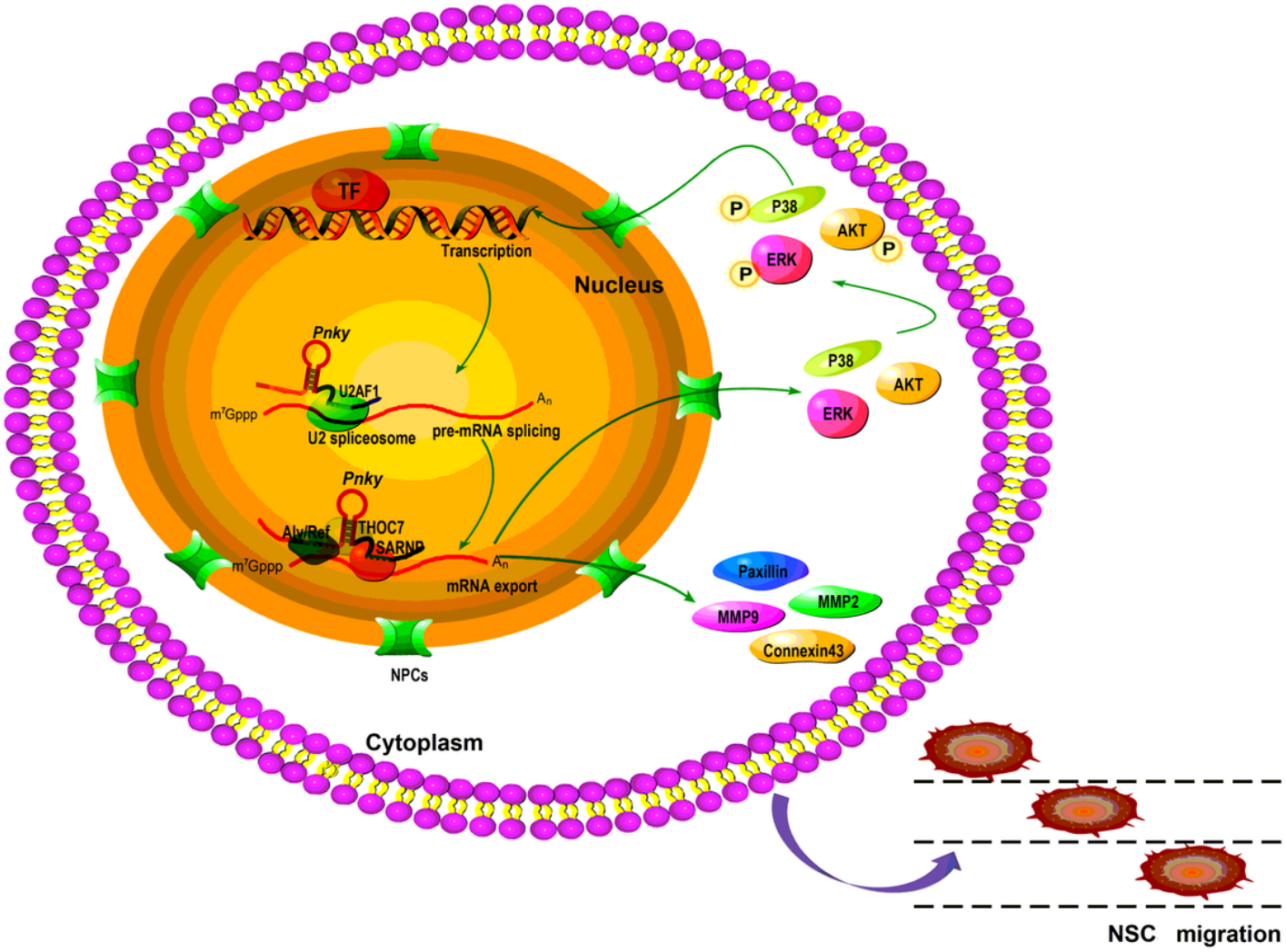

Figure 7

The mechanism of the regulatory network and function of lncRNA Pnky in NSC migration. Through interacting with U2AF1, SARNP, Aly/Ref and THOC7, Pnky couples and modulates mRNA splicing and export process of downstream factors, such as MMP2, MMP9, p38MAPK, and Akt et al., which subsequently facilitating NSC migration

Supplementary Files

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