KLF4 transactivates TRIM29 expression and modulates keratin network

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ABSTRACT

The Krüppel-like factor 4 (KLF4) is well known to be a conserved zinc-containing transcription factor that participates in diverse biological processes such as cell proliferation and differentiation. In this study, we found KLF4 can bind specific site in the promoter of TRIM29 to transactivate its transcription, and sumoylation modification on 278 lysine site was not essential for KLF4 to transactivate TRIM29 transcription. It also was showed that KLF4 promoted cell migration when overexpressed, and knockdown of TRIM29 abrogated the migration triggered by KLF4. In addition, overexpression of KLF4 reduced the phosphorylation level of keratin 8 at 432 amino acid site. Our study demonstrated that KLF4 is an important transcription factor on regulating TRIM29 expression and modulates the keratin network.

1. Introduction

Krüppel-like factor 4 (KLF4), also called gut-enriched Krüppel-like factor (GKLF), is a zinc-containing transcription factor. KLF4 gene is conserved among vertebrate, and get lots of attention not only because of its key role on inducing pluripotent stem cells, but also because of its extensive participation on physiological processes and diseases. KLF4 exerts different physiology functions dependent on cell types. It was showed that KLF4 maintained a low expression level in colorectal cancer compared to normal tissues and overexpression inhibited the progression of CRC [1]. In contrast, KLF4 was in a high level in mammary cancer, and knockdown of KLF4 slowed down cell renewing and migration in vitro [2,3]. KLF4 was considered as a versatile transcription factor regulating many cellular activities [4]. However, the regulation on cell migration and cellular bone skeleton has been known little about. In this study, we reported that KLF4 transactivated the transcription of TRIM29 (Tripartite motif-containing 29) and promoted cell migration. Knockdown of TRIM29 attenuated the migration induced by KLF4 overexpression. Sumoylation has been reported to enhance the ability of KLF4 to induce its target genes transcription in macrophages, but our evidence showed that KLF4-mediated TRIM29 transactivation is independent of sumoylation on 278 lysine. Furthermore, overexpression of KLF4 reduced the phosphorylation level of keratin 8 at amino acid 432

site (K8–S432) which is closely associated with the assembly and disassembly of keratin network. Overall, the study revealed that KLF4 is an important transcription factor to regulate TRIM29 expression and modulate keratin network.

2. Materials and methods

2.1. Cell line and plasmid construction

The human embryonic kidney cell line HEK293T which was purchased from the ATCC was cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco), penicillin (100 U/ml), and streptomycin (100 μg/ml) at 37 °C in a humidified incubator with 5% CO_2.

KLF4 ORF was cloned into the pCDNA3.1 with an N-terminal HA (influenza hemagglutinin epitope) tag. The reporter plasmid was build up by ligating the 1437 bp TRIM29 promoter sequence (from-1246 bp to +191 bp) to the pGL3-Basic vector with Kpn I and Hind III sites. The mutant K278R and reporter lacking of KLF4 binding site were constructed with Fast Mutagenesis System (TransGen Biotech) using the wild type plasmid as template.

2.2. RNA extraction and qRT-PCR

Total RNA was extracted using Total RNA Kit I (Omega) according to the manufacturer's protocol. RNA (1500 ng) was reverse-transcribed with the EasyScript one-step gDNA Removel and cDNA Synthesis Super Mix (TransGen Biotech). Real-time PCR was performed with TransStart® Tip Green qPCR SuperMix (TransGen Biotech). PCR procedure was described as followed: 95 °C for 3 min; 95 °C for 15 sec, 60 °C for 30 sec, 72 °C for 15 sec (45 cycles). The primers used for qRT-PCR were showed in Table S1. The relative gene expression levels were determined by the $2^{-\Delta\Delta Ct}$ method with GAPDH as the internal control.

2.3. Protein extraction and Western Blotting analysis

Cells were rinsed with cold PBS (phosphate buffered solution) twice, and total protein was extracted using RIPA buffer supplemented with the protease inhibitor cocktail (Beyotime Biotechnology) on ice for 30 min. The protein concentration of lysis was measured with BCA Protein Assay Kit (ThermoFisher). Proteins were separated by SDS-PAGE and then transferred to nitrocellulose membrane (Millipore). The membranes were blocked in 10% skim milk for 1 hr at room temperature followed by incubating with specific primary antibodies at 4 °C overnight. The antibody used were listed as followed: GAPDH (1:10000; Cell Signaling Technology), HA Rabbit antibody (1:10000, Cell Signaling Technology), TRIM29 (1:500, Santa Cruz), keratin 8 (1:10000, Proteintech), keratin 18 (1:10000, Proteintech), keratin 8-phops 432 (1:10000, Abcam), keratin 8-phops 52 (1:10000, Abcam). After four times washing with PBST (PBS containing 0.5% Tween-20), membranes were incubated with Goat anti-mouse-HRP antibody or Goat anti-rabbit-HRP antibody (1:10000, Cell Signaling Technology) for 1 hr at room temperature. After four times washing, protein bands were detected by enhanced chemiluminescence reagent (ThermoFisher).

2.4. Immunofluorescent assay (IFA)

Cells were washed twice with PBS, fixed with 4% paraformaldehyde for 20 min, permeabilized with 0.2% Triton X-100 in PBS for 15 min, and blocked with normal goat serum (Boster) for 1 hr at room temperature. Following, cells were incubated with HA tag antibody (1:500, Cell Signaling Technology) or pan-keratin antibody (1:500, Cell Signaling Technology). After 2 hr incubation, cells were washed for four times (5 min per washing) with PBST on table concentrator. Then secondary antibody conjugated 488 or 594 nm fluorophore (ThermoFisher) was incubated for 1 hr, and nuclei were stained with Hoechst 33342 (ThermoFisher). Images were captured with Leica confocal microscope.

2.5. Transient transfection and transwell assay

Cells seeded in 6 cm-diameter Petri dish with 50%–60% confluence were transfected with 2 μg plasmid or 100 nM siRNA (final concentration). The plasmids and siRNA were transfected by lipo 8000 (Beyotime Biotechnology) and RNAi Max (ThermoFisher) respectively.

Cell layers were digested with 2.5% trysin and suspended with FBS-free DMEM. 24-well transwell plates (Corning)with 8-μm pore size polycarbonate filter were used for transwell assay.

10^5 cells in 200 μl volume were seeded onto the upper cell and 600 μl DMEM with 20% FBS was injected into the down cell. 24 hr later, cells was fixed with fixed with 4% paraformaldehyde and stained with crystal violet. The cells on upper surface of filter were erased with cotton swab, and after rinsing with PBS, the cells on lower surface were photographed at magnification of 200×.

2.6. Promoter reporter and dual-luciferase assay

5×10^4 cells were seeded into 24-well plates and transfected with 100 ng expression plasmids as indicated along with 20 ng reporter and
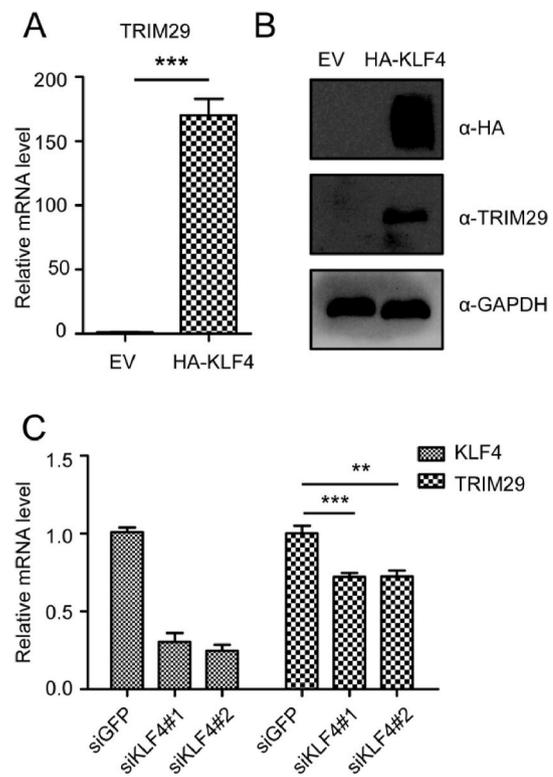
Fig. 1. KLF4 modulates TIRM29 expression. Transfection of KLF4 expression plasmid into HEK293T cells upregulated TRIM29 both at mRNA (A) (mean ± SD, n = 3) and protein level (B) after 48 hr. (C) Knockdown of KLF4 with siRNA downregulated the TRIM29 mRNA level (mean ± SD, n = 3). The statistical analysis of p values was performed using two-tailed Student's test (***, P < 0.001; **, P < 0.01).

20 ng pRL-TK. 30 hr later, cells were lysed with 200 μl lysis buffer, and the firefly and Renilla luciferase activities with Dual-luciferase Reporter Assay kit (Promega). Each experiment was repeated at least three times.

2.7. Chromatin immunoprecipitation assay

The ChIP Kit (Abcam) was use for ChIP assays. The specimens were prepared according to the manufacture's protocol. The specimens were incubated with 4 μg anti-HA antibody (Cell Signaling Technology) overnight at 4 °C, and IgG served as a negative control. The purified DNA fragments were analyzed using real-time qPCR.

2.8. Binding activity of KLF4 to promoter site

The nuclear extract was isolated using Nuclear and Cytoplasmic Protein Extraction Kit (Beyotime). The interaction between KLF4 and probes was determined using EpiQuik™ General Protein-DNA Binding Assay Kit (Colorimetric) (EPIGENTEK) according to manufacturer's protocol. Briefly, the reaction system contains 23 μl binding buffer, 6 μg nuclear extract, 40 ng probes. For determining specificity, 2 μl unlabeled probes (cold-probe) has been added in reaction. The reaction time was 60 min at room temperature. After 60 min incubation, three times washing were performed and primary antibody was added and incubated for 1 hr. After four times washing, HRP-conjugated secondary antibody was added and incubated for 30 min. After four times washing, 100 μl developing solution was added for reacting 2–10 min. 50 μl stop solution was used to stop the chromogenic reaction and the OD value was determined at 450 nm.
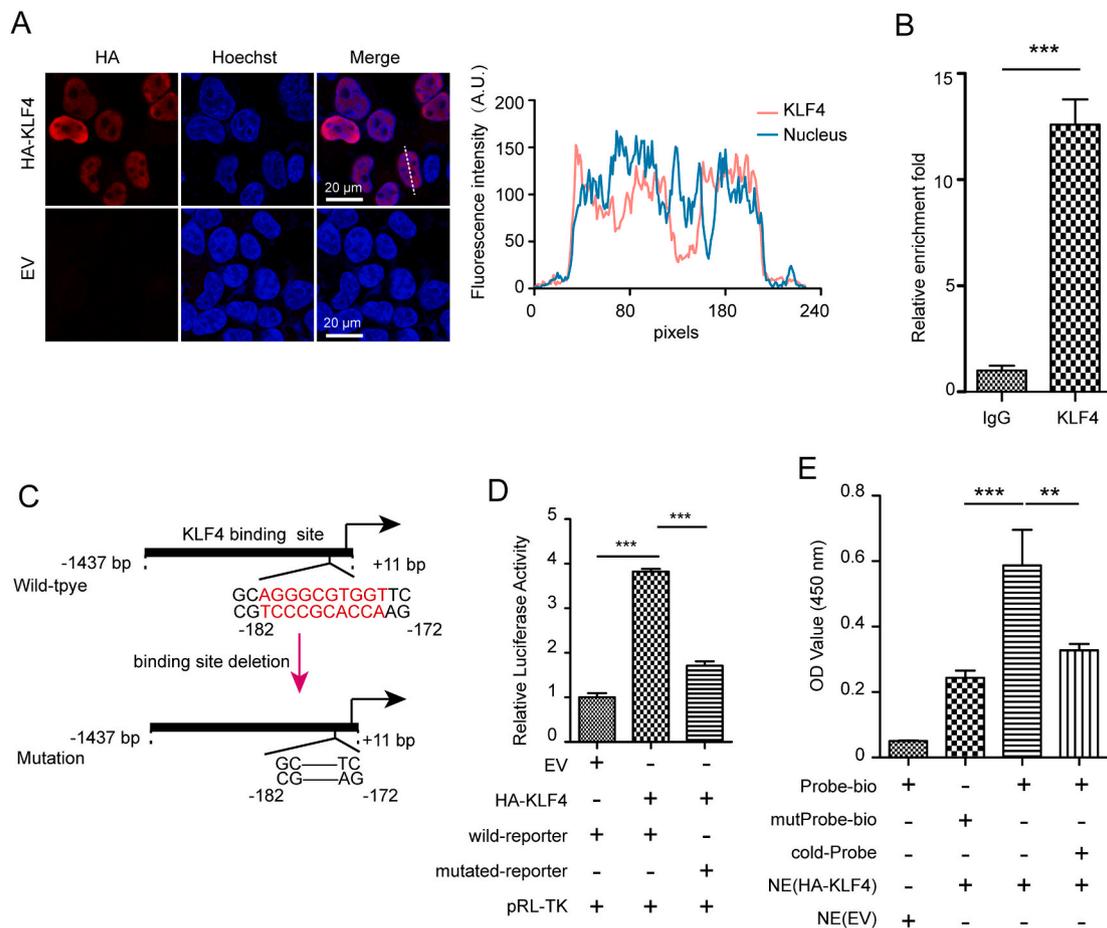
Fig. 2. KLF4 binds the specific site in promoter domain to modulate TRIM29 expression. (A) KLF4 located in the nucleus. (B) KLF4 enriched in the promoter region of TRIM29 in Chip assay (mean ± SD, n = 3). (C) Schematic map of reporters in luciferase assay. The sequence in red indicated KLF4 binding site. (D) Overexpression of KLF4 upregulated the TRIM29 promoter activity and deletion of KLF4 binding site reduced the promoter activity (mean ± SD, n = 3). (E) The binding of KLF4 and promoter site was determined by Colorimetric assay. NE(HA-KLF4) &NE(EV), nuclear extract from cells transfected with HA-KLF4 plasmids or empty vectors (mean ± SD, n = 4). The statistical analysis of p values was performed using two-tailed Student's test (***, P < 0.001; **, P < 0.01). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

2.9. Statistical analysis

Data was analyzed with GraphPad Prism 5 software and presented as the mean ± standard deviation. Two-tailed unpaired Student's t-test was used to evaluate the statistical differences of two groups. It was considered to be statistically significant where p < 0.05. The asterisks indicate differences that are statistically significant (***, P < 0.001; **, P < 0.01; *, P < 0.05).

3. Results

3.1. TRIM29 is transactivated by KLF4

It was showed that overexpression of KLF4 in HEK293T cell upregulated TRIM29 expression both in mRNA and protein level (Fig. 1A and B). In consistence, knockdown of KLF4 significantly downregulated the mRNA level of TRIM29 (Fig. 1C). The results suggested that KLF4 may modulate the expression of TRIM29 at transcription process.

3.2. KLF4 transactivates TRIM29 by binding the specific site in promoter

KLF4 is considered as a transcriptional factor. In fact, we also found that KLF4 mainly located in nucleus (Fig. 2A). In order to confirm that KLF4 upregulate TRIM29 expression dependent on its transcription factor activity, we performed the Chromatin Immunoprecipitation

(ChIP) assay. As shown, immunoprecipitation of KLF4 significantly enriched TRIM29 promoter fragment (Fig. 2B). Using JASPAR tool, a database of transcription factor binding profiles, we found there is a specific KLF4 binding site in TRIM29 promoter region with high score (Fig. 2C). It was shown that KLF4 was able to upregulate the reporter signal in dual-luciferase assay, whereas deletion of the binding site significantly reduced the raising extent of reporter signal (Fig. 2D). In addition, the direct binding of KLF4 to specific site was confirmed by protein-DNA binding assay (Fig. 2E). These evidences indicated that KLF4, as a transcriptional factor, activates TRIM29 transcription by binding the specific site in promoter.

3.3. KLF4 transactivates TRIM29 independent on the SUMOylation

TRIM29 was reported to be involved in migration and invasion of numerous solid tumors, like breast cancer, lung cancer and liver cancer [5–7]. In fact, we also found that TRIM29 promoted movement in transwell assay when it was stably expressed in HEK293T cells (Fig. S1A). To know whether overexpression of KLF4 will promote the migration like TRIM29, we transfected the KLF4 plasmid into HEK293T cells and performed transwell assay. It was demonstrated that overexpression of KLF4 also enhanced cell migration, which was abrogated by knockdown TRIM29 (Fig. 3A and B). Transactivating the downstream genes by KLF4 was reported to be dependent on the sumoylation. However, substitution of lysine with arginine on 278 sites of KLF4, the
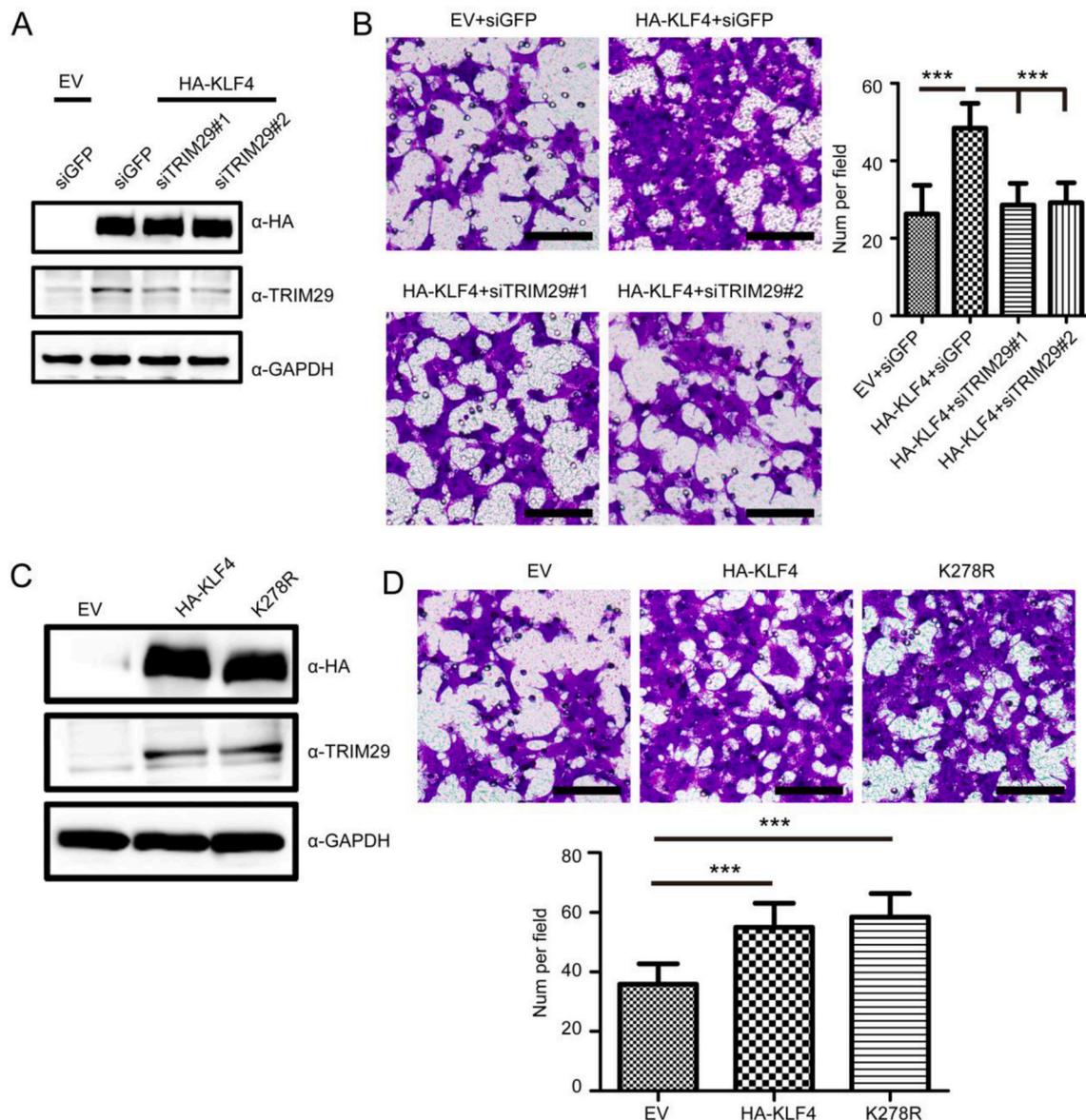
Fig. 3. Overexpression of KLF4 promotes cell migration. (A and B) HEK293T cells were transfected with KLF4 expression plasmid and siRNAs, and transwell assays was performed after 30 hr. (C and D) KLF4 wildtype and muant (K278R) expression were infected into HEK293T cells, and transwell assays was performed after 30 hr. KLF4 and TRIM29 were detected by Western Blotting. The statistical analysis of p values was performed using two-tailed Student's test (***, P < 0.001). Scale bar = 20 μm.

most possible sumoylation site predicted by on-line tools (Fig. S1B and 1C), did not harm the transactivation on TRIM29 and the promotion on migration (Fig. 3C and D). The results above indicated that KLF4 activates the transcription of TRIM29 to promote cell migration, and sumoylation is not necessary for its transcription-activating function on TRIM29.

3.4. KLF4 modulates keratin network

Teruki Yanagi et al. has reported that loss of TRIM29 was able to alter the distribution of keratin in squamous cell carcinoma and promote migration [8]. We also found that TRIM29 co-localized with and changed the distribution of keratin in HEK293T Cells when overexpressed (Fig. S2). We wanted to know whether KLF4 may modulate the keratin network by inducing TRIM29 expression. As results shown, overexpression of KLF4 did not influence the total protein level of keratin 8 and keratin 18 but reduced the phosphorylation level of K8–S432 (Fig. 4). We has determined the change of keratin 18 phosphorylation

level at 52 amino acid site (K18–S52), but no signal has been detected in the specimens (data not shown). Overall, our results demonstrated that KLF4 might modulate the keratin network by influencing the phosphorylation modification of keratin 8.

4. Discussions

Previous studies on KLF4 demonstrated its diverse functions in numerous tissues and biological processes, whereas we know little about the role of KLF4 in cell migration and keratin network dynamic. In this report, we indicated that KLF4 transactivated TRIM29, promoted cell migration and modulated the phosphorylation of keratin 8.

KLF4 is a versatile transcription factor, which can induce or suppress target gene expression dependent on context [9,10]. Our results showed that KLF4 was able to bind the specific site in TRIM29 promoter, resulting in upregulation of TRIM29. KLF4-mediated genes transactivation is regulated precisely through phosphorylation, acetylation, sumoylation, methylation and ubiquitination. Our evidence indicated
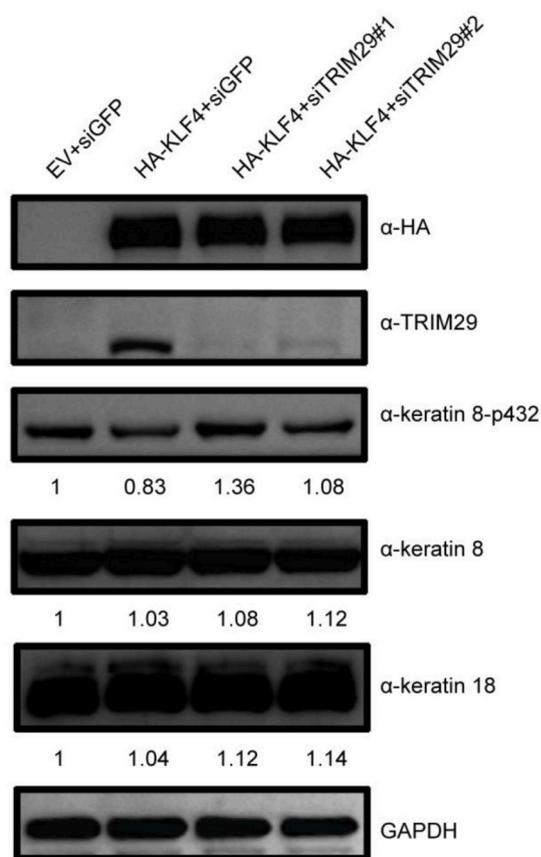
Fig. 4. KLF4 modulates the kertain network. HEK293T cells were transfected with KLF4 expression plasmid, followed by transfection with 100 nM final concentration siRNA indicated. The total keratin 8/18 and the keratin 8 phosphorylation level at 432 site was detected after 30 hr.

that KLF4 transactivation function to TRIM29 is independent of sumoylation on 278 lysine site, but whether other modification on KLF4 is necessary to this process needs more investigation.

TRIM29, also known as ataxia-telangiectasia group D-associated protein (ATDC) is the member of the TRIM family. Although TRIM29 has been shown to has E3 ligase activity [11,12], the function of it has not been fully clarified so far. TRIM29 was reported not only to involve in controlling antiviral immune response and mediating stem cell-like characteristics in pancreatic ductal adenocarcinomas, but also to alter keratin distribution in squamous cell carcinoma [8,13,14]. In fact, we also found that TRIM29 co-localized with keratin when it was overexpressed. The dynamic turnover and restructuring of keratin network is required for cell moving and cancer metastasis [15,16]. It seemed that KLF4 promoted cell migration by inducing TRIM29, leading to the reconstruction of keratin network, because knockdown TIRM29 abrogated the migration triggered by KLF4. The phosphorylation of K8-432 was reported to be closely associated with the assembly and disassembly of keratin network [17]. Actually, it was demonstrated that overexpression of KLF4 downregulated the phosphorylation level of K8–S432. It was worth noting that knockdown of TRIM29 recovered the phosphorylation level of K8-432, even slightly exceeded the background level. This implied that there is another unknown mechanism by which KLF4 modulates the keratin phosphorylation. Because both the total protein level of keratin 8 and keratin 18 had no change in the context of KLF4 overexpression, KLF4 may mostly modulate the keratin network by interfering in the phosphorylation of keratin.

TRIM29 also was demonstrated to negatively regulate innate immune response in dendritic cells, macrophages and natural killer cells dependent on its E3 ligase activity [18,19]. Adaptors of immune-related

signaling pathway such as STING, NEMO were modified by ubiquitin mediated by TRIM29 and degraded through proteasomes [11–13]. There was no evidence to confirm that TRIM29 modulated keratin stability despite altering keratin distribution [8]. Our results also demonstrated that the protein level was not changed in the context of upregulating TRIM29 triggered by KLF4 (Fig. 4). It is assumed that E3 ligase-independent function of TRIM29 exists. This hypothesis was supported by the evidence that TRIM29 fucntions as scaffold protein to assemble DNA repair proteins into chromatin independent of E3 ligase activity [20]. Whether KLF4 regulates innate immune response by inducing TRIM29 expression needs further investigation in the future.

In conclusion, it was revealed that KLF4 can transactivate TRIM29 and play a role in cell migration as well as modulating the keratin network. The study expands the knowledge of diverse functions of KLF4.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbrep.2021.101117.

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