

Overexpression of hCLP46 enhances Notch activation and regulates cell proliferation in a cell type-dependent manner

Q. Chu*, L. Liu* and W. Wang*,†

*College of Life Sciences, University of Chinese Academy of Sciences, Beijing, 100049, China and †School of Medical Sciences, Edith Cowan University, Perth, WA, 6027, Australia

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Abstract

Objectives: Human CAP10-like protein 46 kDa (hCLP46), also known as Poglut1, has been shown to be an essential regulator of Notch signalling. hCLP46 is overexpressed in primary acute myelogenous leukaemia, T-acute lymphoblastic leukaemia samples and other leukaemia cell lines. However, effects of hCLP46 overexpression, up to now, have remained unknown.

Materials and methods: In this study, we established stable 293TRex cell lines inducibly overexpressing hCLP46, and knocked down hCLP6 with a specific small interfering RNA to explore function of the protein in Notch signalling and cell proliferation.

Results: hCLP46 overexpression enhanced Notch1 activation in 293Trex cells in a ligand-dependent manner, with increased Notch signalling enhancing Hes1 expression. We further verified that overexpression of hCLP46 inhibited proliferation of 293TRexs and was correlated with increases in cyclin dependent kinase inhibitors p21 and p27, whereas reduced hCLP46 expression moderately increased cell proliferation. In addition, p21 and p27 protein levels were higher when Notch signalling was activated by EDTA treatment.

Conclusions: Taken together, hCLP46 enhanced Notch activation and inhibited 293TRex cell proliferation through CDKI signalling.

Introduction

Previously we have identified *hCLP46* (human Cap10-like protein 46 kDa) in human acute myelogenous leukaemia, transformed from myelodysplastic syndrome CD34⁺ cells (1), and proved it to be a potential biomarker for leukaemia (2). Acar *et al.* have reported that Rumi is *Drosophila* homologue of hCLP46 and adds a glucose moiety to serine residues in Notch EGF repeats (3). Later, hCLP46 was also shown to be an *O*-glucosyltransferase required for embryonic development and Notch signalling in mammals (1,4). Lack of hCLP46 leads to impaired ligand-induced Notch activation without affecting Notch ligand binding (2,5). Recently, hCLP46 also has been proven to be an *O*-xylosyltransferase and an inverting enzyme (6). These studies demonstrate the important role of hCLP46 in development and pathogenic outcome when misregulated in leukaemia.

Core components of Notch signalling are transmembrane ligands (Delta and Jagged) and receptors (Notch1-4 in mammals) and transcription factor CSL (C-promotor-binding factor in mammals) (7). Notch receptors can be modified by two unusual forms of *O*-glycosylation, *O*-glucose and *O*-fucose. Fringe glycosyltransferases (LFng, lunatic fringe; MFng, manic fringe; and RFng, radical fringe) extend *O*-fucose on Notch by adding additional sugar moieties (8). In canonical Notch signalling, ligand–receptor interactions promote two proteolytic cleavage events in Notch receptors, which are separately mediated by ADAM (disintegrin and metalloprotease) and γ -secretase (7,9–11). Second cleavage releases the Notch intracellular domain, which subsequently translocates to the nucleus and associates with CSL to form a transcription complex that activates downstream target genes. Basic helix-loop-helix (bHLH) genes of HES/HEY families are best characterized Notch targets and the first genes demonstrated to have altered transcription following Notch activation,

Correspondence: L. Liu, College of Life Science, University of Chinese Academy of Sciences, #19A Yuquan Road, Beijing 100049, China; and W. Wang, School of Medical Sciences, Edith Cowan University, Joondalup Drive 100, Perth, WA 6027, Australia. Tel.: +86 10 8825 6454; Fax: +86 10 8825 6454; E-mails: lxliu@ucas.ac.cn; wei.wang@ecu.edu.au

providing a key paradigm for unravelling the Notch pathway (11). Notch activation elicits diverse outputs, such as proliferation, apoptosis and cell fate decisions (12).

Cell cycle progression is governed by a series of cyclin-dependent kinases (CDKs), which are in turn regulated by CDK inhibitors (CDKIs) (13). p21 and p27 proteins (Kip/Cip members of the CDKI family), are capable of binding to and inhibiting most cyclin/CDK complexes (13). Expression of p21 and p27 is dependent on up-stream signalling events, such as Notch signalling (12).

Our previous work has suggested that overexpression of hCLP46 might be an early event in pathogenesis of acute myelogenous leukaemia (AML) and T-acute lymphoblastic leukaemia (T-ALL) (2); however, causative mechanisms are still largely unknown. Here, we aimed to explore molecular effects elicited by hCLP46 overexpression.

Materials and methods

Materials

hCLP46 open reading frame (ORF) was amplified by PCR and inserted into the pcDNA4/TO/Myc vector (T-Rex System; Invitrogen, Carlsbad, CA, USA) using BamHI and XhoI sites incorporated into primers (underlined) (forward primer, 5'-AAAGGATCCGCCACCATG GAGTGGTGGGCTAGC-3', and reverse primer, 5'-ATC CTCGAGCGTAGTTCAGT TTTCAA-3'). An expression construct encoding human Jagged1 (hJ1) in an LZRS-linker-IRES-enhanced green fluorescent protein (eGFP) retrovirus and the empty vector (Emp) were kindly provided by Dr Leonor Parreira (14). All constructed plasmids were prepared using Plasmid Midi Kits (Qiagen, Hilden, Germany). hCLP46-specific siRNA and negative-control siRNA (Nc) were produced as previously described (5).

Antibodies against the following proteins were used: cleaved Notch1 (NICD, Val1744) and Myc-tag (Cell Signaling Technology, Danvers, MA, USA); p21 and p27 (Santa Cruz Biotechnology, Santa Cruz, CA, USA); β -actin (Proteintech Group, Chicago, IL, USA); and Hes-1 (Aviva Systems Biology, San Diego, CA, USA). Anti-rabbit and anti-mouse horseradish peroxidase-conjugated antibodies were also used (KPL, Gaithersburg, MD, USA).

Cell culture and transfections

293TRex and 293T cells (Invitrogen) were cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco

BRL, Gaithersburg, MD, USA) supplemented with 10% FBS. Cells were maintained at 37 °C under humidified conditions and 5% CO₂. siRNA and plasmids were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.

Establishment of inducible hCLP46 cells

293TRex cells, which stably express the repressor of the Tet (tetracycline) operon, were transfected with pcDNA4/TO/myc-hCLP46 and chosen for zeocin resistance. Individual clones were selected, expanded and tested for tetracycline-inducible hCLP46 overexpression, by immunoblotting.

Immunoblotting

Cell lysates were prepared by resuspending cell pellets in lysis buffer containing 20 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.25% NP-40, 10% glycerol, 1 mM EDTA, 1 mM DTT, 5 mM sodium fluoride, 1 mM sodium orthovanadate, and 1 mM phenylmethyl sulphonyl fluoride. Equal amounts of protein were loaded on SDS-PAGE gels, electrophoretically separated and transferred to PVDF membranes using standard procedures. Membranes were blocked with 5% non-fat milk then probed with indicated antibodies overnight at 4 °C. Membranes were then washed and incubated for 1 h with horseradish peroxidase (HRP)-conjugated secondary antibody at room temperature for 1 h. Signals were detected using a chemiluminescence detection system. Experiments were repeated at least three times.

Cell viability/proliferation

293TRex-hCLP46 cells were inoculated in 24-well culture plates (Greiner, Frickenhausen, Germany), and cell viability/proliferation was evaluated by reduction of MTT tetrazolium salt [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (0.5 mg/ml) by viable cells to form purple formazan product after 4 h incubation. Experiments were performed in triplicate on days 1–7, as previously described (15), and repeated at least three times.

Apoptotic analysis of hCLP46-overexpressing and down-regulated 293TRex cells

Apoptosis was assessed using annexin V, a protein that binds to phosphatidylserine (PS) residues that become exposed on surfaces of apoptotic cells. Cells derived from stable cell lines, transfectants and controls were washed in PBS and stained with fluorescent

isothiocyanate-labelled annexin V and PI (Annexin V-FITC Apoptosis Detection Kit; Biosea, Beijing, China). Cells were immediately analysed by fluorescence-activated cell sorting (FACS; BD, San Jose, CA, USA) for PI⁺, PI⁻, annexin V⁺ and annexin V⁻ cells, and the analysis was repeated to ensure accuracy of results. The experiments were performed in triplicate.

EDTA treatment of cells

EDTA treatment to activate Notch signalling was performed as previously described (8). Briefly, cells were washed once in PBS and incubated in either PBS or PBS containing 5 mM EDTA, for 10 min at 37 °C. After being washed three times in PBS, cells were incubated in DMEM + 10% FBS at 37 °C for 30 min, the protein level of NICD was analysed by immunoblotting. To measure EDTA-induced p21 and p27 expression, incubation time was increased to 6 h before proteins were extracted.

Ligand-dependent Notch signalling assay

A co-culture experiment was utilized, as previously described (8). In brief, 293T cells were transfected with hJ1 or Emp, 43 h before co-culturing, and 293TRex-hCLP46 was administered with or without Tet as indicated. At time of co-culture, hJ1 or Emp cells were removed by trituration in DMEM + 10% FBS and overlaid on 293TRex-hCLP46 cells. Co-cultures were incubated for 5 h at 37 °C.

Immunofluorescence

For immunostaining, cells were rinsed once in PBS and fixed for 15 min in 4% paraformaldehyde/PBS. Then they were rinsed three times in PBS and stained in DAPI for 15 min. After rinsing three times in PBS, permeabilization was achieved in 0.4% Triton X-100/PBS for 20 min. Antibody (NICD, Val1744) incubation was performed in PBS for 1 h, following blocking in 10% serum/PBS for 10 min. After being rinsed in PBS, cells were incubated in FITC-coupled goat anti-rabbit IgG for

45 min, then rinsed three times in PBS. Confocal images were taken using a Confocal Laser Scanning Biological Microscope FV1000-IX81 (Olympus, Tokyo, Japan).

RNA extraction and expression analysis

Total RNA from 293TRex cells that had undergone indicated treatment was isolated using Trizol (Invitrogen). For reverse transcription and polymerase chain reaction (RT-PCR), first-strand cDNA was synthesized from 1 µg of total RNA using the ReverTraAceqPCR RT Kit (Toyobo, Osaka, Japan) according to the supplier's manual. Quantitative RT-PCR was performed as described previously (2) using a QuantiTect™ SYBR green PCR method (Toyobo) and an Mx3000P instrument (Stratagene Laboratories, La Jolla, CA, USA). Sequences of primers for qPCR are listed in Table 1. Expression levels of detected genes were normalized to the internal control, β-actin. Experiments were performed in triplicate and repeated at least three times.

Statistical analysis

Differences between control and target data sets were determined using independent sample *t*-tests, with *P*-values less than or equal to 0.05 being considered statistically significant.

Results

Generation of a stable cell line overexpressing hCLP46

Stable zeocin-resistant clones expressing high levels of hCLP46 on addition of Tet were isolated and characterized (Fig. 1a). Clone 1, which displayed pronounced induction of hCLP46, at both the protein and mRNA levels, only in the Tet-treated cells (Fig. 1b and 1c), was called 293TRex-hCLP46 and used in the subsequent experiments. hCLP46 was reproducibly increased to a similar level in the same concentration of Tet in 293TRex-hCLP46 cells (Fig. S1).

Table 1. Sequences of the primers for qPCR

Gene	Forward primers	Reverse primers
<i>p21</i>	5'-GGACAGCAGAGGAAGACCATGT-3'	5'-TTTCGACCCTGAGAGTCTCCAG-3'
<i>p27</i>	5'-GGCTAACTCTGAGGACACGCAT-3'	5'-TGCAGGTCGCTTCTTATTC-3'
<i>hCLP46</i>	5'-GATATCATGTATCCTGCTTG-3'	5'-TTTTCCATGGCCACTGTGCTG-3'
<i>Hes1</i>	5'-TGATTTGGATGCTCTGAAGAAAGATA-3'	5'-GCTGCAGGTTCCGGAGGT-3'
<i>β-actin</i>	5'-GTGACGTGGACATCCGCAAAGAC-3'	5'-TCAAGAAAGGGTGTACGCAACTAA-3'

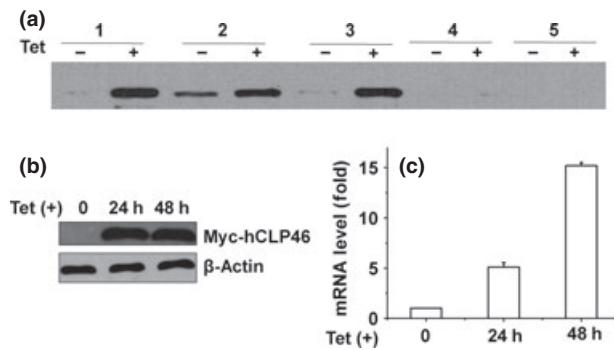


Figure 1. Generation of a stable cell line inducibly overexpressing hCLP46. (a) Proteins from five 293Trex-hCLP46 cell clones incubated in absence or presence of 0.5 µg/ml Tet, were subjected to immunoblotting analyses to detect exogenous hCLP46, using an anti-Myc antibody. Protein level of exogenous hCLP46 (b) and mRNA level of hCLP46 (c) were assayed in clone 1 after 24 and 48 h of Tet induction.

hCLP46 enhanced Notch activation in a ligand-dependent manner

Effects of hCLP46 overexpression on Notch signalling, particularly Notch1 activation, were examined. Increased levels of Notch1 intracellular domain (NICD) were detected in cells overexpressing hCLP46 (Fig. 2a). EDTA treatment acted as positive control to increase NICD levels (16) that did not alter obviously in cells with or without hCLP46 overexpression (Fig. 2a). Furthermore, hCLP46 had the ability to augment Jagged1-induced Notch1 activation (Fig. 2c). Immunostaining results showed that hCLP46 increased NICD when cells were at high confluence (Fig. 2e), suggesting that overexpression of hCLP46 activated Notch1 signalling in a ligand-dependent manner, in 293Trex cells. Consistently, protein and mRNA levels of Hes1, effector of Notch activation (17), were increased with hCLP46 overexpression (Fig. 3a and 3c) and reduced with hCLP46 knock-down by specific siRNA (Fig. 3d and 3f). In 293Trex cells, hCLP46-specific siRNA decreased mRNA level of hCLP46 to approximately 43% compared to NC (Fig. 3f). These results support the hypothesis that hCLP46 enhanced Notch activation in 293Trex cells.

hCLP46 inhibited cell proliferation, and hCLP46 knock-down promoted cell proliferation

It is interesting to note that hCLP46-overexpressing cells had markedly lower population growth rates compared to control cells (Fig. 4a). We then examined expression of two cell proliferation regulators, CDKIs p21 and p27, and results showed that protein and mRNA levels of p21 and p27 were elevated in hCLP46-overexpressing

cells (Fig. 4b and 4d). Furthermore, down-regulation of hCLP46 by specific siRNA, moderately but significantly increased growth rate (Fig. 5a) and led to decreases in p21 and p27 (Fig. 5b and 5d). These results suggest that hCLP46 might regulate proliferation of 293Trex cells, through CDKI signalling.

hCLP46 did not influence cell apoptosis

To determine possible influence of hCLP46 on apoptosis, 293Trex cells induced with Tet or treated with siRNA-hCLP46 were analysed by FACS. Fig. 6 shows that no significant alteration in apoptosis occurred when hCLP46 expression was up-regulated or down-regulated.

Effects of hCLP46 on CDKI signalling were mediated through the Notch pathway

As Notch pathway signalling affects a variety of cell processes, we investigated whether hCLP46 played a role in CDKI signalling through Notch. To address this, we examined function of Notch activation on p21 and p27 expression and found accumulation of p21, p27 and Hes1 proteins when Notch was activated by EDTA treatment (Fig. 7). However, hCLP46 overexpression did not elicit this effect with EDTA treatment, which was consistent with release of NICD (Fig. 2a).

Discussion

Using inducible overexpression and RNAi target reduction of hCLP46, we confirmed that aberrant hCLP46 expression could lead to deregulated Notch signalling and cell behaviour.

Deregulation of Notch can lead to development of haematological malignancies (7,9–11). More than 50% of human T-ALLs have activating mutations of *Notch1* (18), and both *Notch1* and *Jagged1* are overexpressed in AML (12). Here, our results indicated that overexpression of hCLP46 enhanced Notch1 activation (Figs 2,3), which might provide a clue to explain high levels of hCLP46 in primary AML and T-ALL samples.

Treating cells that express Notch receptors, with EDTA, resulted in shedding of the Notch extracellular domain and release of NICD (16). We have previously proven that down-regulated hCLP46 does not affect cell surface expression of Notch1 receptors (5). Consistent with this finding, in present study we have shown that overexpression of hCLP46 did not alter EDTA-induced NICD release (Fig. 2a). As EDTA-induced Notch signalling was not significantly affected, we hypothesized that hCLP46 might regulate Notch signalling in a ligand-dependent manner. Thus, we performed a co-culture assay

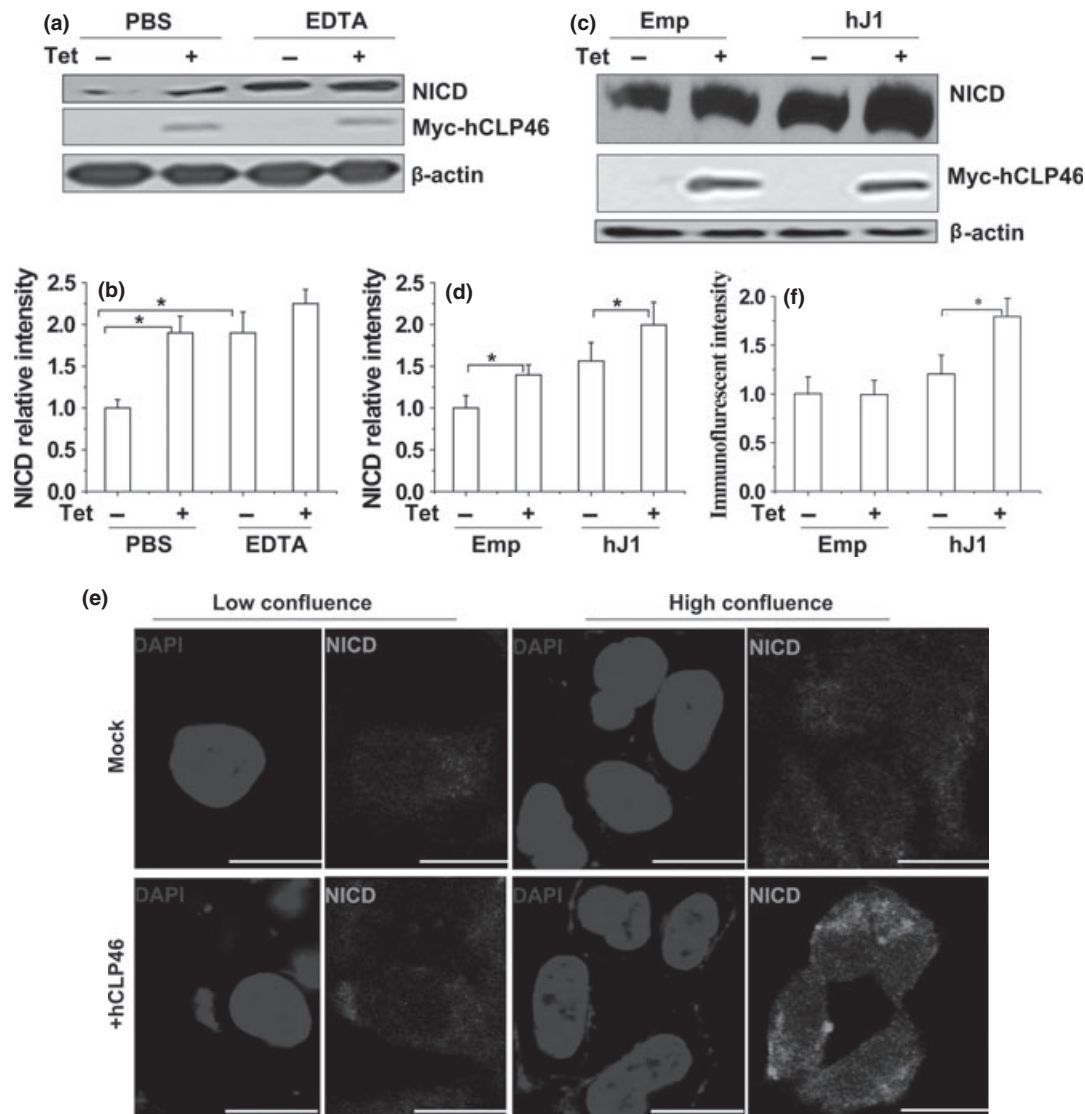


Figure 2. Overexpression of hCLP46 enhanced Notch activation. (a) 293Trex-hCLP46 cells were incubated for 48 h in absence (–) or presence (+) of 0.5 μg/ml Tet. Cells were treated with 5 mM EDTA or PBS and then cultured for additional 30 min. (c) 293Trex-hCLP46 cells were incubated for 42 h in absence or presence of Tet and then co-cultured for 5 h with 293T cells transfected with human Jagged1 (hJ1) or empty vector (Emp) for 36 h. Immunoblot band intensities were quantified using loading controls (b, d). (e) 293Trex-hCLP46 cells were seeded at low confluence (low) or high confluence (high) and were incubated for 48 h in absence or presence of Tet. Green fluorescence indicates that NICD and nuclei were counterstained with DAPI (blue). Confocal images are representative of results obtained in three separate sets of experiments. Bar = 20 μm. Immunofluorescence intensities were quantified (f) and data represent mean ± SD of three independent experiments. *P ≤ 0.05.

as previously described by Yang *et al.* (8), and compared effects of hCLP46 on Notch1 activation when cells were at low confluence and when they were highly confluent. Because presence of endogenous Notch ligands in 293T cells (19), when cells are highly confluent Notch receptors can be stimulated and activated by ligands on adjacent cells. The results showed that hCLP46 overexpression enhanced Notch1 signalling in a ligand-dependent manner (Fig. 2c and 2e). Yang *et al.* had reported that Jagged1-induced Notch1 signalling was enhanced by RFng and

suppressed by LFng and MFng in 293T cells (8). Our results and previous studies demonstrate that Notch signalling is regulated by glycosylation and that fucosylation and glycosylation play different roles in modulating Notch signalling.

No significant apoptosis occurred when hCLP46 expression was up-regulated or when it was reduced (Fig. 6). NaAsO₂ treatment induced significant apoptosis in 293Trex cells (Fig. S2), indicating that our assay worked well. MTT assay measures cell proliferation

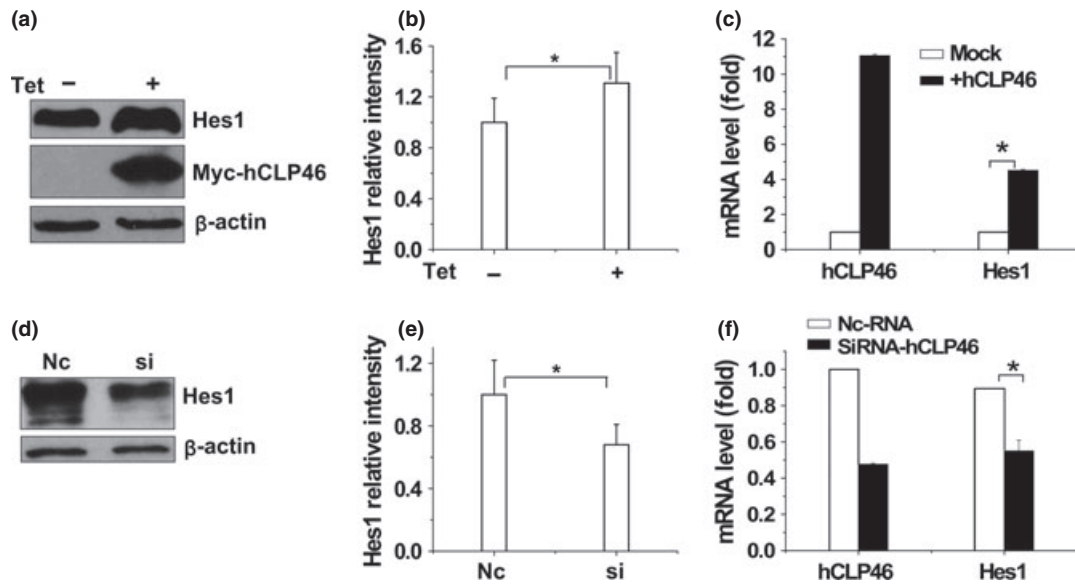


Figure 3. hCLP46 regulated Notch signalling effector Hes1 expression. (a, c) 293Trex-hCLP46 cells were incubated for 48 h in absence or presence of 0.5 $\mu\text{g/ml}$ Tet. (d, f) 293Trex-hCLP46 cells were transfected with hCLP46-siRNA (Si) or negative control-RNA (Nc). Cells were then subjected to immunoblotting analyses to detect indicated proteins (a, d) and RT-PCR to detect mRNAs (c, f). Immunoblot band intensities were quantified using loading controls (b, e). Data represent mean \pm SD of three independent experiments. $*P \leq 0.05$.

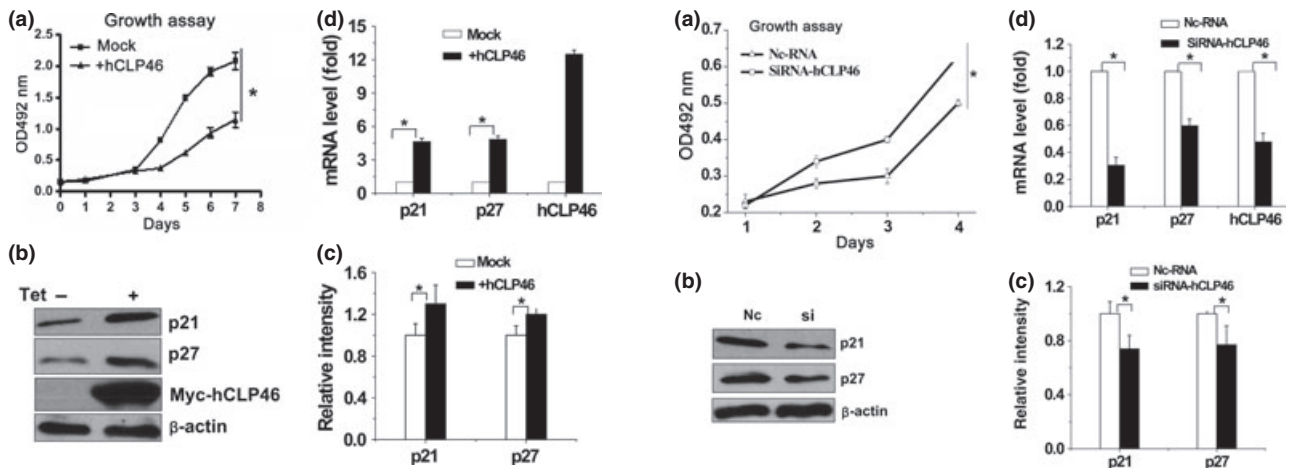


Figure 4. hCLP46 inhibited cell proliferation and increased CDKIs p21 and p27. (a) 293Trex-hCLP46 cells were incubated in absence or presence of 0.5 $\mu\text{g/ml}$ Tet. At indicated time points, cells were harvested and incubated with MTT; absorbance at 492 nm was determined. All time points were assayed in triplicate. Cells were incubated for 48 h in absence or presence of Tet (b) and cell lysates were immunoblotted with indicated antibodies. (c) Immunoblot band intensities were quantified using loading controls. (d) Total RNA was subjected to RT-PCR analysis for p21, p27 and hCLP46. Data represent mean \pm SD of three independent experiments. $*P \leq 0.05$.

Figure 5. Inhibition of hCLP46 promoted cell proliferation and reduced CDKIs p21 and p27. (a) 293Trex-hCLP46 cells were transfected with hCLP46-siRNA (Si) or negative control-RNA (Nc); cells were harvested and incubated with MTT, and absorbance at 492 nm was determined. All time points were assayed in triplicate. (b) Cell lysates were immunoblotted with indicated antibodies. (c) Immunoblot band intensities were quantified against loading controls. (d) Total RNA was subjected to RT-PCR analysis for p21, p27 and hCLP46. Data represent the mean \pm SD of three independent experiments. $*P \leq 0.05$.

level when reduction in cell viability caused by apoptosis or necrosis is excluded (20); results showed that hCLP46 expression affected 293Trex cell proliferation

(Figs. 4a and 5a). Indeed, overexpression of hCLP46 resulted in significant inhibition of 293Trex proliferation (Fig. 4a), which could be a result of activation of Notch

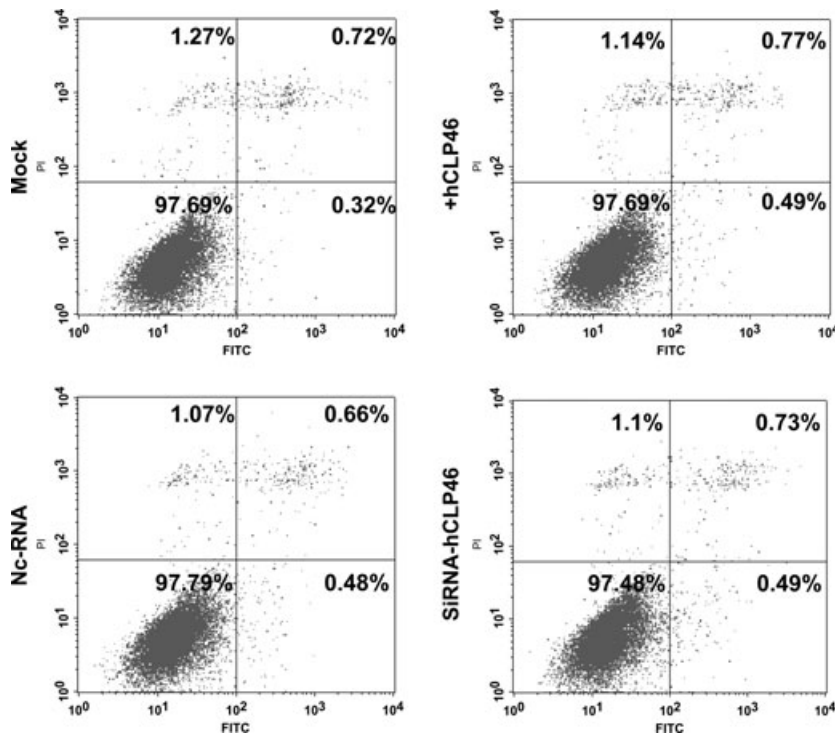


Figure 6. hCLP46 had no significant effect on apoptosis. 293Trex-hCLP46 cells were incubated in absence or presence of 0.5 µg/ml Tet or transfected with hCLP46-siRNA (Si) or negative control-RNA (Nc) for 48 h. Apoptosis was measured using annexin-V and PI double staining. FITC axis denotes annexin-V, and PI axis denotes PI.

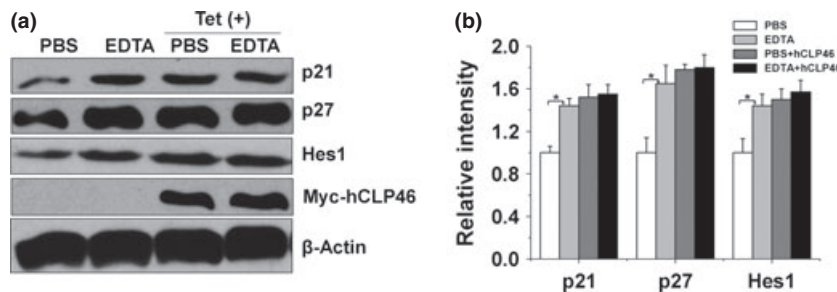


Figure 7. Effects of hCLP46 on CDKI signalling were mediated through the Notch pathway. (a) 293Trex-hCLP46 cells were incubated for 42 h in absence or presence of 0.5 µg/ml Tet, then treated with PBS or 5 mM EDTA. After treatment, cells were cultured for additional 6 h and analysed by immunoblotting with indicated antibodies. (b) Immunoblot band intensities were quantified against loading controls. **P* ≤ 0.05.

signalling (Figs 2,3). Active Notch proteins, but not Hes1, cause profound population growth arrest associated with up-regulation of p21 and p27 (15,21); in contrast, inhibition of Notch signalling by Notch1-specific siRNA resulted in reduced p21 levels (22). In agreement with these studies, we showed that overexpression of hCLP46 caused increases in p21 and p27 (Fig. 4) and that knock-down caused the reverse effect (Fig. 5), indicating involvement of hCLP46 in the CDKI pathway. However, Hes1 also has the ability to directly control cell proliferation by regulating expression levels of p21 and p27 (23,24). Because Hes1 protein was increased in hCLP46-overexpressing cells and reduced in Hes1-knocked-down cells (Fig. 3), cell inhibition

through Notch activation may be dependent on or independent of Hes1.

The inhibitory role of hCLP46 in 293Trex cell proliferation is contrary to our previous study, which showed that hCLP46 stimulated U937 human monocytic leukaemia cell proliferation (1). With repeated titration and expression studies, we believe that differences in results stem from existence of intrinsically different machinery between U937 and 293Trex cells, rather than from bias between the experiments. These results therefore indicate that function of hCLP46 in cell proliferation is highly dependent on cell type. The established theory is that Notch signalling can regulate gene transcription activation and repression and either block

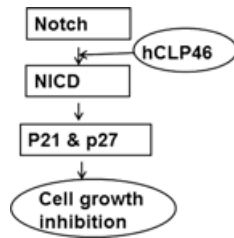


Figure 8. Schematic diagram of regulation of 293Trex cell proliferation by hCLP46. hCLP46 increased expression of p21 and p27 through enhanced Notch signalling, leading to cell proliferation arrest in 293Trex cells.

or promote cell population growth in a cell type-specific manner (12). For example, Notch1 inhibits epidermal, endothelial and small lung cancer cell proliferation (21,25), and active forms of Notch have been shown to have oncogenic activity in acute lymphoblastic T-cell leukaemia and fibroblasts (26,27). As 293 cells were derived from human embryonic kidney and U937 cells were derived from a lymphoma, specific functions of hCLP46 in these two cell lines is possibly due to context-dependent Notch signalling. Additionally, in normal adult human tissues, hCLP46 is highly expressed in liver and is also expressed in low mitotic index tissues (1). Cell type-dependent functions and different expression levels in normal tissues reveal the important role of hCLP46 in mammalian development, particularly in pathogenesis of hCLP46-related haematological diseases (1,2,5).

On the basis of this study, we propose a role for hCLP46 in regulating 293Trex cell proliferation (Fig. 8). We provide evidence that hCLP46 exerts its function in a cell-specific manner, which is useful to understand the mechanism leading to haematological tumorigenesis.

Acknowledgements

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Conflict of interest

The authors declare that there is no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. The expression of exogenous hCLP46 in 293Trex-hCLP46 cell line. 293Trex-hCLP46 cells were incubated for 48 h in the absence or presence of 0.5 $\mu\text{g/ml}$ Tet. The experiments were repeated two times.

Figure S2. NaAsO₂ induced 293Trex cells apoptosis. 293Trex-hCLP46 cells were incubated for 48 h or 24 h in the absence or presence of 8 $\mu\text{mol/l}$ NaAsO₂. Apoptosis was measured by annexin-V and PI double staining. The FITC axis denotes annexin-V, and the PI axis denotes PI. The experiments were performed in triplicate and repeated three times. Values represent an average of triplicate samples of a representative experiment.