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hCLP46 regulates U937 cell proliferation via Notch signaling pathway

Wenzhan Ma [a], Jie Du [a], Qiaoyun Chu [a], Youxin Wang [b], Lixin Liu [a], Manshu Song [b], Wei Wang [a,b,*]

[a] College of Life Science, Graduate University of Chinese Academy of Sciences, Beijing 100049, China
[b] School of Public Health and Family Medicine, Capital Medical University, Beijing 100069, China

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ABSTRACT

Human CAP10-like protein 46 kDa (hCLP46) is the homolog of Rumi, which is the first identified protein *O*-glucosyltransferase that modifies Notch receptor in *Drosophila*. Dysregulation of hCLP46 occurs in many hematologic diseases, but the role of hCLP46 remains unclear. Knockdown of hCLP46 by RNA interference resulted in decreased protein levels of endogenous Notch1, Notch intracellular domain (NICD) and Notch target gene Hes-1, suggesting the impairment of the Notch signaling. However, neither cell surface Notch expression nor ligand binding activities were affected. In addition, down-regulated expression of hCLP46 inhibited the proliferation of U937 cells, which was correlated with increased cyclin-dependent kinase inhibitor (CDKI) CDKN1B (p27) and decreased phosphorylation of retinoblastoma (RB) protein. We showed that lack of hCLP46 results in impaired ligand induced Notch activation in mammalian cell, and hCLP46 regulates the proliferation of U937 cell through CDKI-RB signaling pathway, which may be important for the pathogenesis of leukemia.

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1. Introduction

hCLP46 (human CAP10-like protein 46 kDa), also known as Poglut1 (protein *O*-glucosyltransferase1), is a CAP10 domain protein that abnormally expressed in many hematopoietic malignancies [1–3]. Although the role of hCLP46 remains unclear, the homolog of hCLP46 in *Drosophila* named Rumi has been well studied as the first identified protein *O*-glucosyltransferase (Poglut) that modulates Notch signaling [4]. Recently, hCLP46 has been demonstrated to be able to hydrolyze UDP-Glc *in vitro* [5], which provides strong evidence that hCLP46 acts as an *O*-glucosyltransferase as Rumi.

Notch signaling pathway is highly conserved from *Drosophila* to mammalian, and regulates cell fate decisions in a wide range through direct cell–cell contact [6,7]. Of the four mammalian Notch receptors, Notch1 is the most important one. A 300 kDa full-length Notch1 is cleaved in Golgi body (namely S1 cleavage), then forms a heterodimer containing Notch extracellular domain (NECD) and Notch transmembrane-intracellular (NTM) subunits. The NECD is removed upon ligand binding (Delta and Serrate/Jagged in *Drosophila* and mammals), so that the NTM can be cleaved and release the Notch intracellular domain (NICD). NICD transits to nucleus, binds to DNA binding protein CSL (CBF1/RBPjκ/Su(H)/Lag-1), and activates transcription of target genes such as hairy and enhancer of split 1(Hes-1) [6], which is an important transcriptional repressor [8–10].

Notch signaling pathway is preciously regulated by many post-translational modifications, especially glycosylation [7,11]. There are two unusual forms of modification on NECD: *O*-fucosylation and *O*-glucosylation [12]. The *O*-fucose is added by protein *O*-fucosyltransferase (Ofut1/Pofut1 in *Drosophila* and mammalian) and elongated by Fringe, both of which have been extensively studied for their role in Notch signaling [13–16]. However, the role of *O*-glucosylation has only been revealed until the recent discovery of Rumi [4]. Rumi regulates folding and/or trafficking of Notch receptor and allows signaling at the cell membrane [4]. Considering the evolutionary conservation of Notch pathway [7], hCLP46 has great potential in modulating Notch receptor, and is a good model to study the role of *O*-glucosylation [2,11].

Our previous work demonstrated that overexpression of hCLP46 stimulates human monocytic leukemia cell line U937 cell proliferation [1]. In this study, we aim to clarify whether hCLP46 is essential for Notch signaling and to identify the mechanism that hCLP46 regulating U937 cells proliferation.

Abbreviations: hCLP46, human CAP10-like protein 46 kDa; Poglut, protein *O*-glucosyltransferase; Poglut1, protein *O*-glucosyltransferase1 (mammalian); CDKI, cyclin-dependent kinase inhibitor; CDKN1B (p27), cyclin-dependent kinase inhibitor 1B; RB, retinoblastoma; CSL, CBF1/SuH/LAG-1; NECD, Notch extracellular domain; NICD, Notch intracellular domain; NTM, Notch transmembrane-intracellular; EGF, epidermal growth factor-like; ER, endoplasmic reticulum; Pofut, protein *O*-fucosyltransferase; Ofut1, protein *O*-fucosyltransferase1 (*Drosophila*); Pofut1, protein *O*-fucosyltransferase1 (mammalian); HSPCs, hematopoietic stem/progenitor cells; MDS, myelodysplastic syndrome.

* Corresponding author at: College of Life Science, Graduate University of Chinese Academy of Sciences, #19A Yuquan Road, Beijing 100049, China. Fax: +86 10 83911501.

E-mail addresses: wei6014@yahoo.com, wei6014@gucas.ac.cn (W. Wang).

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2. Materials and methods

2.1. Materials

The hCLP46 specific siRNA and negative-control siRNA (NS) were chemically synthesized by GenePharm (Shanghai, China) and listed as below:

hCLP46-siRNA: 5′-GGCGUAGCUGCAAGUUUCCGGUUUATT-3′;
Negative control siRNA (NS): 5′- UUCUCCGAACGUGUCACGUTT-3′.

An expression construct encoding human full length Notch1 (hN1) in pcDNA3 was kindly provided by Dr. Spyros Artavanis-Tsakonas of Harvard Medical School [17] and prepared with Plasmid Midi Kits (Qiagen, Hilden, Germany).

The soluble forms of recombinant human Notch ligands Jagged1 that fused in frame with human IgG Fc sequences (J1-Fc) and Delta-like protein 1 that fused in frame with 6-His tag (D1-his) were purchased from R&D Systems (Minneapolis, MN, USA). Human IgG (California Bioscience, Coachella, CA, USA) was used as a negative control for J1-Fc.

The following antibodies were used: NTM, NICD (Cell Signaling Technology, Danvers, MA, USA); CDKN1B (Santa Cruze Biotechnology, Santa Cruze, CA, USA); Caspase8 (MBL, Woburn, MA, USA); Hes-1(Aviva Systems Biology, San Diego, CA, USA); pRB(S780) (Bioworld, Minneapolis, MN, USA); His-tag (California Bioscience, Coachella, CA, USA); β-actin (Proteintech Group, Chicago, IL,USA); horseradish peroxidase (HRP) conjugated goat anti-mouse (rabbit or rat), fluorescein isothiocyanate conjugated goat anti-Human IgG Fc (FITC-anti-Fc), DyLight 649 conjugated goat anti-mouse IgG (KPL, Gaithersburg, MD, USA).

2.2. Cell culture and transfection

293T cell line was cultured in DMEM (Gibco BRL, Gaithersburg, MD, USA) with 10%FBS, Jurkat human lymphoblastic T-cell line and U937 human monocytic leukemia cell line (including its subclones) were grown in RPMI1640 (Gibco BRL, Gaithersburg, MD, USA) supplemented with 10% FBS. Cells were maintained at 37 °C in humidified conditions of 5% CO_2.

siRNA and plasmids transfection were carried out using Lipofectamine 2000 (Invitrogen, Carlsbad, CA,USA) according to manufacturer's instruction.

To establish the U937 cell lines constantly expressing hCLP46-specific-shRNA, U937 cells were transfected with pGPU6/GFP/Neo-hCLP46-siRNA plasmid (GenePharma, Shanghai, China), seeded in 96-well plate with limited dilution and selected using 800 μg/mL G418 (Sigma, St. Louis, MO, USA) as described before [2]. Stable clones were obtained and verified by qRT-PCR.

2.3. Western blot

Western blot was performed essentially as described in [1,2].

2.4. Quantitative real time PCR (qRT-PCR) analysis

Total RNA from U937 cells and Jurkat cells was extracted by Trizol reagent (Invitrogen, Carlsbad, CA, USA) and reverse transcription was carried out using ReverTra Ace® qPCR RT Kit (Toyobo, Osaka, Japan) according to user's manual. qRT-PCR was carried out using the SYBR green method (Toyobo, Osaka, Japan) on a Mx3000P instrument (Stratagene Laboratories, La Jolla, USA).

2.5. Ligand-independent Notch signaling assay

The EDTA induced ligand-independent Notch activation was performed by the well established method [17,18]. U937 cells were washed one time with PBS and incubated in 5 mM EDTA or PBS for 15 min. After washing three times with PBS, cells were incubated in RPMI1640 with 10% FBS at 37 °C for 30 min, and the protein level of NICD was analyzed by Western blot. To measure EDTA-induced Hes-1 transcription, the incubation time was increased to 1 h before the mRNA was extracted.

2.6. Soluble ligand binding assay

Soluble ligand binding assay was performed according to the methods of Stahl [18] and Yang [16]. 293T cells were incubated for 45 min at 37 °C in blocking media (DMEM containing 10% FBS and 1% bovine serum albumin (BSA)). 1 μg/ml soluble ligands J1-Fc and D1-His were clustered respectively with FITC-anti-Fc antibody or mouse anti-His tag antibody in ligand binding buffer (10 mM HEPES, 150 mM NaCl, 1 mM MgCl, 5 mM $CaCl_2$, 0.05% NaN_3) with 1% BSA for 30 min at 4 °C. The pre-clustered ligands were added to cells for 30–60 min at room temperature. For D1-His, 293T cells were washed three times after binding and incubated with DyLight 649 conjugated goat anti-mouse antibody for 30 min at room temperature. The cells were removed by gently pipetting up and down and washed in ligand binding buffer for three times. The mean fluorescence intensity (MFI) was analyzed by FACScan (BD Biosciences, San Jose, CA, USA).

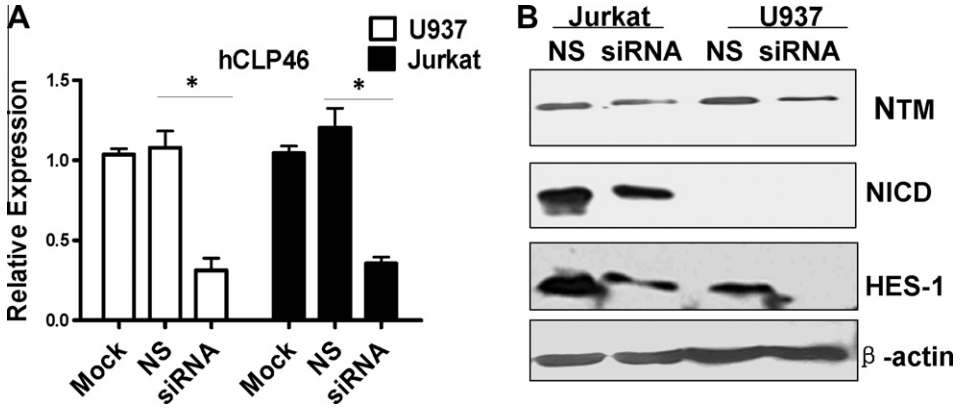

Fig. 1. Knock-down of hCLP46 impairs Notch signaling pathway. U937 cells and Jurkat cells were untreated, transfected with negative-control siRNA (NS) or hCLP46 specific siRNA. (A) The mRNA level of hCLP46 was examined by qRT-PCR and normalized to β-actin. mRNA levels were expressed as -fold changes relative to untreated mock samples and shown as mean ± S.D. of three independent experiments $^{*}p < 0.01$. (B) The amounts of NTM, NICD and Hes-1 were examined by Western blot. β-actin was used as a loading control.

2.7. Growth assays

The hCLP46–shRNA-expressing cells and control cells were inoculated in 48-well culture plates (Greiner, Frickenhausen, Germany). Viable cells are assayed using the MTT assay on days 1–7 as described previously [1]. Experiments were performed in triplicates and repeated at least twice.

2.8. Statistical analysis

Results were presented as mean ± S.D. The differences between control and target data sets was tested by Student's *t*-test, and *P*-values $\leqslant 0.05$ were considered to be of statistical significance.

3. Results

3.1. Knock-down of hCLP46 impairs endogenous Notch signaling

For both U937 and Jurkat cells, hCLP46 specific siRNA decreased the mRNA level of hCLP46 to around 30% compared to NS (Fig. 1A). NICD was detectable in Jurkat cell line but not in U937 cell line (Fig. 1B). Down-regulation of hCLP46 led to decreased protein levels of NTM and Hes-1 in both cell lines, which was correlated with decreased NICD (Fig. 1B). These results showed a suppression of endogenous Notch signaling in the lack of hCLP46.

3.2. Cell surface expression of Notch1 and ligand binding is not significantly affected

As shown in Fig. 2A, the EDTA induced NICD release in U937 cells was not appreciably affected, with Jurkat cells as a positive control. Although the mRNA level of endogenous Hes-1 was largely reduced by hCLP46 specific siRNA, the EDTA induced Hes-1 transcription was not reduced at all and even slightly elevated (Fig. 2B), which indicated a similar amount of cell surface expression of Notch1.

The MFI represented the extent of Notch ligands Delta1 or Jagged1 binding to 293T cells that transfected with exogenous full length human Notch1. The binding of Notch1 receptors to its ligands was not significantly affected by hCLP46 (Fig. 2C), which additionally demonstrated an equivalent cell surface expression of Notch1 receptors.

3.3. hCLP46 affects the proliferation of U937 cells through CDKI-RB signaling

To test the effect of down-regulated hCLP46 on U937 cells proliferation, a stable cell line that constantly expresses hCLP46 specific shRNA were established. qRT-PCR showed that the mRNA level of hCLP46 was reduced to 30% compared to the control. The mRNA level of Notch1 was not affected (Fig. 3A), further demonstrating that hCLP46 regulated Notch receptor in a posttranscriptional manner. The growth-rate of hCLP46–shRNA-expressing U937 cell significantly decreased compared to that of the control (Fig. 3B). To further investigate the underlying mechanism, we detected several key proteins in regulating cell cycle and/or apoptosis. CDKN1B was accumulated in siRNA-treated U937 cells, which is accompanied by decreased phosphorylated RB protein (Fig. 3C). No detectable changes appeared on the apoptosis related protein Caspase8. These results suggested that hCLP46 regulate the proliferation of U937 cells through CDKI-RB signaling.

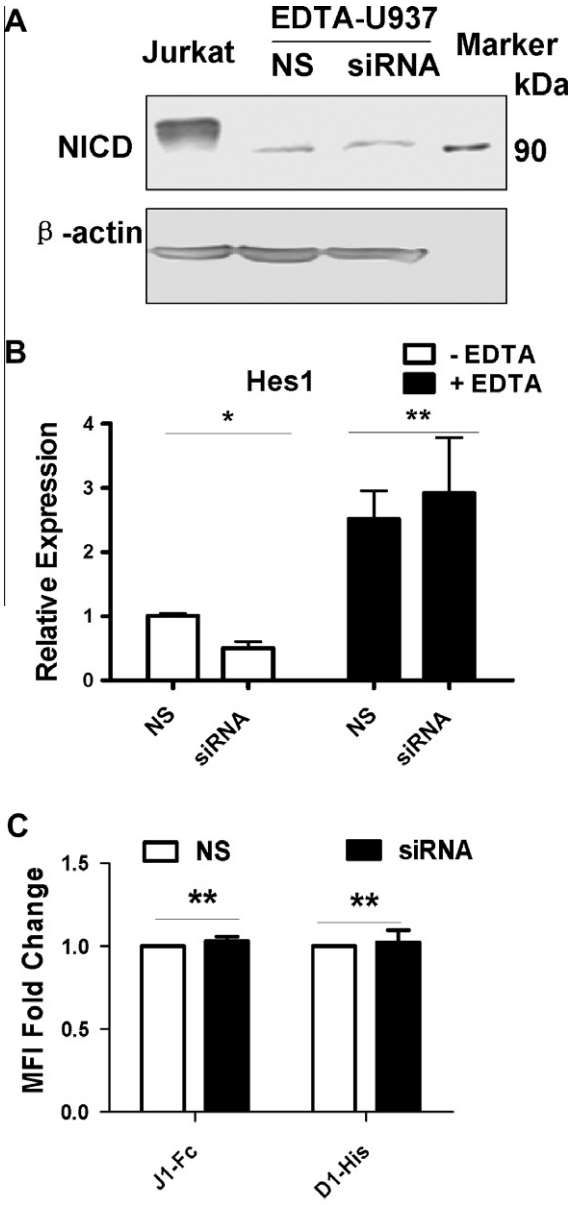

Fig. 2. Cell surface expression and ligand binding to Notch1 in the absence of hCLP46 is not significantly affected. (A) U937 cells were transfected with negative-control siRNA (NS) or hCLP46 specific siRNA. Ligand-independent Notch activation was induced by incubating cells in 5 mM EDTA or PBS for 15 min, and cultured at 37 °C in the incubator. After 30 min, the amount of NICD was examined by Western blot. Jurkat serves as a positive control. β-actin was used as a loading control. Western marker was used to reference of the molecular weight. (B) After cultured for 1 h, the mRNA level of Hes-1 was examined by qRT-PCR and normalized to β-actin. mRNA levels were expressed as -fold changes relative to PBS-treated NS samples and shown as mean ± S.D. of three independent experiments. $^{*}p < 0.01$; $^{**}p > 0.05$. (C) Binding of preclustered J1-Fc (1 μg/ml) or D1-His (1 μg/ml) to 293T cells that transfected with hN1 plasmid along with hCLP46 specific siRNA or negative-control siRNA (NS) was analyzed by flow cytometry. The mean fluorescence intensity (MFI) of three independent experiments was normalized as fold changes relative to NS transfected 293T cells. Data represent the mean ± SD. $^{**}p > 0.05$.

4. Discussion

Using RNAi targeted reduction of hCLP46, we confirmed that hCLP46 is essential for mammalian Notch signaling and is necessary for the proliferation of U937 cells. Dysregulated hCLP46, thus, may affect hematopoietic stem/progenitor cells (HSPCs) proliferation, leading to hematologic tumorigenesis.

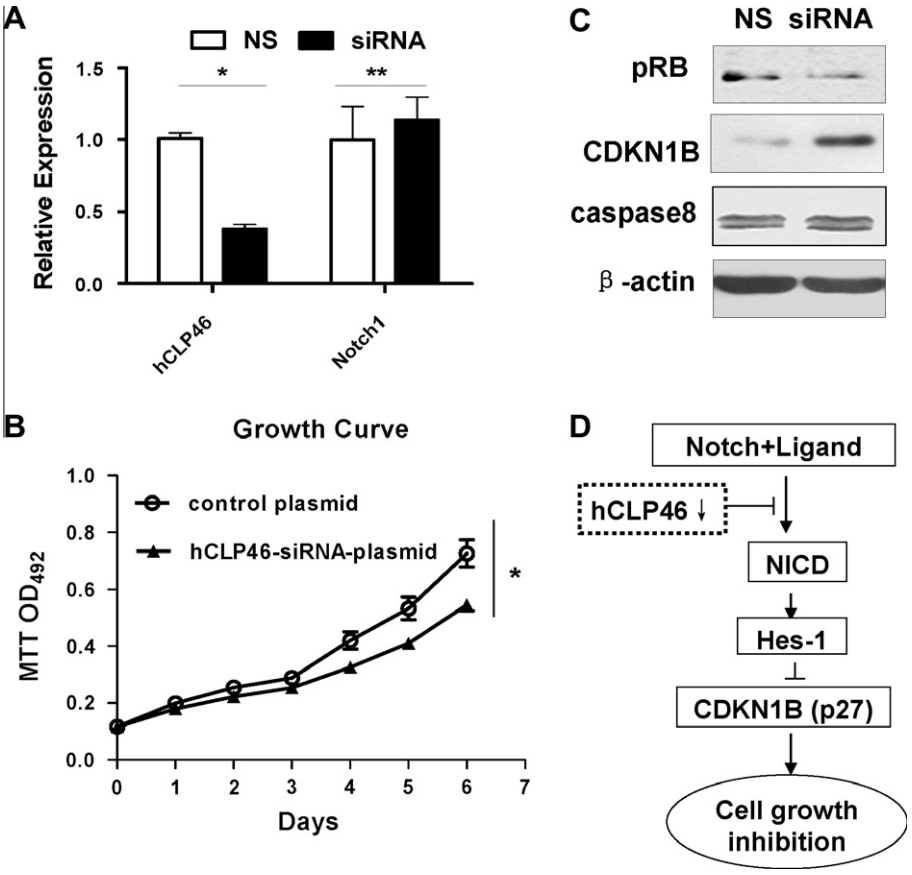

Fig. 3. Knock-down of hCLP46 inhibits U937 cell proliferation. U937 cells were stably transfected with hCLP46-specific-shRNA expression plasmid or control plasmid. (A) The mRNA level of hCLP46 and Notch1 was examined by qRT-PCR and normalized to β-actin. mRNA levels were expressed as -fold changes relative to untreated mock samples and shown as mean ± S.D. of three independent experiments $^{*}p < 0.01$; $^{**}p > 0.05$. (B) The same amount of both cells was seeded into a 48-well plate. After 12 h incubation, the cell number was determined every 24 h utilizing MTT assay. Data represent the mean ± S.D. $^{*}p < 0.01$. (C) The amount of Caspase8, pRB and CDKN1B (p27) was examined by Western blot. β-actin was used as a loading control. (D) A model for hCLP46 in regulating U937 cells proliferation.

Although Notch signaling is highly conserved from *Drosophila* to mammalian, conclusions drawn from *Drosophila* cannot necessarily be applied to mammalian [18]. Deletion of Rumi leads to accumulated Notch inside cells and on the membrane [4], while in mammalian the total Notch1 was decreased (Fig. 1B). This difference might due to that mammalian have more strict quality control mechanism, and incorrectly glycosylated proteins prone to degradation [19].

The NICD can be released and detected after ligand binding or EDTA treatment [17]. Jurkat cells constantly express detectable NICD, because both PTEN and FBW7 that mediate NICD for proteasomal degradation are inefficient [20,21]. Now that the EDTA-induced Notch signaling is not significantly affected (Fig. 2A and B), we deduce that hCLP46 regulates Notch signaling in a ligand-dependent manner. U937 cells express high level of cell surface Fc receptors [22], thus we performed ligand binding assay with 293T cells according to Yang et al [16]. hCLP46 did not affect the binding of cell surface Notch1 to either Delta1 or Jagged1 (Fig. 2C), which further revealed that *O*-fucosylation and *O*-glucosylation have different role in modulating Notch signaling, i.e., *O*-fucosylation is essential for ligand binding to Notch1 receptors [18], while *O*-glucosylation is essential for ligand induced cleavage of Notch1 receptors [4,23]. The expressing of hCLP46 is different in adult multiple tissues [1], which may partly contribute to the tissue-specific Notch activation [7]. Our results expand the knowledge of how the glycosylation state of a receptor can regulate a signaling event, and hCLP46 provides a novel target to explore the role of O-glucosylation in mammalian.

Reduced expression of hCLP46 resulted in significant but modest inhibition of U937 cell proliferation (Fig. 3B), which can be resulted from decreased Hes-1 expression (Figs. 1B and 2B). Hes-1 stimulates cell proliferation by repressing CDKN1B transcription in many tissues [8,9,24]; reversely, inhibition of Notch signaling by GSI or DAPT leads to growth inhibition through cell cycle arrest [25,26]. In consistent with these studies, we evidenced that knock down of hCLP46 resulted in increased CDKN1B (p27) and decreased phosphorylation of RB protein (Fig. 3C), indicating an involvement of hCLP46 in CDKI-RB pathway. The Caspase8 was not affected, which was consistent with our previous work that knock-down of hCLP46 did not affect the apoptosis of U937 cells [2]. The CDKN1B competitively inhibits the formation of CDK2-cycinE complex, thus inhibits the phosphorylation of RB, which results in inhibited cell growth [27]. Based on these facts, a model for hCLP46 in regulating U937 cells proliferation was proposed as Fig. 3D. This can be an important mechanism for tumor genesis, especially for the pathogenesis of hCLP46 related hematology diseases, e.g., myelodysplastic syndrome (MDS). It should be noted that we can not deny the possibility that hCLP46 is able to regulate p27 independent of Notch signaling, which may provide an alternative pathway for hCLP46 in regulating hematologic tumorigenesis.

In the study, we confirmed that hCLP46 is an essential component for Notch signaling and revealed that hCLP46 affects ligand-induced cleavage of Notch1 receptor. Additionally, hCLP46 regulates the proliferation of U937 cells through CDKI-RB signaling pathway.

Acknowledgments

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