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Acetylation of cyclin-dependent kinase 5 is mediated by GCN5

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ABSTRACT

Cyclin-dependent kinase 5 (CDK5), a member of atypical serine/threonine cyclin-dependent kinase family, plays a crucial role in pathophysiology of neurodegenerative disorders. Its kinase activity and substrate specificity are regulated by several independent pathways including binding with its activator, phosphorylation and S-nitrosylation. In the present study, we report that acetylation of CDK5 comprises an additional posttranslational modification within the cells. Among many candidates, we confirmed that its acetylation is enhanced by GCN5, a member of the GCN5-related N-acetyl-transferase family of histone acetyltransferase. Co-immunoprecipitation assay and fluorescent localization study indicated that GCN5 physically interacts with CDK5 and they are co-localized at the specific nuclear foci. Furthermore, liquid chromatography in conjunction with a mass spectrometry indicated that CDK5 is acetylated at Lys33 residue of ATP binding domain. Considering this lysine site is conserved among a wide range of species and other related cyclin-dependent kinases, therefore, we speculate that acetylation may alter the kinase activity of CDK5 via affecting efficacy of ATP coordination.

1. Introduction

CDK5 is a proline-directed serine/threonine kinase which plays an important role in the nervous system. In the physiological condition, CDK5 modulates many cellular processes including neuronal migration, survival, differentiation and synaptic function [1–3]. However, aberrant CDK5 activity has been implicated in pathogenesis of many neurological disorders [4,5]. It has been shown that the dysregulated CDK5 activation is closely associated with cleavage of a neuronal activator of CDK5, p35. Under neurotoxic stresses that trigger increased intracellular free calcium, p35 is cleaved by calpain to generate p25. The p25 forms a complex with CDK5, leading to change of its subcellular localization and kinase activity, and eventually causing neuronal cell death [6,7]. In addition, it has been demonstrated that several other independent mechanisms are involved in regulation of CDK5 activity. For example, phosphorylation at Thr14 of CDK5 via c-Abl, Fyn or EPHA4 leads to decreased kinase activity whereas phosphorylation at Tyr15 by unidentified kinases increases its kinase activity [8–11]. Ser159-phosphorylated CDK5 casein kinase 1 or CDK7/cyclin H complex also exhibits an increased kinase activity [3,12]. More recently, S-nitrosylation also emerged as a novel regulatory mechanism for CDK5 activity [13].

Acetylation received much attention as a major posttranslational regulatory mechanism of numerous proteins including histones and non-histone proteins [14–16]. It also affects broad signaling pathways thereby alters cellular function via regulation of protein properties such as stability, activity and subcellular localization. Among over 30 histone acetyltransferases (HAT), GCN5 was first identified in yeast as a transcription-related HAT, providing an evidence for a link between histone acetylation and gene activation [17]. GCN5 acetylates free or nucleosomal histones, allowing cell to maintain open chromatin structure for nucleosome assembly, transcriptional activation and DNA repair. Many non-histone targets of GCN5 have been recently reported. These include CDK2, CDK9, CDC6, Swi2/Snf2, E2A-PBX1 and Ifh1 [18–23]. Therefore, the goals of this study is to (i) investigate the possibility that CDK5 is acetylated; (ii) identify the acetyltransferase involved in this event; and (iii) determine a site of acetylation in CDK5. In the present study, we firstly provide evidence indicating that CDK5 is an acetylated protein. GCN5 physically binds to acetylate CDK5 at Lys33 residue essential for ATP coordination, raising the possibility that acetylation may comprise an alternative route for regulation of CDK5's kinase activity.

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2. Materials and methods

2.1. Cell culture and transfection

HEK293 cells were cultured in DMEM (GenDEPOT, Barker, TX, USA) supplemented with 10% heat-inactivated fetal bovine serum (GenDEPOT) and maintained in an atmosphere of 5% CO_2. For transient transfection, HEK293 cells were first plated at a density of 1.5×10^6 cells on P-100 culture plates. At 3 DIV, transfection was performed using polyethylenimine (PEI; Sigma, St. Louis, MO, USA) as recommended by the supplier. Briefly, 9 μl PEI (1 μg/μl) was mixed with 3 μg of DNA in DMEM and incubated at RT for 20 min. Mixtures were added to cells, remained on the cells for 3–4 h, and were replaced with a fresh medium. To inhibit deacetylase activity, cells were incubated with 1 μM of trichostatin A (TSA; Sigma) and 5 mM nicotinamide (NA; Sigma) for 24 h.

2.2. Acetylation assays

For detecting acetylation of exogenous CDK5, transfected cells were washed in PBS and lysed into 0.5 ml RIPA buffer containing complete protease inhibitor cocktail (Roche, Basel, Switzerland) followed by sonication on ice. The lysates were then collected by centrifugation at 15,000*g* for 20 min at 4 °C. Protein contents in the supernatant were quantified using Bradford protein assay reagent (Bio-Rad, Hercules, CA, USA). For immunoprecipitation, 2 mg of protein was incubated with 10 μl of anti-FLAG M2 affinity gel (Sigma) in RIPA buffer at 4 °C overnight. The conjugates were collected by centrifugation at 400*g* for 2 min at 4 °C and the resulting pellets were washed with RIPA buffer. Proteins eluted from the beads by an addition of 45 μl of 1 × sample buffer were subjected to denaturation by boiling and separation on 11.5% SDS–PAGE. Immunoblot analyses were performed using anti-acetyl lysine antibody (Cell signaling, Beverly, MA, USA). For detection of endogenous CDK5 acetylation, 2 mg of cellular lysates were pre-cleared with mouse IgG-conjugated agarose (Sigma) at 4 °C for 2 h and incubated with 2 μg of CDK5 antibody (J3; Santa Cruz Biotechnology, Dallas, TX, USA) overnight. After incubation with protein A-agarose (Upstate Biotechnology, Lake Placid, NY, USA) for 2 h at 4 °C, the immunoprecipitates were washed, eluted as described above and subjected to an immunoblot analysis. *In vitro* acetylation assay was performed according to the methods previously reported [24]. Briefly, His-tagged mouse CDK5 WT or K33R were expressed and purified from *Escherichia coli* BL21 DE3 using nickel nitrilotriacetic acid (Ni^{2+}-NTA) bead as recommended by the supplier (Invitrogen, Carlsbad, CA, USA). One microgram of each protein was incubated with 1 μg of human GCN5 catalytic domain (Enzo Life Sciences, Inc., Farmingdale, NY, USA) and 0.1 mM acetyl-CoA (Sigma) in 1 × HAT buffer (50 mM Tris–HCl pH 8.0, 10% glycerol, 0.1 mM EDTA, 5 mM DTT) for 1 h at 30 °C. Reaction mixtures were denatured with 1 × sample buffer by boiling for 5 min and immunoprobed with anti-acetyl lysine antibody.

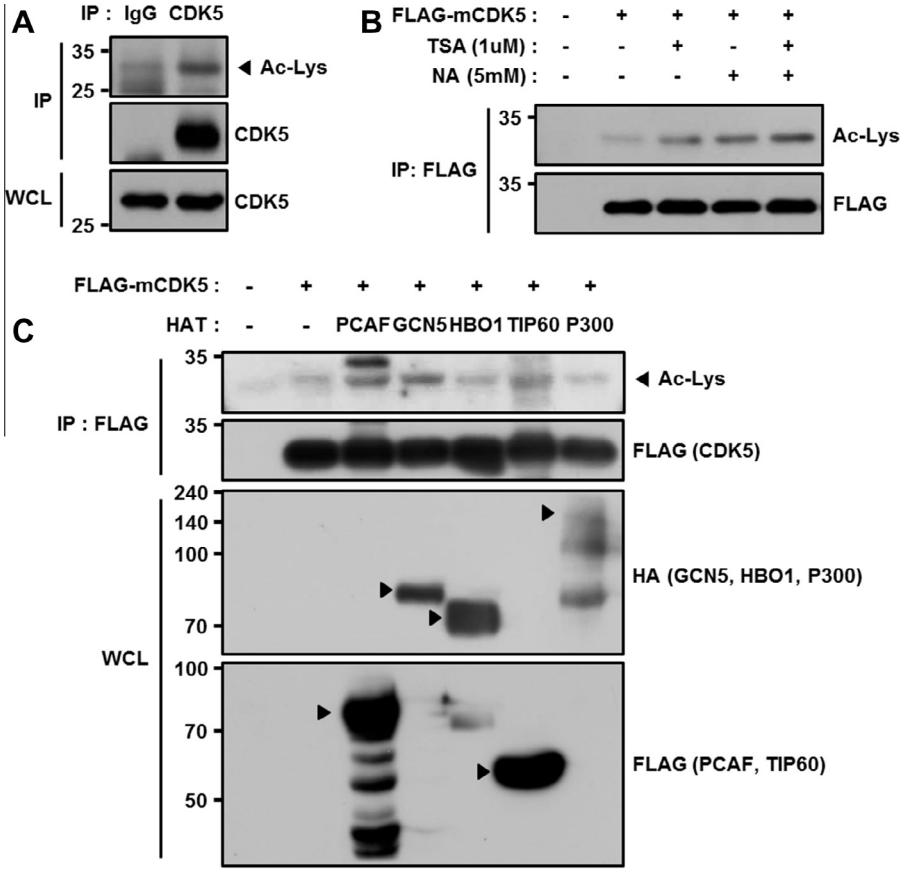

Fig. 1. CDK5 is acetylated by a subset of HATs. (A) HEK293 cell lysates were immunoprecipitated with anti-CDK5 antibody or control IgG followed by immunoblotting with anti-acetyl lysine antibody to detect endogenous level of the acetylated CDK5 (Ac-Lys). (B) HEK293 cells transfected with FLAG-tagged mouse CDK5 were treated with or without 1 μM TSA or 5 mM NA alone or in combination for 24 h. The whole cell lysates were subjected to immunoprecipitation with FLAG-M2 affinity gel followed by immunoblotting with anti-acetyl lysine antibody or with anti-FLAG HRP antibody. (C) HEK293 cells were transiently transfected with FLAG-tagged mCDK5 and the indicated human HATs. Whole cell lysates were immunoprecipitated with FLAG-M2 affinity gel and blotted with anti-acetyl lysine antibody. The exogenously expressed HA-tagged or FLAG-tagged HATs were immunoprobed with anti-HA or anti-FLAG antibody and indicated using arrowheads at the two lower panels.

2.3. Confocal microscopy

Cells plated on cover glass were rinsed with PBS and fixed with 4% paraformaldehyde for 10 min at RT. Cells were then rinsed with PBS for 5 min and permeabilized with PBS containing 0.5% triton X-100 for 10 min at RT with gently shaking. To counterstain the nuclei, cells were further incubated at RT for 1 h with Hoechst 33258 (2 μg/ml; Invitrogen) in PBS containing 0.1% triton X-100. After several washes, cells were mounted with Vectashield mounting medium (Vector Laboratories, Burlingame, CA, USA). Fluorescence images were observed under an LSM 510 META confocal microscope equipped with epifluorescence and a LSM digital image analyzer (Carl Zeiss, Zena, Germany).

2.4. Mass spectrometry

To map GCN5-mediated acetylation site(s) of CDK5, HEK293 cells were transfected with FLAG-tagged mouse CDK5 along with or without FLAG-human GCN5S as described above. Twenty-four hours after transfection, cells were treated with 1 μM TSA and 5 mM NA for 24 h. Twelve milligrams of the whole cell lysates were immunoprecipitated with 120 μl anti-FLAG M2 affinity gel. The precipitate was resolved on SDS–PAGE and stained with 0.1% Coomassie brilliant blue G-250. A band corresponding to CDK5 was excised and subjected to in-gel digestion with trypsin. The resulting tryptic peptides were analyzed by nano-LC MS/MS using a hybrid triple quadrupole-ion trap (QTRAP 5500) and a high-resolution hybrid FT-ICR mass spectrometry (LTQ-FT) as described previously [25].

3. Results

The previous reports predicted that CDK may be acetylated in several independent mammalian cell lines as well as *Drosophila* cell line [26,27]. Recent studies illustrating acetylation of CDK2 and CDK9, members of the CDK family involved in regulation of cell cycle and transcription, also raise the possibility of CDK5 acetylation [18,19]. Therefore, we specifically attempted to clearly determine whether CDK5 can be acetylated in cultured cell. Immunoprecipitation followed by an immunoblot analysis revealed that the acetylated form of endogenous CDK5 is present in HEK293 cells (Fig. 1A). When HEK293 cells transiently overexpressing FLAG-tagged mouse CDK5 were treated with or without empirically determined concentration of trichostatin A (TSA; a broad-spectrum HDAC inhibitor) and nicotinamide (NA; an inhibitor for NAD^+-dependent sirtuins) either alone or in combination, we found that levels of the acetylated CDK5 are significantly increased (Fig. 1B), suggesting the presence of CDK5 as an acetylated form and its level regulated by a specific set of acetyltransferases in cells. To identify the lysine acetyltransferase(s) responsible for CDK5 acetylation, we screened several histone acetyltransferases (HAT) for CDK5 acetylation. Among the 5 HATs chosen for screening, P300/CBP-associated factor (PCAF), GCN5 and TIP60 significantly increased the level of acetylated CDK5 (Fig. 1C). In consideration of previous reports demonstrating that acetylation of CDK2 and CDK9 are mediated by GCN5 and PCAF [18,19], and the PCAF is expressed in a tissue-specific manner in rodent [28,29], we chose GCN5 for further study.

GCN5 belongs to the GCN5-related N-acetyltransferases (GNAT) super family that is critically involved in histone and non-histone

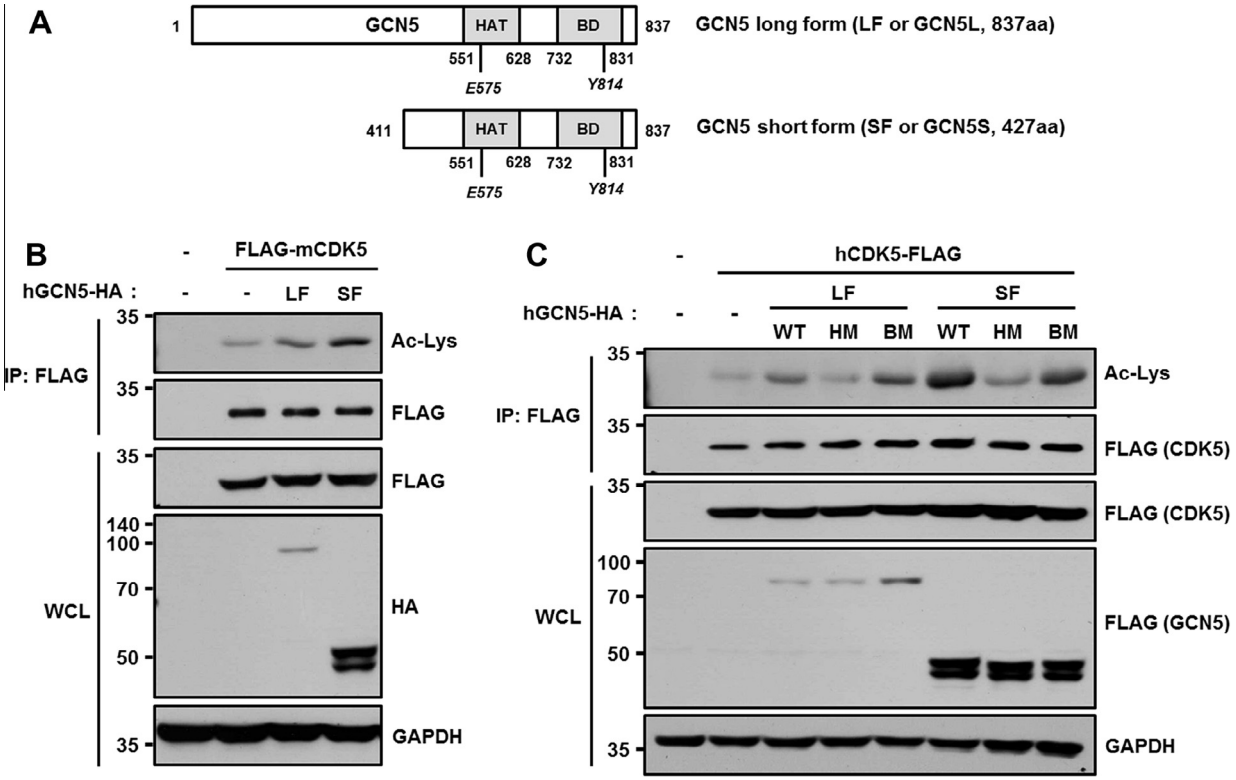

Fig. 2. GCN5 isoforms acetylate CDK5. (A) Schematic representation of two isoforms of human GCN5. (B) HEK293 cells were transfected with the indicated combination of FLAG-tagged mouse CDK5 and HA-tagged human GCN5 isoforms (LF; long form and SF; short form). The lysates were subjected to immunoprecipitation using FLAG-conjugated beads followed by an immunoblot analysis with anti-acetylated lysine antibody. Anti-GAPDH antibody was used as a loading control. (C) FLAG-tagged human CDK5 and HA-tagged human GCN5 isoforms bearing the indicated mutation were used to transfect HEK293 cells: WT (wild-type); HM (HAT domain mutant); and BM (bromodomain mutant) as depicted in (A). The lysates were subjected to immunoprecipitation followed by an immunoblot analysis as described above.

acetylation [30,31]. GCN5 transcript had undergone an alternative splicing, generating a predominantly expressed long form (GCN5L; LF) and a shorter isoform (GCN5S; SF) lacking N-terminal PCAF homology domain (Fig. 2A) [28]. First, we compared acetylation efficacy of these isoforms in HEK293 cells after transfection with GCN5L or GCN5S along with FLAG-tagged CDK5. The rate of the CDK5 acetylation was greater in GCN5S-expressing cell than in GCN5L-expressing cells (Fig. 2B). However, this may be due to greater transfection efficiency of GCN5S form. Mutation analysis demonstrated that GCN5-mediated CDK5 acetylation is not enhanced in cells transfected with a catalytically inactive GCN5 mutant (E575Q; HM) whereas a bromodomain mutant (Y814A; BM) still acetylates CDK5 at a quite similar level found in GCN5 wild type (WT; Fig. 2C). These results indicate that two isoforms of GCN5 can acetylate CDK5 and HAT activity domain is necessary for acetylation (Fig. 2C). To determine their localization and their binding, we performed fluorescent imaging study and co-immunoprecipitation assay in cells overexpressing tagged proteins. As shown in Fig. 3A, fluorescence microscopy revealed colocalization of both isoforms of GCN5 with CDK5 within the nuclei, suggesting that GCN5-mediated acetylation of CDK5 occurred at the specific nuclear foci. Co-immunoprecipitation study revealed that GCN5L and GCN5S interact with CDK5 (Fig. 3B). To further confirm whether this modification is a consequence of direct binding of these two proteins, *in vitro* acetylation assay was performed by incubating the purified CDK5 proteins with a recombinant six-Histidine-tagged HAT domain of GCN5. As shown in Fig. 3C, incubation with GCN5 markedly increased CDK5 acetylation. Collectively, these data clearly indicate that CDK5 is a direct target of acetylation and its acetylation is mediated by GCN5.

To determine which lysine residue(s) within CDK5 is a target of GCN5-mediated acetylation, the lysates from cell expressing

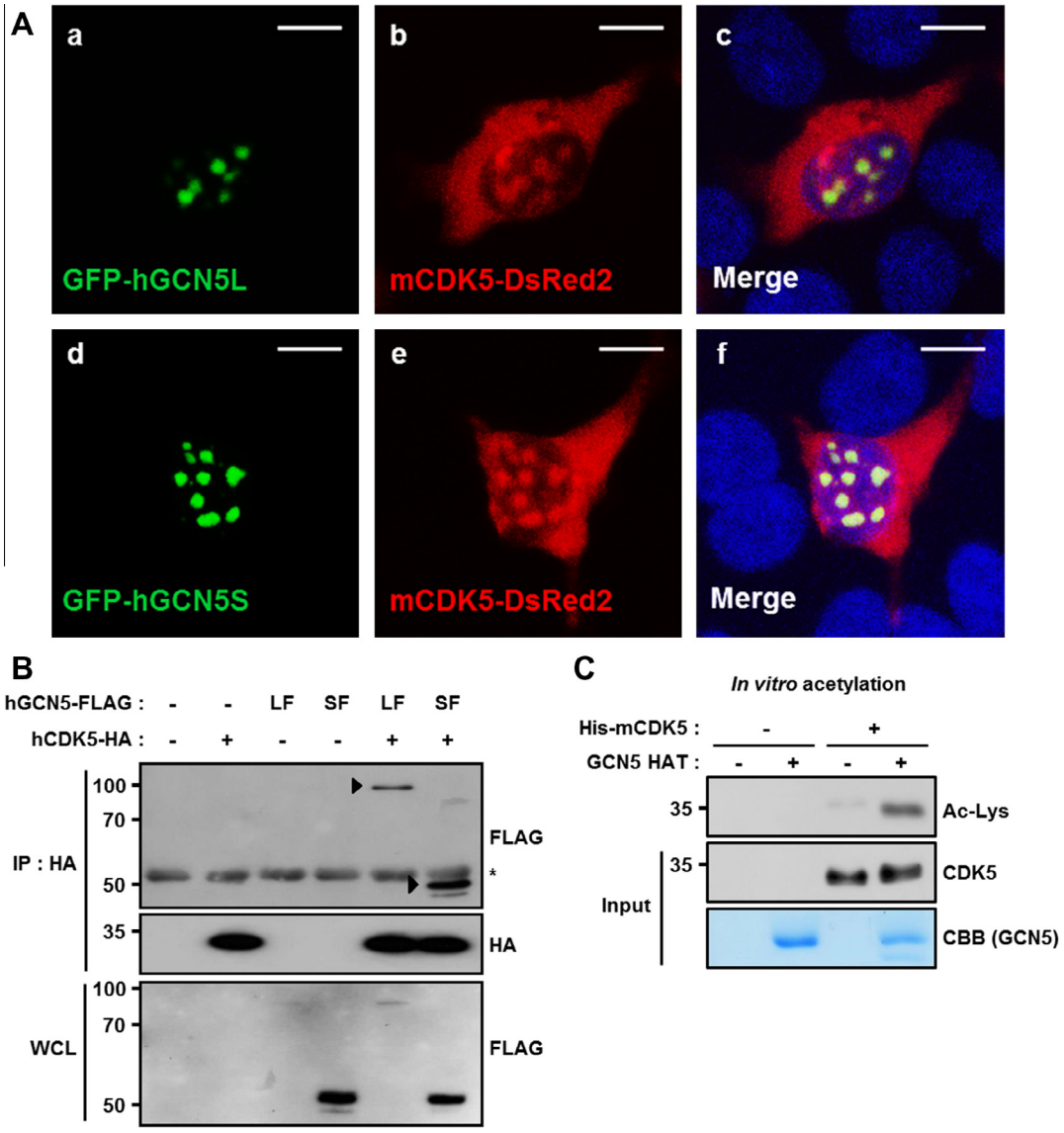

Fig. 3. Subcellular localization and binding of GCN5 and CDK5. (A) HEK293 cells were transiently transfected with DsRed2-tagged mouse CDK5 and GFP-tagged human GCN5L or GCN5S. Cells were examined under a confocal microscope. Hoechst 33258 was used to visualize nuclei. Scale bar represents 10 μm. (B) HEK293 cells were transfected with the indicated combination of vectors. The lysates were subjected to binding analyses using co-immunoprecipitation. Arrowheads indicate long and short forms of GCN5 bound to CDK5. Asterisk indicates signal from the heavy chain band of the antibody used for immunoprecipitation. (C) Bacterially prepared six histidine (His)-tagged mouse CDK5 was incubated with a recombinant GCN5 HAT domain protein. Acetylation was visualized by immunoblotting with anti-acetyl lysine antibody. Input was verified by immunoblotting with anti-CDK5 antibody or staining with 0.1% Coomassie Brilliant Blue G-250 for GCN5 HAT.

FLAG-tagged mouse CDK5 with or without GCN5 were prepared and subsequently immunoprecipitated with anti-FLAG M2 bead. After confirmation of expression and successful acetylation of CDK5 in this condition (Supplementary Fig. 1A), the remaining immunoprecipitates were separated by a gel electrophoresis, subjected to enzymatic digestion, and then analyzed using a tandem mass spectrometry. Although CDK5 was found to be acetylated at several lysine residues, mass spectral analysis indicated that the site which was predominantly acetylated using GCN5 is at Lys33 (K33; Supplementary Fig. 1B and Fig. 4A). In support of these data, both cell-based and *in vitro* acetylation assay showed that GCN5 acetylates only wild type CDK5 but not K33R mutant (Fig. 4B and C). Although previous study predicted that K56 is another acetylation site of CDK5 [26,27], we found that GCN5 quite effectively acetylates CDK5 K56R mutant (Supplementary Fig. 2).

4. Discussion

In this study, we found that CDK5 is presented as an acetylated form in cells. Since acetyltransferases exhibit remarkable specificity for acetyl acceptor, we tested several candidate proteins for its efficacy and found that GCN5, PCAF and TIP60 are involved in acetylation of CDK5. We further confirmed that both GCN5 long and short forms are responsible for acetylation of CDK5 at the specific nuclear foci. Study using mutant constructs indicated that HAT domain but not bromodomain of GCN5 is absolutely necessary for its activity. Mass spectral analysis maps the acetylation site of CDK5 at K33. As shown in Supplementary Table 1, the K33 of CDK5 lies within the catalytic cleft and is highly conserved among species. Intriguingly, other lysine sites as K41, K43, K48, and K52 of human CDK family correspond to K33 of CDK5 and are responsible for ATP and magnesium ion binding [32,33], indicating that acetylation of CDKs at these sites may comprise a critical way of regulating their kinase activity. Indeed, it has been shown that the kinases including p38, JNK1, CDK2 and CDK9, which commonly share the structure of the ATP binding region, undergo acetylation at the same position [18,19,34]. However, consequence of their acetylation differs in that the acetylation of CDK2 (at K33) and CDK9 (at K48) inhibits their kinase activity [18,19] whereas the acetylated p38 (at K53) and JNK1 (at K55) shows enhanced ATP binding affinity and kinase activity *in vitro* [34]. In our unpublished data, CDK5 K33R (acetylation null mutant) and K33Q (mimetic mutant) all abolished its kinase activity, suggesting the importance of K33 residue for maintenance of overall kinase activity of CDK5. In strong support of this idea, a mutant of CDK5 with lysine at K33 substituted to threonine was used as a second dominant negative mutant of CDK5 after D144N mutant [35]. These previous findings along

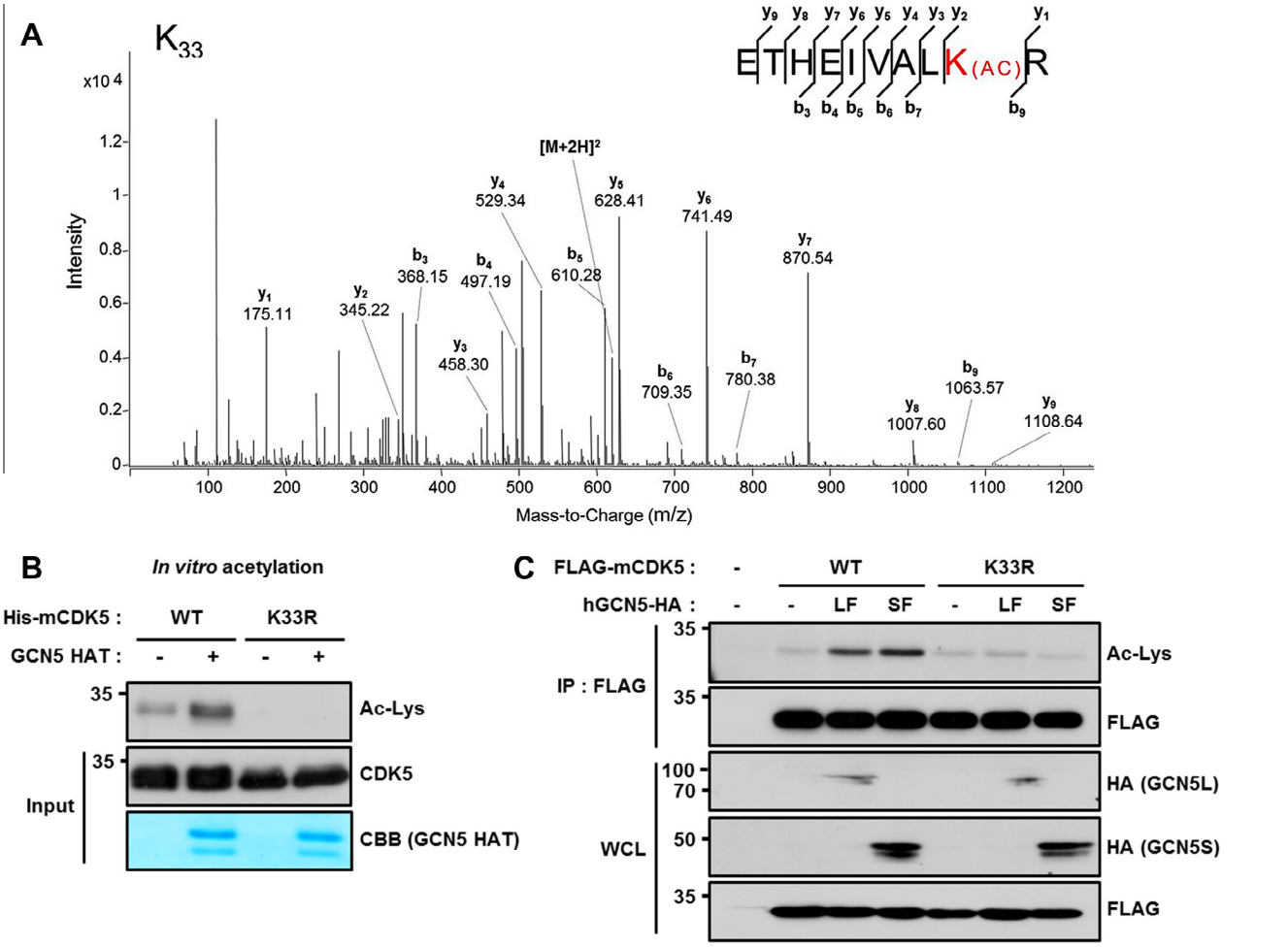

Fig. 4. Acetylation of CDK5 by GCN5 occurs at Lys33 residue. (A) Annotation of a tandem mass spectrum of trypsin-digested CDK5 depicts acetylation site at K33. (B) Bacterially expressed six histidine (His)-tagged mouse CDK5 WT or K33R protein was incubated with a recombinant GCN5 HAT domain protein. Acetylation was visualized by immunoblotting with anti-acetyl lysine antibody. (C) The cell lysates from HEK293 cells transiently transfected FLAG-mouse CDK5 WT or K33R along with HA-tagged human GCN5 isoforms were subjected to immunoprecipitation followed by immunoblot analysis using the indicated antibodies.

with our present data prompt us to speculate that CDK5 acetylation by GCN5 affects ATP binding capability and subsequently alters its kinase activity. As shown in Fig. 1C, acetylation of CDK5 is increased by TIP60 acetyltransferase. In our preliminary study, however, we found that TIP60 seems to acetylate other lysine residues than K33 or K56, suggesting that another regulatory lysine residue may exist in CDK5.

It has been demonstrated that CDK5 plays an important role in neuronal death and survival by phosphorylating its target substrates. Interestingly, GCN5 and CDK5 are co-localized within the nucleus as shown in Fig. 3A. CDK5 has a number of nuclear substrates such as myocyte-specific enhancer (MEF2D), Ataxia telangiectasia mutated (ATM), nuclear p53, huntingtin and apurinic/apyrimidinic endonuclease 1 (APE1) [36]. There is strong evidence to demonstrate that phosphorylation of these substrates by CDK5 may facilitate the progression of apoptosis. For example, the phosphorylated MEF2D at Ser444 using CDK5 is preferentially degraded by caspase, causing lack of survival gene expression and subsequently leading to neuronal cell death [37]. ATM, a member of phosphatidylinositol-3-kinase-like kinase family, is phosphorylated at Ser794 by nuclear CDK5/p25 complex in postmitotic neurons exposed to DNA damage [38]. The CDK5-mediated ATM activation in response to DNA damage triggers rH2AX and p53 phosphorylation as well as subsequent apoptotic gene expression such as PUMA and BAX, stimulating cell apoptosis via caspase cascades. All of these studies described above suggest that aberrant CDK5 activity in the nucleus is closely associated with neuronal death. Although too much or too little CDK5 activity all contributes to neuronal death, silencing or inhibition of overactivated nuclear CDK5 may promote rescuing neurons from dying. Previous reports demonstrating subcellular redistribution and overactivation of CDK5 in an array of neurodegenerative disorders support a role for CDK5 in their pathogenesis and strengthen importance of regulation of CDK5 activity as a potential therapeutic strategy for acute and chronic neurodegenerative disorders [5,36].

In sum, regulation of CDK5 activity plays a key role in determining neuronal survival and death. It has been demonstrated that CDK5 activity is determined using several independent mechanisms via posttranslational modifications including availability of p35 via a balance between synthesis and degradation, as well as CDK5 phosphorylation [39]. We extend this notion by raising the possibility that acetylation of CDK5 at lysine residue within the catalytic ATP binding domain may be an additional route for regulating CDK activity and its versatile role in neuronal physiology and pathophysiology. Further study delineating a close correlation between acetylation state and kinase activity in disease models would provide significant insights into its role as a key regulator of neurodegeneration.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bbrc.2014.03.118.

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