ISSN : 1225-9918
Journal of Life Science **2011** Vol. 21. No. 8. 1067~1075 DOI : http://dx.doi.org/10.5352/JLS.2011.21.8.1067

Coiled-Coil Domain-Containing Protein 98 (CCDC98) Regulates Cyclin B1 Expression by Affecting WTAP Protein Stability

Yun Jung Oh[1†], Eun Hee Lee[1†], Il Kyu Lee[2†], Kyung-Soo Kim[2] and Hongtae Kim[1]*

[1]*Department of Biological Sciences, Sungkyunkwan University, 300 Cheoncheon-dong, Jangan-gu, Suwon 440-746, Korea*
[2]*Department of Family Medicine, The Catholic University of Korea, Seoul 137-701, Korea*

Received May 9, 2011 / Revised July 6, 2011 / Accepted July 8, 2011

Coiled-coil domain-containing protein 98 (CCDC98) plays a role in G2/M DNA damage checkpoint pathways by recruiting breast cancer 1 (BRCA1)-A complex to the DNA-damaged sites. However, the molecular mechanism of CCDC98 on the DNA damage-induced G2/M checkpoint pathways is unclear. In this study, we identifed Wilms tumor 1-associating protein (WTAP) as a novel CCDC98-binding protein, using tandem affinity purification. We confirmed the association between CCDC98 and WTAP using *in vivo* and *in vitro* binding assays. We demonstrated that CCDC98 regulates cyclin B1 expression by affecting WTAP protein stability. Based on these results, we suggest that CCDC98 may act as a novel cell cycle regulator by regulating the expression level of cyclin B1.

Key words : DNA damage, Coiled-coil domain-containing protein 98 (CCDC98), Wilms tumor 1-associating protein (WTAP), cyclin B1, cell cycle

Introduction

Genomic instability is characterized by an increased rate of chromosome aberrations and mutations. Genomic instability can arise due to multiple causes, including DNA damage [1]. DNA damage is induced by external and internal factors. External factors include ultraviolet radiation (UV), ionizing radiation (IR), and numerous chemical and internal factors, including reactive oxygen species (ROS), replication errors, and mitotic errors [4]. The DNA damage-inducing factors activate the cell cycle checkpoint to protect genomic stability through DNA lesion repair and apoptosis. Many proteins are involved in the DNA damage - induced cell cycle checkpoint, including Ataxia-telangiectasia (ATM), Rad3-related protein (ATR) kinases, mediator of DNA damage checkpoint protein 1 (MDC1), the product of breast cancer susceptibility gene 1 (BRCA1), receptor associated protein 80 (RAP80), coiled-coil domain-containing protein 98 (CCDC98), p53-binding protein 1 (53BP1), DNA topoisomerase II binding protein 1 (TopBP1), and the checkpoint kinases 1 and 2 (Chk1 and Chk2) [8,11].

CCDC98 plays an important role in targeting the BRCA1 complex to DNA damage sites by binding with RAP80 [6,7]. CCDC98 is a transducer of the DNA damage checkpoint response, and it regulates the DNA damage signaling pathway mediated by BRCA1 in order to participate in G2/M checkpoint regulation [5,6,7,10,12,13]. Although convincing evidence has established that CCDC98 is involved in the G2/M checkpoint, regulation of this event in the cell remains to be elucidated. Here, we demonstrate that WTAP is as a novel CCDC98-binding protein, and we illustrate the association between CCDC98 and WTAP using *in vivo* and *in vitro* binding assays. We also demonstrate that CCDC98 may act as a novel cell cycle regulator by regulating cyclin B1 expression levels.

Materials and Methods

Plasmids

SFB tagged, wild-type CCDC98 and serial deletion mutant expression vectors (SFB-CCDC98 WT, D1, D2, D3, and △ SPTF) were described previously [6]. Myc-tagged, WTAP wild type and a deletion mutant expression vector (Myc-WTAP) were generated by PCR. Deletion mutants of WTAP were generated by site-directed mutagenesis and were subcloned into a Myc-tagged mammalian expression vector. GST-tagged CCDC98 and SFB-tagged RAP80 expression vectors were described previously [6].

Cell culture

HeLa and human embryonic kidney (HEK) 293T cell lines

†These three authors contributed equally to this paper
*Corresponding author
Tel : +82-31-299-4497, Fax : +82-31-290-7015
E-mail : khtcat@skku.edu

were purchased from American Type Culture Collection (ATCC, Manassas, VA, USA) and maintained in DMEM medium (WelGENE, Dalseogu, Daegu, Korea) supplemented with 10% fetal bovine serum at 37°C in 5% CO_2 (v/v).

siRNAs

Control, CCDC98 [6], and WTAP siRNAs [3] were described previously. siRNAs were transfected into the cells using oligofectamine (Invitrogen Corporation, Carlsbad, CA, USA).

Antibodies, transfection, and immunoprecipitation

Anti-CCDC98 antibody was described previously [6]. Anti-Flag, -Myc, -WTAP, and-β-actin antibodies were purchased from Sigma Inc (St, Louis, MO, USA), and human cyclin B1 antibodies were purchased from Cell Signaling. Transient transfection was performed using the Fugene 6 reagent (Roche Applied Science, Hague Road, IN, USA). Cell transfection was performed using Fugene 6 (Roche. Inc). For immunoprecipitation and immunoblotting, cells were washed with ice-cold PBS and lysed in NETN buffer containing 0.5% Nonidet P-40, 20 mM Tris [pH 8.0], 50 mM NaCl, 50 mM NaF, 100 μM Na_3VO_4, 1 mM DTT, and 50 μg/ml PMSF at 4°C for 10 min. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 5 min, and supernatants were incubated with protein G-Sepharose-conjugated anti-FLAG or protein A-agarose-conjugated anti-Myc antibodies. The immunocomplexes were washed twice with NETN buffer and were subjected to SDS-PAGE. Western blotting was performed using the antibodies.

Establishment of stable cell lines and affinity purification of S-Flag-SBP (SFB)-tagged, CCDC98-containing complexes

To construct a stable cell line, HEK 293T cells were transfected with SFB-CCDC98 or SFB plasmid as a control. After 48 hr, the cells were split at a 10:1 ratio and were cultured in a medium containing 1 mg puromycin (Sigma) for three weeks. The individual puromycin-resistant colonies were isolated and confirmed by western blotting for the over-expressed CCDC98.

Affinity purification of S-Flag-SBP (SFB)-tagged, CCDC98-containing complexes and mass spectrometry

The 293T cells stably expressing SFB-CCDC98 protein were lysed with 4 ml of NETN buffer on ice for 10 min. Crude lysates were cleared by centrifugation at 14,000 rpm at 4°C for 10 min, and supernatants were incubated with 300 μl of streptavidin-conjugated beads (Amersham Biosciences, Heights, IL, USA). The immunocomplexes were washed three times with NETN buffer, and bead-bound proteins were eluted with 500 μl of NETN buffer containing 2 mg/ml biotin (Sigma). The eluted supernatant was incubated with 60 μl of S-protein beads (Novagen, Madison, WI, USA). The immunocomplexes were washed three times with NETN buffer and subjected to SDS-PAGE. Silver staining was performed to visualize the protein bands. Specific bands were excised and digested, and the peptides were analyzed by a mass spectrometer.

Purification of the GST-fusion proteins

The GST fusion protein was expressed in *Escherichia coli* and purified as described [2].

Immunofluorescence staining

Cells grown on coverslips were fixed with 3% paraformaldehyde at room temperature for 15 min. After 24 hr, the cells were rinsed with 1× PBS and fixed with 3% paraformaldehyde for 15 min. Then, the cells were permeabilized in PBS containing 0.5% Triton X-100 for 5 min, and the coverslips were blocked with 5% goat serum in PBS for 30 min. The coverslips were incubated with anti-Flag (1:200) and anti-Myc (1:1,000) antibodies at room temperature for 20 min. After washing with PBS, cells were incubated with secondary antibodies, fluorescein isothio-cyanate-conjugated goat anti-mouse IgG, rhodamine-conjugated goat anti-rabbit IgG, or rhodamine-conjugated goat anti-mouse IgG (Jackson ImmunoReserach Laboratories, Inc., West Grove, PA, USA) at room temperature for 20 min. 4,6-diamidino-2-phenylindole (DAPI) was using to counterstain the nuclei. After a final wash with PBS, coverslips were mounted with glycerin containing p-phenylenediamine. All images were obtained with a Nikon ECLIPSE E800 fluorescence microscope.

FACS analysis

FACS analysis was performed as described previously [6].

Results

Identification of WTAP as a CCDC98-binding protein.

To find the CCDC98-binding partners, we performed

large scale-affinity purification using the SFB-CCDC98 stably expressing HEK 293T cell lines (Fig. 1A). After sequential affinity chromatography with streptavidin agarose and S-agarose beads and mass spectrometry analysis using the cell lysates prepared from cells expressing SFB- CCDC98, we identified several putative CCDC98 binding proteins (Table 1). Among these proteins, we focused on WTAP because peptides from this protein were abundantly recovered from the mass spectrometry analysis. First, we checked for binding between CCDC98 and WTAP by overexpression in 293T cells. As shown in Fig. 1B, CCDC98 associated strongly with WTAP and showed no binding to RAP80. A GST-pull

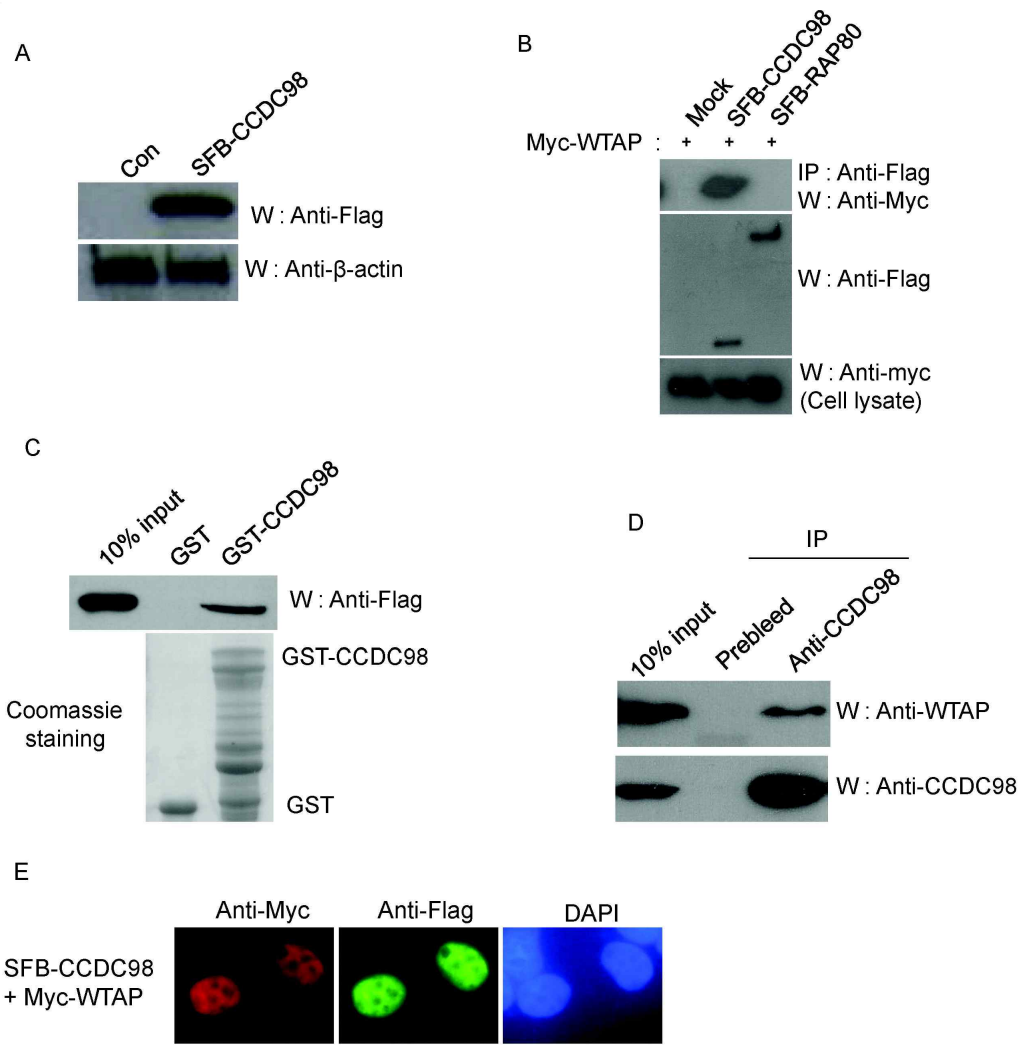

Fig. 1. Identification of WTAP as a novel CCDC98-binding protein. A, establishment of human embryonic kidney 293T cell lines stably expressing SFB-CCDC98. Cell extracts prepared from 293T cells stably expressing a control (Con) plasmid or SFB-CCDC98 fusion protein were subjected to Western blotting analysis (W) using anti-FLAG antibody. B, interaction between exogenous Myc-tagged WTAP and SFB triple-tagged CCDC98. Immunoprecipitation (IP) reactions were performed using anti-Flag antibody and were subjected to Western blot analysis (W) using anti-Myc and anti-Flag antibodies. C, GST pulldown assay. GST only or GST-CCDC98 protein was incubated with cell lysates containing transiently transfected, SFB triple-tagged, wild-type WTAP (SFB-WTAP). After extensive washing, bound WTAP proteins were analyzed by Western blotting analysis (W) with anti-Flag antibody. The levels of GST and GST-CCDC98 are shown in the lower panel. D, binding between endogenous WTAP and CCDC98. Immunoprecipitation (IP) reactions were performed using preimmune serum or anti-CCDC98 antibodies and were subjected to Western blot analysis (W) with the antibodies listed on the right. E, colocalization of CCDC98 with WTAP. 293T cells were transfected with Myc-tagged WTAP and SFB triple-tagged CCDC98 expression plasmids. Next, immunofluorescence assays were performed using anti-Flag and-Myc antibodies. 4,6-Diamidino-2-phenylindole (DAPI) was used as an indicator for the nucleus.

Table. 1. List of proteins associated with SFB-CCDC98 identified by mass spectrometry analysis

Protein name	*No. of peptides obtained*
	Experiment
Laminin gamma 1	21
BRCA1/BRCA2-containing complex subunit 45	17
Wilms tumor 1 associated protein	17
Heat hock cognate 71 kDa protein	15
Coiled-coil domain-containing protein 98	15
MRNA encoding beta-tubulin	14
Tubulin alpha-2 chain	12
Aspartate-tRNA ligase	10
Receptor-associated protein 80	9
26S protease regulatory subunit 6A	8
BRCA1/BRCA2-containing complex subunit 36	8
Sex co mb on midleg-like 1	5

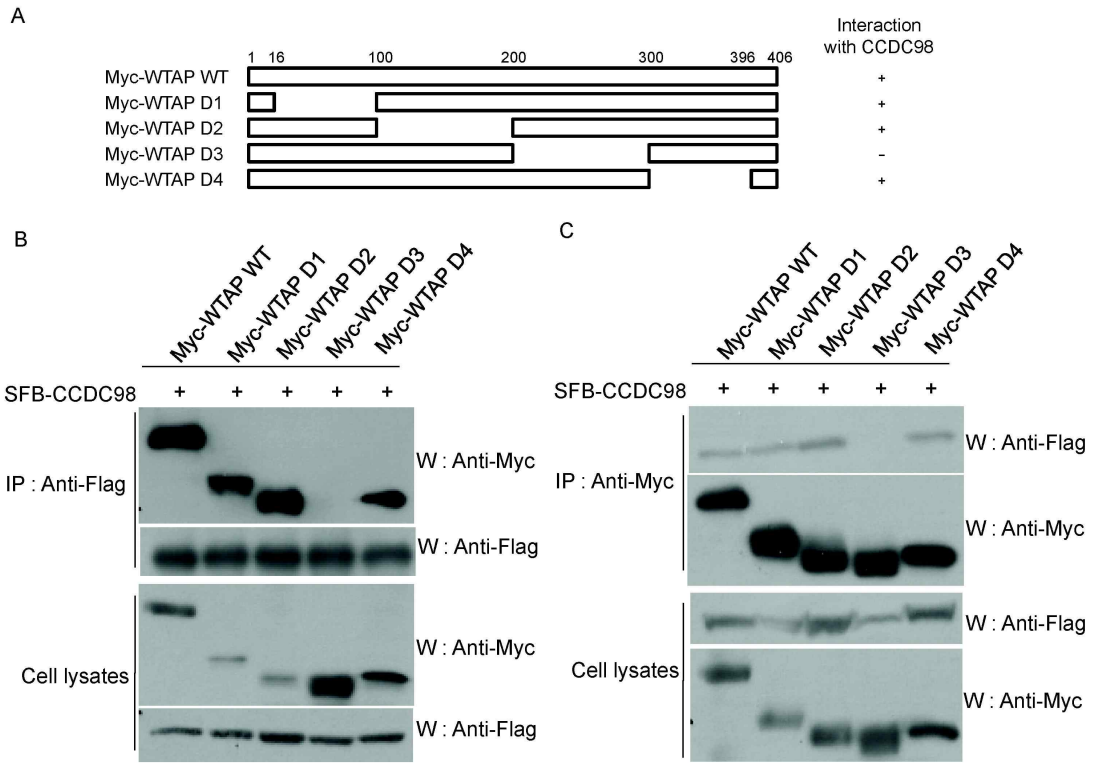

Fig. 2. Identification of the CCDC98-binding regions of WTAP. A, diagram of wild type (WT) WTAP and serial deletion mutants (D1 – D4). B and C, 293T cells were transfected with plasmids encoding SFB-CCDC98 and wild type Myc-WTAP or serial deletion mutants. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag (B) or anti-Myc (C) antibodies and were immunoblotted (W) with the antibodies listed on the right. The levels of SFB triple-tagged CCDC98 and Myc-tagged WTAP in the lysates were analyzed by immunoblotting and shown in the bottom panels.

down assay using a GST-CCDC98 fusion protein and cell lysates of 293T cells overexpressing WTAP showed that GST-CCDC98 specifically bound to overexpressed WTAP, in contrast to GST only (Fig. 1C). Immunoprecipitation analysis using an anti-WTAP antibody also showed that CCDC98 specifically bound to WTAP at endogenous levels (Fig. 1D). CCDC98 colocalized with WTAP in the nuclear neoplasm (Fig. 1E). These data indicate that WTAP is a *bona fide* CCDC98-binding partner.

Identification of binding regions of CCDC98 and WTAP

To identify the CCDC98-binding regions of WTAP, internal serial deletion mutants of WTAP were constructed (Fig. 2A). WTAP wild type protein and deletion mutants were tested for their ability to interact with full-length CCDC98 by coexpression in 293T cells. As shown in Fig. 2B and C, WTAP amino acid residues 201 – 300 were important for association with the full-length CCDC98 protein. We further confirmed that the WTAP amino acid residues 201-234 are a CCDC98 binding region (Fig. 3A, B and C). Similarly, we generated a series of CCDC98 deletion mutants (Fig. 4A) and examined which regions of CCDC98 might be required for interaction with WTAP. We found that CCDC98 amino acid residues 301 – 405 are capable of binding to WTAP (Fig. 4B and C).

CCDC98 affects the cell proliferation

A recent paper showed that WTAP knockdown reduced expression of many genes related to cell cycle progression, including cyclins A2, B1, B2, and CDC20, thereby regulating G2/M cell-cycle transition [3]. These findings suggest that CCDC98, WTAP-binding partner, also affects the expression of proteins for regulating cell cycle progression. We therefore checked the effect of the CCDC98 knockdown on the expression level of cyclins A2, B1, D1 or E and Cdk1. Among these proteins, expression levels of cyclin A2 and B1 proteins decreased in HeLa cells transfected with WTAP or CCDC98 siRNA (Fig. 5A). We also found that treatment with either siRNA can efficiently downregulate both CCDC98 and WTAP expression (Fig. 5A). To investigate whether or not CCDC98 is involved in the turnover of WTAP, HeLa cells were depleted for CCDC98 using siRNA. As a measure of protein turnover, cells were treated with cycloheximide (CHX), a protein synthesis inhibitor, and were processed for Western blotting analysis. Figure 5B showed that the depletion of CCDC98 led to a dramatic decrease in the half-life of WTAP compared to that for control siRNA transfected

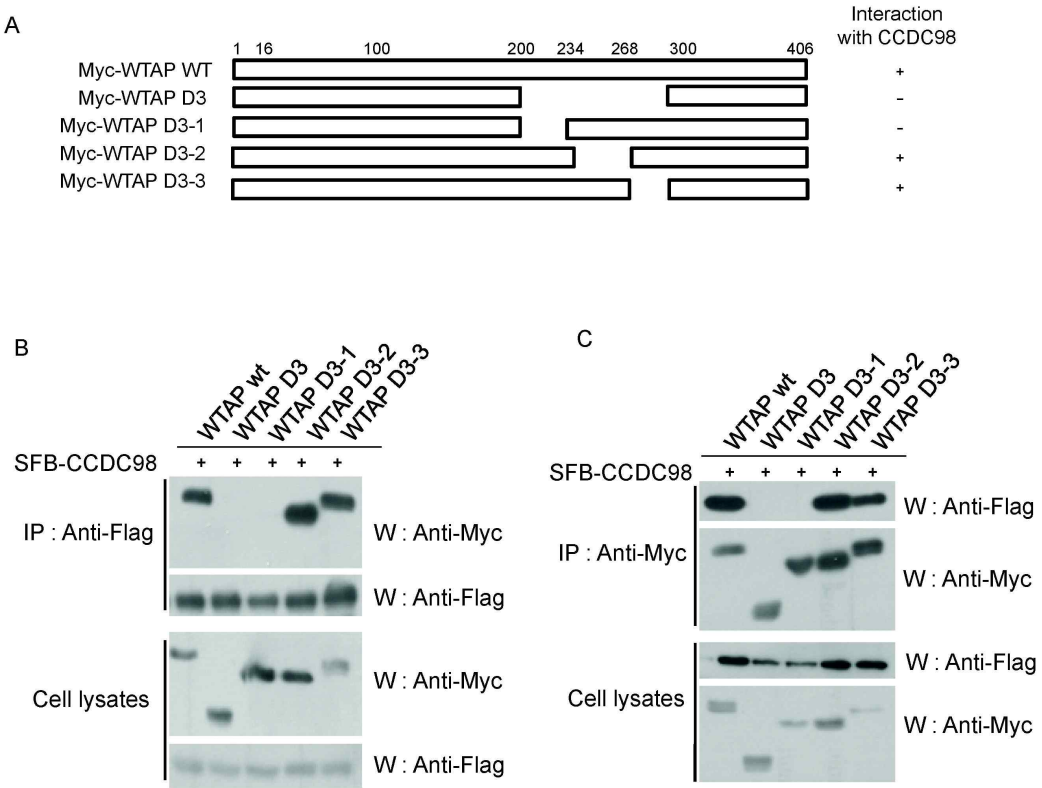

Fig. 3. Identification of the CCDC98-binding regions of WTAP. A, diagram of wild type (WT) WTAP and serial deletion mutants (D3-1 – D3-3). B and C, 293T cells were transfected with plasmids encoding SFB-CCDC98 and wild type Myc-WTAP or serial deletion mutants. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag (B) or anti-Myc (C) antibodies and immunoblotted (W) with the antibodies on the right. The levels of SFB triple-tagged CCDC98 and Myc-tagged WTAP in the lysates were analyzed by immunoblotting and shown in the bottom panels.

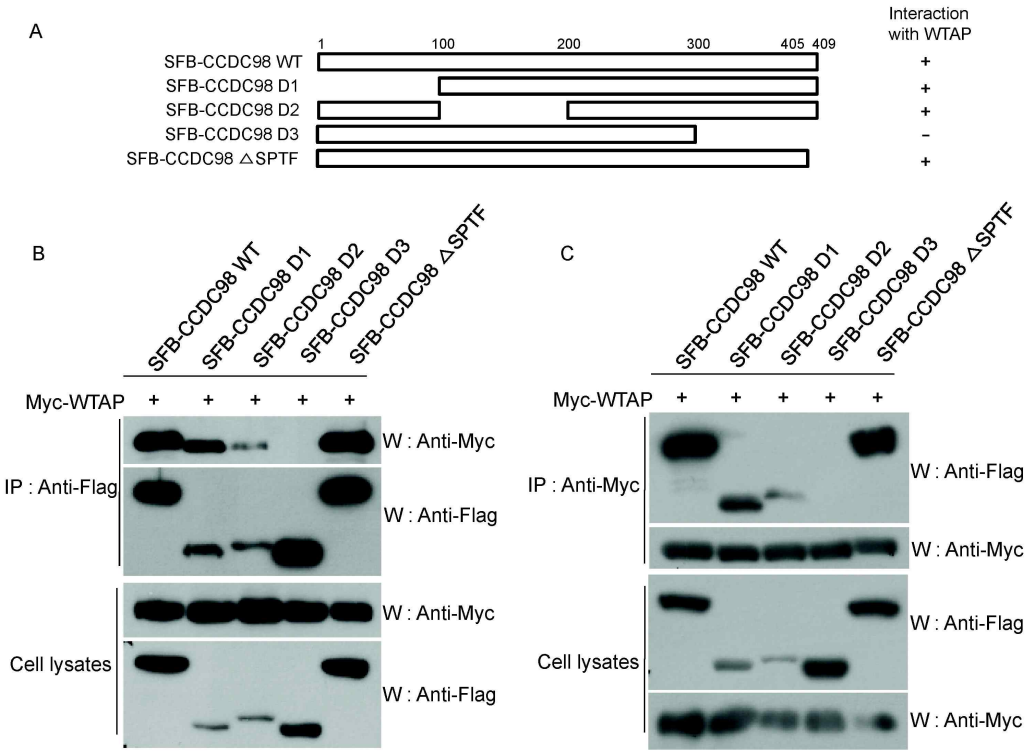

Fig. 4. Identification of the WTAP-binding regions of CCDC98. A, diagram of wild type (WT) CCDC98 and serial deletion mutants (D1 – D3 and △SPTF). B and C, 293T cells were transfected with plasmids encoding Myc-WTAP and wild type SFB-CCDC98 or serial deletion mutants. Cell lysates were subjected to immunoprecipitation (IP) with anti-Flag (B) or anti-Myc (C) antibodies and immunoblotted (W) with the antibodies on the right. The levels of SFB triple-tagged CCDC98 and Myc-tagged WTAP in the lysates were analyzed by immunoblotting and shown in the bottom panels.

HeLa cells. Previous data showed the ubiquitination of WTAP [9]. These data indicate that WTAP protein is degraded by CCDC98 via the ubiquitination/proteosome pathway. It has been reported that WTAP knockdown led to a decrease in cyclin A2 at the mRNA and protein levels [9]. Therefore, we investigated whether or not CCDC98 knockdown also affects cyclins A2 and B1 at the mRNA levels.

Quantitative real-time PCR analysis revealed that the cyclin A2 and B1 mRNA levels decreased to 30% those of controls within 48 hr after siRNA transfection (Fig. 5C and data not shown), and consistent reduction in the cyclin A2 and B1 protein levels was observed by Western blot analysis (Fig. 5A). To further demonstrate the functional importance of the WTAP and CCDC98 proteins on cell cycle progression using cell proliferation assays and FACS analysis, we down-regulated each protein in the HeLa cells because cyclins A2 and B1 are very important proteins for G2/M cell cycle progression. As shown in Fig. 5D, the control siRNA transfected cells showed moderate growth for the first 48 hr compared to WTAP or CCDC98 siRNA transfected cells after siRNA transfection, and the cell number increased four-fold by 96 hr. Moreover, FACS analysis revealed that WTAP or CCDC98 siRNA-treated cells had a significantly lower proportion of cells in mitosis compared with the proportion of control siRNA transfected cells (Fig. 5D).

Discussion

In this study, we identified WTAP as a novel CCDC98-binding protein using the tandem repeat affinity purification method. We confirmed the physical association between WTAP and CCDC98 by overexpression in 293T cells and at endogenous levels. CCDC98 or WTAP knockdown decreased cyclin A2 and B1 expression and mitotic cell population leading to reduction of cell proliferation. These data strongly suggest that CCDC98 may function as a new regulator of cell cycle progression by affecting WTAP

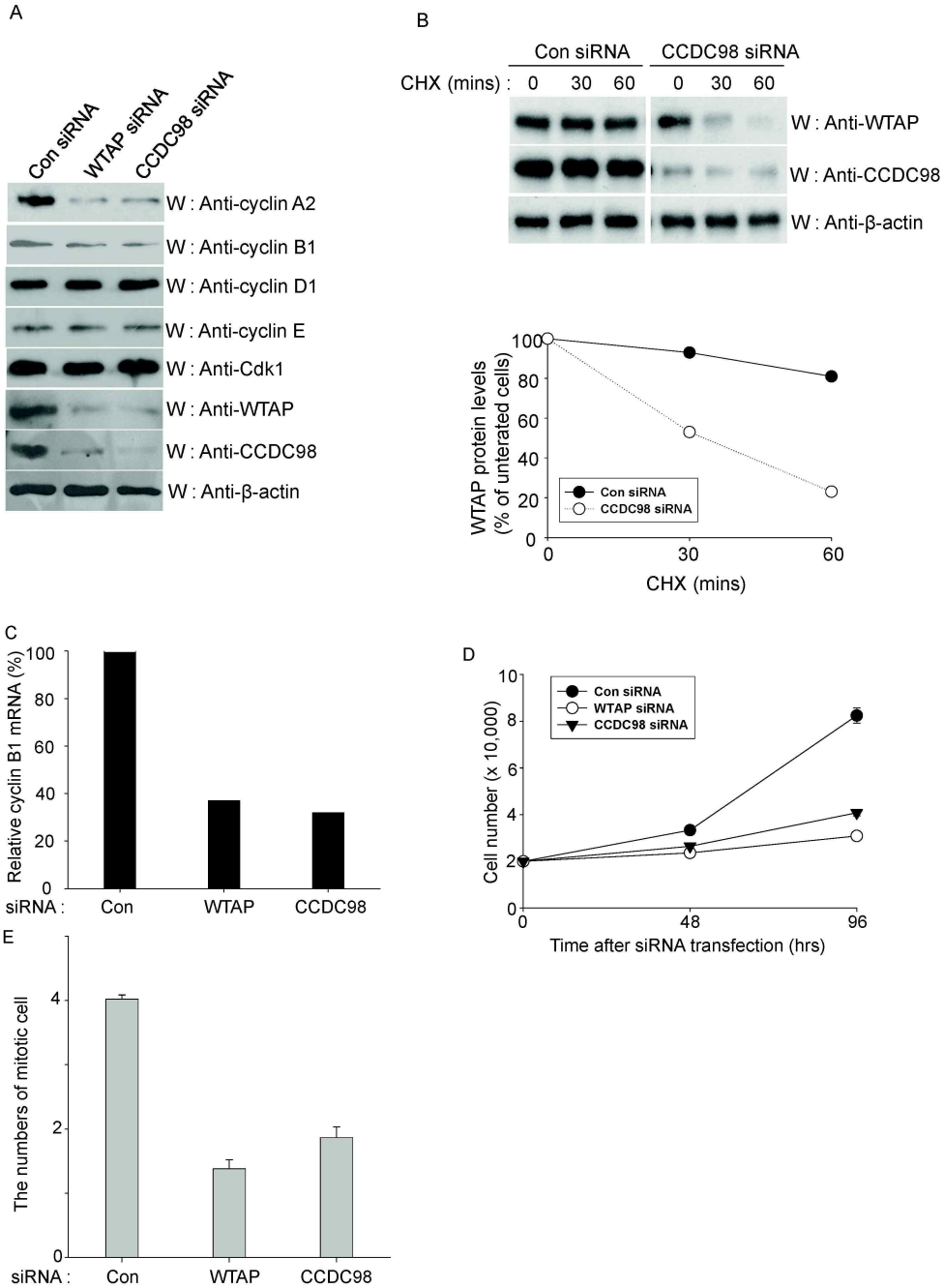

Fig. 5. WTAP and CCDC98 are important for regulation of cell cycle progression. A, the knockdown of WTAP or CCDC98 reduced the cyclin A2 or B1 expression. Control (con), WTAP or CCDC98 siRNAs were transfected into HeLa cells. The transfected cell lysates were subjected to immunoblotting (W) with the antibodies indicated on the right. B, knockdown of CCDC98 decreases WTAP turnover. Control (Con) or CCDC98 siRNA transfected HeLa cells were treated with cycloheximide (CHX). At each time point (0, 30, 60 min) after incubation, cells were harvested for analysis. Cell lysates were immunoblotted (W) with the antibodies listed on the right. Densitometric analysis was performed on WTAP protein normalized to actin using an image reader. C, quantitative real-time PCR analysis of cyclin B1 expression. Cyclin B1 mRNA levels were decreased to 30% those of controls 24 hr after siRNA transfection. The results are normalized to beta-actin mRNA levels. D, cell growth rates in CCDC98, WTAP, or control siRNA-treated HeLa cells. Cell numbers were determined using a hemacytometer at the indicated times. E, knockdown of WTAP or CCDC98 reduced the mitotic cell population. HeLa cells transfected with indicated siRNAs were fixed and stained with histone-specific anti-pH3 (a mitotic marker) and propidium iodide. Percentages of mitotic cells were determined by FACS analysis. Data shown are averages of two independent experiments; error bars indicate s.d.

and cyclin A2 and B1 expression levels. While WTAP colocalizes with CCDC98 in the nuclear neoplasm without ionizing radiation, only CCDC98 localizes to the DNA damage site with IR (data not shown). These data suggest that the WTAP may function with CCDC98 except for localization of DNA damage sites. Here, we show that the CCDC98 knockdown downregulates the cyclin B1 expression and reduces mitotic cell population. These data show the novel function of CCDC98 to inhibit cell cycle progression by reducing cyclin A2 and B1 expression. The data in this study also show that the expression levels of the WTAP and CCDC98 proteins decreased after knockdown of each protein. These results suggest that these two proteins are required for mutual stabilization. We think that one or more ubiquitin E3 ligase proteins may ubiquitinate these proteins and may have a major role in the interplay between WTAP and CCDC98. The identification of the E3 ligase(s) and the molecular mechanisms underlying the interplay between WTAP and CCDC98 remain to be determined. WTAP was previously shown to reduce expression in IGF-treated cell lines [9]. These data provide additional evidence that WTAP expression may be regulated in the protein levels. We also show that the knockdown of CCDC98 downregulates cyclin B1 expression and reduces mitotic cell population. These data show the novel function of CCDC98 on the regulation of the cell cycle to maintain WTAP stability. In conclusion, the identification of WTAP, a new CCDC98-binding protein, provides new implications for CCDC98-mediated regulation of the cell cycle.

Acknowledgements

This work was supported by the Korea Research Foundation Grant funded by the Korean Government (MOEHRD, Basic Research Promotion Fund) (KRF-2008-313-C00257) and the National R&D Program for Cancer Control, Ministry for Health, Welfare and Family Affairs, Republic of Korea (0920070) to H.K.

References

1. Elledge, S. J. 1996. Cell cycle checkpoints: preventing an identity crisis. *Science* **274,** 1664-1672.
2. Hofer, B., S. Backhaus, and K. N. Timmis. 1994. The biphenyl/polychlorinated biphenyl-degradation locus (bph) of Pseudomonas sp. LB400 encodes four additional metabolic enzymes. *Gene* **144,** 9-16.
3. Horiuchi, K., M. Umetani, T. Minami, H. Okayama, S. Takada, M. Yamamoto, H. Aburatani, P. C. Reid, D. E. Housman, T. Hamakubo, and T. Kodama. 2006. Wilms' tumor 1-associating protein regulates G2/M transition through stabilization of cyclin A2 mRNA. *Proc. Natl. Acad. Sci. USA* **103,** 17278-17283.
4. Kim, H. and J. Chen. 2008. New players in the BRCA1-mediated DNA damage responsive pathway. *Mol. Cells* **25,** 457-461.
5. Kim, H., J. Chen, and X. Yu. 2007. Ubiquitin-binding protein RAP80 mediates BRCA1-dependent DNA damage response. *Science* **316,** 1202-1205.
6. Kim, H., J. Huang, and J. Chen. 2007. CCDC98 is a BRCA1-BRCT domain binding protein involved in the DNA damage response. *Nat. Struct. Mol. Biol.* **14,** 710-715.
7. Liu, Z., J. Wu, and X. Yu. 2007. CCDC98 targets BRCA1 to DNA damage sites. *Nat. Struct. Mol. Biol.* **14,** 716-720.
8. Rouse, J. and S. P. Jackson. 2002. Interfaces between the detection, signaling, and repair of DNA damage. *Science* **297,** 547-551.
9. Small, T. W. and J. G. Pickering. 2009. Nuclear degradation of Wilms tumor 1-associating protein and survivin splice variant switching underlie IGF-1-mediated survival. *J. Biol. Chem.* **284**, 24684-24695.
10. Sobhian, B., G. Shao, D. R. Lilli, A. C. Culhane, L. A. Moreau, B. Xia, D. M. Livingston, and R. A. Greenberg. 2007. RAP80 targets BRCA1 to specific ubiquitin structures at DNA damage sites. *Science* **316**, 1198-1202.
11. Su, T. T. 2006. Cellular responses to DNA damage: one signal, multiple choices. *Annu. Rev. Genet.* **40**, 187-208.
12. Wang, B., S. Matsuoka, B. A. Ballif, D. Zhang, A. Smogorzewska, S. P. Gygi, and S. J. Elledge. 2007. Abraxas and RAP80 form a BRCA1 protein complex required for the DNA damage response. *Science* **316**, 1194-1198.
13. Yan, J., Y. S. Kim, X. P. Yang, L. P. Li, G. Liao, F. Xia, and A. M. Jetten. 2007. The ubiquitin-interacting motif containing protein RAP80 interacts with BRCA1 and functions in DNA damage repair response. *Cancer Res.* **67,** 6647-6656.

초록 : WTAP 단백질의 안정성을 통한 CCDC98 단백질의 cyclin B1 발현 조절

오윤정[1] · 이은희[1] · 이일규[2] · 김경수[2] · 김홍태[1]*
([1]성균관대학교 생명과학과, [2]가톨릭대학교 가정의학과)

CCDC98 단백질은 BRCA1-A 복합체를 DNA 손상 부위로 이동시킴으로써 DNA손상에 의하여 유도되는 G2/M cell cycle checkpoint에 중요한 역할을 한다고 알려져 있다. 하지만 많은 연구에도 불구하고 CCDC98 단백질의 기능에 대해서 알려진 바가 거의 없다. 본 연구는 CCDC98 단백질의 기능을 밝히고자 tandem affinity purification 방법을 수행하였다. 그 결과 Wilms tumor 1-associating protein (WTAP)을 CCDC98의 결합 단백질로 분리 동정하였다. 이들 단백질의 결합을 *in vivo* and *in vitro* binding assays를 통하여 확인하였다. 또한, CCDC98 단백질이 cyclin B1의 발현을 억제함을 확인하였는데, 이는 WTAP 단백질의 발현을 조절함으로써 이루어진다는 것을 확인하였다. 이는 CCDC98 단백질이 새로운 세포주기 조절자라는 것을 증명하는 결과이다.