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Title: Physical and functional interactions between ZIP kinase and UbcH5

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Running title: Interactions between ZIPK with UbcH5

Abstract

Zipper-interacting protein kinase (ZIPK) is a widely expressed serine/threonine kinase that has been implicated in cell death and transcriptional regulation, but its mechanism of regulation remains unknown. In our previous study, we showed that leukemia inhibitory factor stimulated threonine-265 phosphorylation of ZIPK, thereby leading to phosphorylation and activation of signal transducer and activator of transcription 3. Here, we identified UbcH5c as a novel ZIPK-binding partner by yeast two-hybrid screening. Importantly, we found that UbcH5c induced ubiquitination of ZIPK. Small-interfering RNA-mediated reduction of endogenous UbcH5 expression decreased ZIPK ubiquitination. Furthermore, coexpression of UbcH5c with ZIPK influenced promyelocytic leukemia protein nuclear body (PML-NB) formation. These results suggest that UbcH5 regulates ZIPK accumulation in PML-NBs by interacting with ZIPK and stimulating its ubiquitination.

Keywords: ZIPK, UbcH5, ubiquitination, PML nuclear body

Introduction

Zipper-interacting protein kinase (ZIPK) was originally identified as a binding partner of activating transcription factor 4 (ATF4), a member of the activating transcription factor/cyclic AMP-responsive element-binding family of transcription factor proteins [1,2]. ZIPK aggregates through its C-terminal leucine zipper (LZ) structure, thereby becoming an active enzyme. Ectopic expression of ZIPK in NIH 3T3 cells induced apoptosis, whereas a kinase-inactive mutant protein, ZIPK K42A, failed to induce apoptosis, indicating that ZIPK stimulates apoptosis via its catalytic activity [1]. Previous studies showed that ZIPK, in collaboration with Daxx and Par-4, induced apoptosis from promyelocytic leukemia protein nuclear bodies (PML-NBs) [3]. However, the mechanisms responsible for the activation of ZIPK and the downstream substrates that mediate its apoptotic activity remain unknown. Recently, we demonstrated that ZIPK specifically interacted with signal transducer and activator of transcription (STAT) 3, phosphorylated STAT3 on serine-727 and enhanced its transcriptional activity [4]. We further demonstrated that leukemia inhibitory factor (LIF) induced threonine-265 (Thr265) phosphorylation of ZIPK, which is critical for its kinase activation [5], suggesting that LIF signaling mediates ZIPK/STAT3

activation through phosphorylation of Thr265.

To investigate the regulatory mechanisms of ZIPK, we sought to identify ZIPK-interacting proteins by yeast two-hybrid screening with the kinase domain of ZIPK as bait.

We identified UbcH5c as a novel binding partner of ZIPK. UbcH5c is a ubiquitously expressed E2 ubiquitin conjugating enzyme that is closely related to UbcH5a and UbcH5b

[6]. Here, we show that UbcH5c acts as a ZIPK ubiquitin ligase and influences ZIPK accumulation in PML-NBs.

Materials and Methods

Reagents and antibodies. Expression vectors for epitope-tagged ZIPK, ZIPK kinase domain (KD) and ZIPK leucine zipper domain (LZ) were described previously [1,2]. Expression vectors for UbcH5a, UbcH5b, UbcH5c and ubiquitin (Ub)-unbound mutant CA [7] were kindly provided by Dr. K. Iwai (Osaka City University, Osaka, Japan). An anti-Myc antibody was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). An anti-FLAG M2 monoclonal antibody was purchased from Sigma (St. Louis, MO). An anti-ZIPK antibody was purchased from BIOMOL Research Laboratories (Plymouth, PA). An anti-UbcH5 antibody was purchased from Boston Biochem (Cambridge, MA). An anti-actin antibody was purchased from Chemicon International (Temecula, CA).

Yeast two-hybrid screening. Gal4-ZIPK was constructed by fusing the coding sequence for the kinase domain (amino acids 1-275) of mouse ZIPK in-frame with the Gal4 DNA-binding domain in the pGBKT7 vector (Clontech, Palo Alto, CA). *Saccharomyces cerevisiae* AH109 cells were transformed with pGal4-ZIPK and mated with Y187 cells containing a pretransformed mouse 11-day embryo MATCHMAKER cDNA library

(Clontech). Approximately 2.6×10^6 colonies were screened as previously described [4].

Plasmid DNAs derived from positive clones were extracted and sequenced.

Cell culture, transfection, siRNAs and RT-PCR. Human cervix carcinoma cell line HeLa and human embryonic kidney carcinoma cell line 293T cells were maintained in DMEM containing 10% FCS. 293T cells were transfected using a standard calcium precipitation protocol [8]. The siRNAs targeting human UbcH5a, UbcH5b and UbcH5c used in the present study were as follows: UbcH5a, 5'-CCAAAGAUUGCUUUCACAATT-3'; UbcH5b, 5'-CAGUGGUCUCCAGCACUATT-3'; and UbcH5c, 5'-UCAAGGCGGUGUAUUCUUUTT-3'. HeLa cells were transfected using jetPEI (PolyPlus-Transfection, Strasbourg, France) according to the manufacturer's instructions.

HeLa cells were plated on 24-well plates at 2×10^4 cells/well and incubated with a siRNA-Lipofectamine 2000 (Invitrogen, Carlsbad, CA) mixture at 37°C for 4 h, followed by the addition of fresh medium containing 10% FCS [9]. At 24 h after transfection, the cells were harvested and subjected to western blot analysis and RT-PCR.

Total RNAs were prepared using the TRI reagent (Sigma-Aldrich) and used for RT-PCR.

RT-PCR was performed using an RT-PCR High -Plus- Kit (TOYOBO, Tokyo, Japan).

The following primers were used for amplification: UbcH5a, 5'-AGCGCATATCAAGGTGGAGT-3' (forward) and 5'-GTCAGAGCTGGTGACCATTG-3' (reverse); UbcH5b, 5'-CAATAATGGGGCCAAATGAC-3' (forward) and 5'-GAGCCTTTTCTTTCCCATCC-3' (reverse); UbcH5c, 5'-CCAGACGACAAGCACACACT-3' (forward) and 5'-TGGGTTTGGATCACATAGCA-3' (reverse).

Immunoprecipitation, immunoblotting and in vivo ubiquitination. Immunoprecipitation and immunoblotting were performed as described previously [8]. The cells were harvested and lysed in lysis buffer (50 mM Tris-HCl pH 7.4, 0.15 M NaCl, 1% NP-40, 1 mM sodium orthovanadate, 1 mM phenylmethylsulfonyl fluoride and 10 µg/ml each of aprotinin, pepstatin and leupeptin). Immunoprecipitates from the cell lysates were resolved by SDS-PAGE, transferred to Immobilon filters (Millipore, Bedford, MA) and immunoblotted with relevant antibodies. Immunoreactive proteins were visualized using an enhanced chemiluminescence detection system (Millipore). For *in vivo* ubiquitination assays of ZIPK, His-tagged proteins were purified as previously described [10] and analyzed by immunoblotting with anti-Myc and anti-ZIPK antibodies.

Indirect immunofluorescence microscopy. HeLa cells (5×10^4) seeded on glass plates were transfected using Metafectene (Biontex Laboratories GmbH, München, Germany). At 48 h after transfection, the cells were fixed with 4% paraformaldehyde and incubated with appropriate antibodies. The cells were then incubated with FITC-conjugated anti-rabbit IgG (Chemicon International) and observed under a confocal laser fluorescence microscope [11]. Images were obtained using a Carl Zeiss LSM 510 laser scanning microscope (Thornwood, NY) equipped with an Apochromat x63/1.4 oil immersion objective and x4 zoom.

Results and Discussion

Molecular interactions between ZIPK and UbcH5c

We performed yeast two-hybrid screening of a mouse embryo cDNA library using the kinase domain of ZIPK (amino acids 1-275) as bait. From a screen of about 2.6×10^6 transformants, we identified several positive clones. Sequence analyses revealed that one of these clones encoded the entire UbcH5c protein (amino acids 1-147). We first examined whether UbcH5c binds to ZIPK in mammalian cells. 293T cells were transfected with His-tagged UbcH5c WT or CA together with Myc-tagged ZIPK. Western blot analysis of immunoprecipitates using an anti-His antibody revealed that ZIPK interacted with both UbcH5c WT and CA, suggesting that UbcH5c binds to ZIPK independently of ubiquitin in 293T cells (Fig. 1A). To further delineate the domains in ZIPK required for the interaction with UbcH5c in 293T cells, we used two sets of mutant ZIPK proteins (ZIPK KD and LZ). 293T cells were transiently transfected with His-tagged UbcH5c and Myc-tagged ZIPK KD or LZ. As shown in Fig. 1B and C, the N-terminal kinase domain of ZIPK (ZIPK KD), but not the C-terminal leucine zipper domain (ZIPK LZ), interacted with UbcH5c, consistent

with the finding that ZIPK KD interacts with UbcH5c in yeast. We further examined whether ZIPK interacted with UbcH5a and UbcH5b, which are closely related to UbcH5c, in 293T cells. His-tagged UbcH5a, UbcH5b or UbcH5c together with Myc-tagged ZIPK were transiently expressed in 293T cells. Immunoprecipitation using an anti-His antibody revealed that ZIPK interacted with all three UbcH5 proteins in 293T cells (Fig. 1D).

UbcH5c promotes ZIPK ubiquitination

Next, we examined whether ZIPK is a target of UbcH5c-mediated ubiquitination. 293T cells were transfected with FLAG-tagged ZIPK and/or His-tagged UbcH5c WT or CA together with His-tagged ubiquitin. After preparation of total cell lysates, ubiquitinated proteins were purified on Ni-NTA beads and subjected to immunoblot analysis using an anti-FLAG antibody. As shown in Fig. 2A, ZIPK was slightly ubiquitinated *in vivo* when coexpressed with ubiquitin. Importantly, UbcH5c WT, but not CA, markedly enhanced ZIPK ubiquitination. We further examined the domains in ZIPK required for ubiquitination by UbcH5c in 293T cells, and used the ZIPK KD and LZ mutants. 293T cells were transiently transfected with His-tagged UbcH5c, Ub and Myc-tagged ZIPK KD or LZ. As

shown in Fig. 2B, ZIPK KD was ubiquitinated by UbcH5c to a greater extent than ZIPK LZ, suggesting that the major sites in ZIPK for UbcH5c-mediated ubiquitination may exist within the kinase domain. Furthermore, overexpression of UbcH5c WT together with ubiquitin promoted endogenous ZIPK ubiquitination in 293T cells (Fig. 2C). Since ubiquitinated proteins are often degraded through a proteasome-dependent pathway, we investigated the potential for UbcH5c to affect the steady-state level of ZIPK protein expression. However, we did not observe any significant alterations in the ZIPK protein contents (Fig. 2C).

Reduction of endogenous UbcH5 reduces ZIPK ubiquitination in HeLa cells

To further explore whether UbcH5c ubiquitinates ZIPK *in vivo*, we used siRNAs to reduce the endogenous expression of UbcH5 in HeLa cells. Specific siRNAs for UbcH5a, UbcH5b and UbcH5c or a control siRNA were transfected into HeLa cells. Total cell lysate, or RNA isolated from the transfected cells was subjected to western blot analysis and RT-PCR, which confirmed reductions in the respective UbcH5 mRNA expression levels. As shown in Fig. 3A (lower panels), an approximately 70% reduction in the UbcH5 protein

contents was observed in HeLa cells following combined expression of the UbcH5 siRNAs.

We then determined the effects of the UbcH5 siRNAs on ZIPK ubiquitination in HeLa cells. As shown in Fig. 3A (upper panel), siRNA-mediated reduced expression of UbcH5 resulted in a significant reduction of ZIPK ubiquitination, indicating that endogenous UbcH5 mediates ZIPK ubiquitination in HeLa cells.

ZIPK ubiquitination influences PML-NB formation within the nucleus

Protein ubiquitination was originally discovered as a signal for proteasomal degradation. Subsequently, however, ubiquitination has also been shown to play important roles in receptor trafficking, immune responses, transcriptional regulation and other cellular processes. In these cellular events, ubiquitination plays important roles by altering the subcellular localizations of target proteins [12]. Therefore, we tested the effect of UbcH5c on the subcellular localization of ZIPK. ZIPK has been shown to localize in the nucleus, especially in PML-NBs. HeLa cells were transiently transfected with FLAG-tagged ZIPK with or without UbcH5c and ubiquitin, and then incubated with an anti-FLAG antibody. ZIPK was localized in a diffuse nuclear pattern in approximately 70% of the transfected

cells (Fig. 3B-I), and a speckled nuclear pattern in approximately 30% of the transfected cells. The latter pattern of ZIPK localization, which is characteristic of PML-NBs, was categorized into two types: 60-100 smaller dot-like structures per nucleus (Fig. 3B-II) and 10-20 larger dot-like structures per nucleus (Fig. 3B-III). PML-NBs are known to aggregate with other NB proteins, such as Daxx and ZIPK, and promote the growth of concentrated PML networks during their maturation process [13]. Therefore, maturation of PML-NBs seems to be determined by their volume, suggesting that the smaller dot-like structures represent an early stage of PML-NB formation. Importantly, coexpression of ZIPK with UbcH5c and ubiquitin enhanced the accumulation of PML-NBs (Fig. 3C-II and -III) in the nucleus compared with expression of ZIPK alone. These results suggest that ZIPK ubiquitination influences PML-NB formation in the nucleus.

Concluding remarks

The present study provides evidence that UbcH5c acts as a ZIPK ubiquitin ligase and influences ZIPK accumulation in PML-NBs. We have also demonstrated that the major ubiquitination sites are located in the N-terminal kinase domain of ZIPK. Ubiquitin is

covalently linked to lysine residues of target proteins. The kinase domain of ZIPK possesses many lysine residues (18 residues in humans; 17 residues in mice). Although we could not determine the precise sites for UbcH5-mediated ubiquitination in ZIPK, their identification will clarify their roles in other ZIPK functions, such as alterations in its localization and kinase activity. Moreover, as shown in Fig. 2A and C, ZIPK ubiquitination by UbcH5c did not promote its proteasomal degradation, although treatment with a proteasome inhibitor, MG132, slightly enhanced the ubiquitination of ZIPK by UbcH5c (data not shown), suggesting that ubiquitination of the ZIPK protein may be partly involved in its degradation.

The kinase domain of ZIPK shows strong homology to that of death-associated protein kinase (DAPK). ZIPK and DAPK are members of a family of related kinases that includes DAPK2/DRP-1, DRAK1 and DRAK2 [14], all of which are implicated in executing apoptosis. Importantly, evidence that the gene encoding DAPK may function as a tumor suppressor has also been presented [15, 16]. Recently, it has been shown that DRAK2 is specifically expressed by lymphocytes and that DRAK2-deficient mice do not show any defects in apoptosis, although DRAK2-deficient T cells are hypersensitive to stimulation

through T-cell receptors [23]. Furthermore, DRAK2-deficient mice are resistant to experimental autoimmune encephalomyelitis but respond normally to infection with lymphocytic choriomeningitis virus. Therefore, DAPK family kinases may play important roles in immune regulation. Indeed, ZIPK phosphorylates an immune disease-related IL-6 signal transducer, STAT3, and regulates its activation. Although we do not have any evidence regarding the involvement of ZIPK in immune regulation, it is possible that ZIPK could be a target candidate for the treatment of autoimmune diseases.

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Figure legends

Fig. 1. Molecular interactions between ZIPK and UbcH5c

A. 293T cells in 10-cm dishes were transfected with Myc-tagged ZIPK (10 μ g) together with His-tagged UbcH5c WT or CA (10 μ g). The cells were lysed at 48 h after transfection, immunoprecipitated with an anti-His antibody and immunoblotted with anti-Myc (upper panel) and anti-His (middle panel) antibodies. Total cell lysates (TCL; 1%) were blotted with the anti-Myc antibody (bottom panel).

B. Schematic diagrams of the domain structures of ZIPK and Myc-tagged mutant fragments.

C. 293T cells in 10-cm dishes were transfected with His-tagged UbcH5c WT (10 μ g) with or without Myc-tagged ZIPK FL, KD or LZ (10 μ g). The cells were lysed at 48 h after transfection, immunoprecipitated with an anti-Myc antibody and immunoblotted with anti-His (upper panel) and anti-Myc (middle panel) antibodies. TCL (1%) were blotted with the anti-His antibody (bottom panel).

D. 293T cells in 10-cm dishes were transfected with Myc-tagged ZIPK (10 μ g) together

with His-tagged UbcH5a, UbcH5b or UbcH5c (10 μ g). The cells were lysed at 48 h after transfection, immunoprecipitated with an anti-His antibody and immunoblotted with anti-Myc (upper panel) and anti-His (middle panel) antibodies. TCL (1%) were blotted with the anti-Myc antibody (bottom panel).

Fig. 2. UbcH5c promotes ZIPK ubiquitination

A. 293T cells in 6-cm dishes were transfected with FLAG-tagged ZIPK (1.0 μ g) with or without His-tagged UbcH5c WT or CA (1.0 μ g) and/or His-tagged Ub (0.5 μ g). The cells were lysed at 48 h after transfection, and total cell lysates (TCL; 1%) were immunoblotted with anti-FLAG (upper panel) and anti-His (lower panel) antibodies.

B. 293T cells in 10-cm dishes were transfected with or without His-tagged UbcH5c WT (10 μ g) with or without Myc-tagged ZIPK FL, KD or LZ (10 μ g), and lysed at 48 h after transfection. His-tagged proteins were purified using Ni-NTA beads and immunoblotted with an anti-Myc antibody (upper panel). TCL (1%) were blotted with anti-Myc and anti-His antibodies (lower panels). NS, non-specific band

C. 293T cells in 10-cm dishes were transfected with His-tagged Ub (5 μ g) with or

without His-tagged UbcH5c WT (10 μ g), and lysed at 48 h after transfection. His-tagged proteins were purified using Ni-NTA beads and immunoblotted with an anti-ZIPK antibody (upper panel). Total cell lysates (TCL; 1%) were also blotted with the anti-ZIPK antibody (lower panel).

Fig. 3. Reduction of endogenous UbcH5 reduces ZIPK ubiquitination in HeLa cells

A. HeLa cells in 24-well plates were transfected with a control siRNA or siRNAs targeting UbcH5a, UbcH5b and UbcH5c as indicated using Lipofectamine 2000. The cells were then transfected with FLAG-tagged ZIPK (0.1 μ g) and His-tagged Ub (0.5 μ g) using JetPEI. The UbcH5 expression levels were quantified by western blot analysis and RT-PCR. The cells were lysed at 36 h after transfection, and total cell lysates (TCL; 1%) were blotted with anti-FLAG (upper panel), anti-UbcH5 (middle panel) and anti-actin (bottom panel) antibodies.

B. HeLa cells in 6-well plates were transfected with FLAG-tagged ZIPK (1.0 μ g) together with His-tagged UbcH5c (1.0 μ g) or Ub (0.5 μ g). The cells were fixed and incubated with an anti-FLAG antibody, following by FITC-conjugated anti-rabbit IgG.

The nuclear localization of ZIPK was observed by indirect immunofluorescence microscopy. Three typical features of ZIPK staining in the nucleus are shown (I, II and III).

C. Approximately 100 cells were quantified according the FITC signals in dotted structures in the nucleus. The results represent the means of three individual experiments, and the error bars represent the SD. *, $p < 0.05$; **, $p < 0.01$.

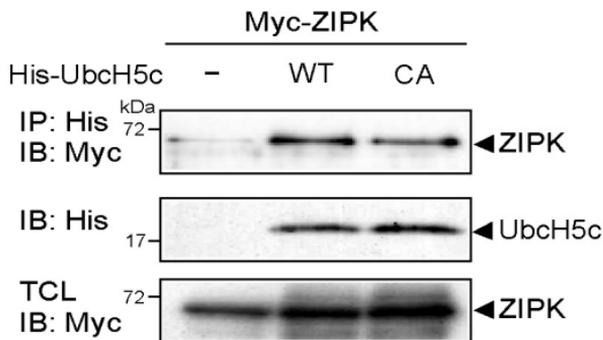
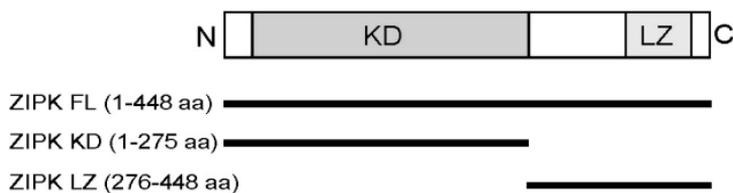
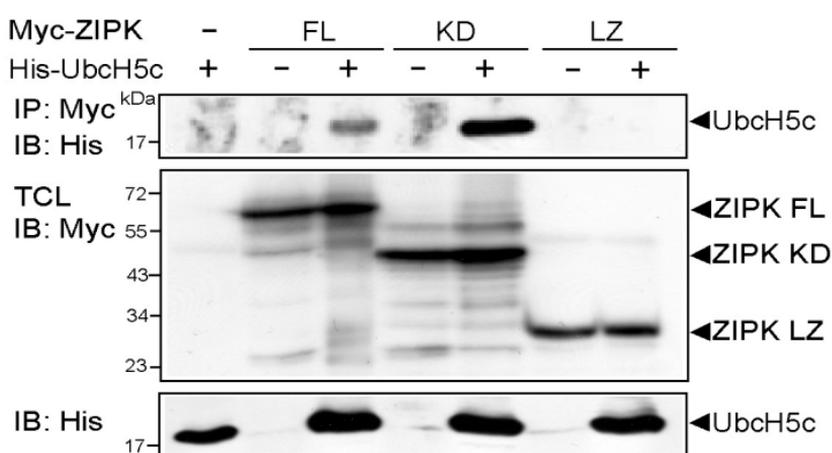
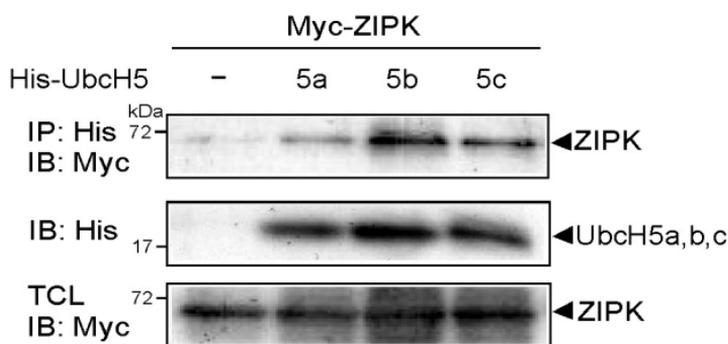
A**B****C****D**

Figure 1

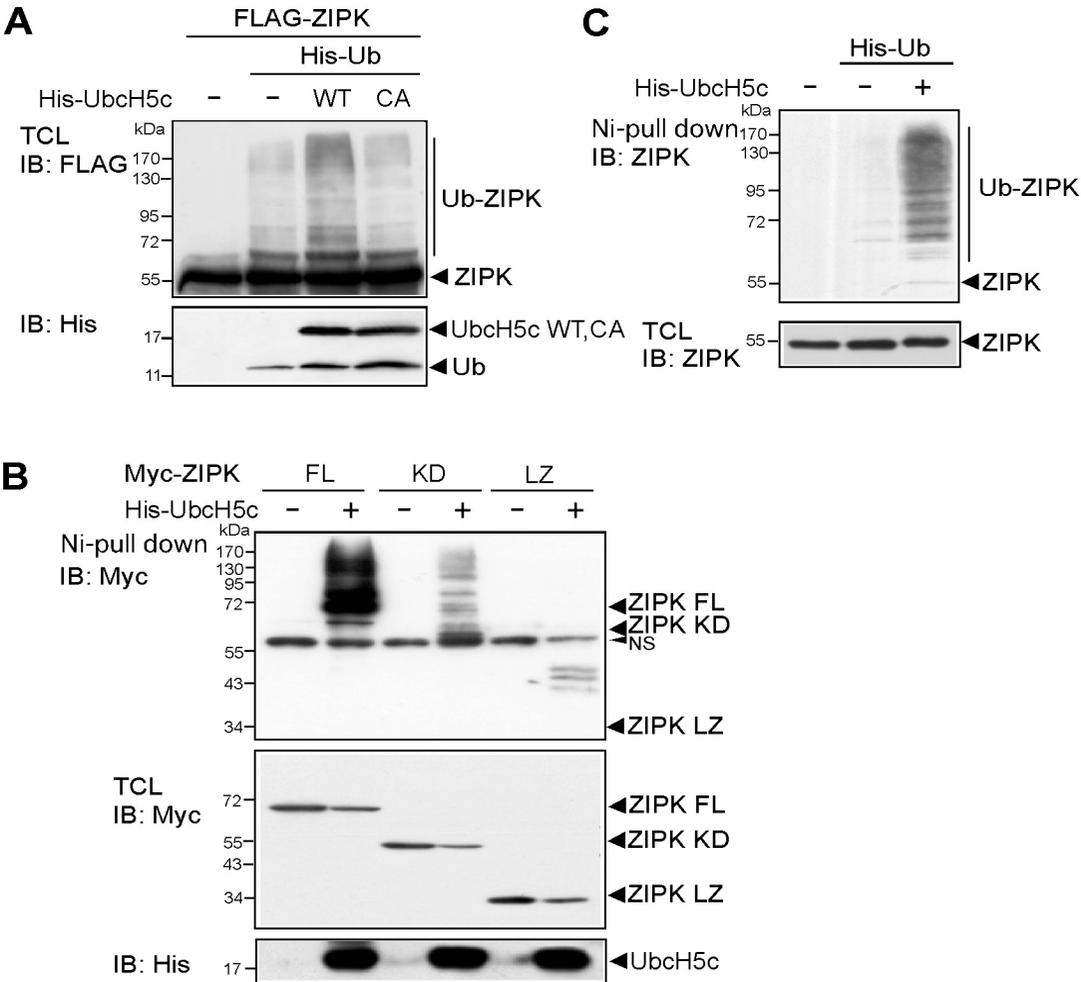
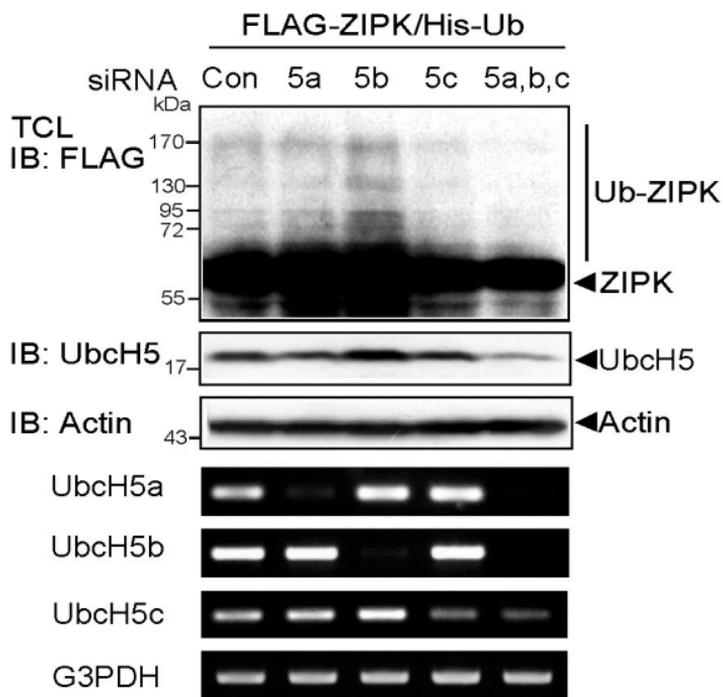
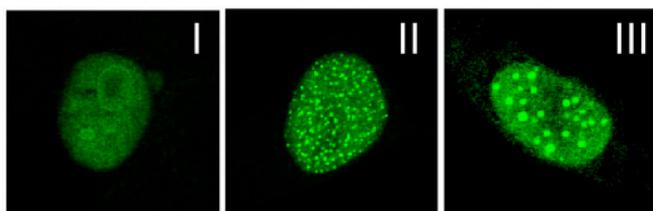
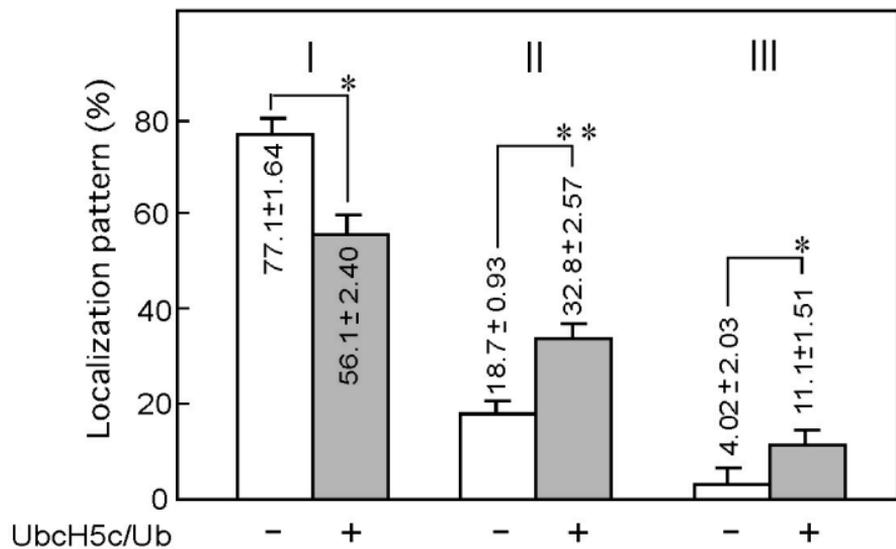


Figure 2

A**B****C****Figure 3**