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βTrCP-mediated ubiquitylation regulates protein stability of Mis18β in a cell cycle-dependent manner

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ABSTRACT

Ubiquitin E3 ligases including SCF complex are key regulators of cell cycle. Here, we show that Mis18β, a component of Mis18 complex governing CENP-A localization, is a new substrate of βTrCP-containing SCF complex. βTrCP interacted with Mis18β exclusively during interphase but not during mitosis and mediated proteasomal degradation of Mis18β leading to the inactivation of Mis18 complex during interphase. In addition, uncontrolled stabilization of Mis18β caused cell death. Together, we propose that βTrCP-mediated regulation of Mis18β stability is a mechanism to restrict centromere function of Mis18 complex from late mitosis to early G1 phase.

1. Introduction

Cell cycle is tightly regulated by various protein complexes which repress or activate specific proteins such as CDKs (cyclin-dependent kinases) [1]. If this regulation fails, the cells are either arrested to specific phase of cell cycle or undergo uncontrolled proliferation which might cause cancer [2,3]. Cell cycle progression is mostly achieved by changing the stability of regulatory proteins through ubiquitin-dependent proteasomal degradation [4–6]. Deregulation of protein stability responsible for the transition of cell cycle phase leads to cell cycle arrest and apoptotic cell death [7,8]. In line with this regulation, it is obvious that many proteins for phase transition in cell cycle turned out to be tumor suppressors [9].

Cullin-based E3 ligases belong to the RING-H2 E3 ligase subfamily and recognize various substrates involved in cell cycle regulation by changing a variable, substrate-recognizing component in the ligase complex [10]. Among Cullin-based complexes, SCF complexes (Skp1/Cul1/F-box protein) are well-characterized, and F-box proteins determine its substrate specificity [11]. As an F-box protein, Skp2 targets CDK inhibitors such as p27 and p21, and promotes CDK activity during G1/S transition [12]. Another F-box protein, βTrCP plays a role in controlling G2/M transition by recognizing a CDK1 inhibitor, WEE1, and in regulating APC/C activity through an APC inhibitor, EMI1 [7]. As these F-box proteins usually target tumor suppressors, verification of substrates is important for cancer study.

The regulation of chromosome segregation during mitosis is another layer of cell cycle regulation. There are fine tuning mechanisms governing centromere function and CENP-A localization [13,14]. Recently, we reported that Mis18α, a component of the Mis18 complex (Mis18α/Mis18β/Mis18BP1), participates in maintaining DNA methylation pattern on centromere region at the specific stage of cell cycle, from late mitosis to early G1, by recruiting DNMT3A/3B, which is critical for centromeric localization of newly synthesized CENP-A [15]. Although we elucidated underlying functional mechanism of Mis18 complex in centromere, it is not clear how the function of Mis18 complex in centromere is restricted to specific cell cycle phases, particularly from late mitosis to early G1 [16]. Recent report showed that massive phosphorylation of Mis18BP1 by CDK1/CDK2 during interphase blocked centromere localization of Mis18 complex, suggesting that it might be one mechanism to keep Mis18 complex out of centromere [17].

In this study, we figured out the functional mechanisms of mitosis-specific regulation of Mis18β. We found that the protein stability of Mis18β was increased in mitosis but, significantly diminished during interphase. Further, we also demonstrated that $SCF^{\beta TrCP}$ complex functioned as an ubiquitin E3 ligase mediating proteasomal degradation of Mis18β during interphase. Finally, we elucidated that the regulation of protein level of Mis18β was crucial for proper cell growth.

Abbreviations: SCF, Skp1/Cul1/F-box protein complex; CDK, cyclin-dependent kinase; CHX, cycloheximide; H3S10ph, phosphorylation at 10th serine residue of histone H3; aa, amino acid.

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2. Materials and methods

2.1. Plasmid construction

Deletion mutants of Mis18α, D1 (38–204 aa) and D2 (119–204 aa) were described in previous report [15]. Mis18α-CA mutants (Mis18α-CA1, Mis18α-CA2, and Mis18α-CA3) were generated by site-directed mutagenesis using nPfu-Forte DNA polymerase (Enzynomics, South Korea) and CA1, 3 mutant was generated by repeating mutagenesis step for CA3 using CA1 as a template. All mutants were verified by sequencing.

2.2. Immunocytochemistry and TUNEL assay

The cells grown on coverslips were washed three times with PBS and then fixed with 2% paraformaldehyde in PBS for 10 min at room temperature. Fixed cells were permeabilized with 0.5% Triton X-100 in PBS (PBS-T) for 10 min at room temperature. Blocking was performed with 10% bovine serum in PBS-T for 30 min. For staining, cells were incubated with anti-Flag M2 IgG (Sigma–Aldrich, USA) overnight at 4 °C, followed by incubation with fluorescent labeled secondary antibodies for 1 h at room temperature (Invitrogen, USA). The TUNEL assay was performed according to the manufacturer's instructions (Roche, Germany).

2.3. Ubiquitylation assay

Ubiquitylation assay was performed as described previously [18]. Briefly, cells were transfected with a combination of plasmids including plasmid expressing HisMax-ubiquitin. After incubation, cells were treated with 10 μM MG132 for 6 h, lysed and incubated with Ni^{2+}-NTA beads (QIAGEN, Germany). After washing the beads, bound proteins were eluted by 200 mM imidazole and subject to immunoblot analysis.

2.4. RNA interference by siRNA and shRNA

For knock-down of specific genes, cells were transfected with siRNA or shRNA as indicated in figures and harvested for experiments 72 h after transfection. The target sequence of siRNA or shRNA against endogenous βTrCP or Mis18α are as follows: shMis18α (human), 5′-GGAACAGAAGCUAUCCAAA-3′; siβTrCP (human), 5′-GUGGAAUUUGUGGAACAUC-3′ [19].

2.5. Cell cycle synchronization

HeLa cells stably expressing either Flag-Mis18α or Flag-Mis18β were synchronized at G1/S phase and released as described previously [15]. For G2/M arrest, cells were incubated with media containing 0.4 μg/ml nocodazole for 12 h and harvested for experiment.

2.6. Statistical analysis

Statistical differences in test and control samples were determined by Student's *t*-test using the Statview package (Abacus Concepts, Inc., USA).

3. Results

3.1. The protein stability of Mis18β is regulated in a cell cycle-dependent manner by ubiquitin–proteasome system

Although we reported that centromeric deposition of CENP-A was regulated by Mis18/DNMT3A/3B complex during mitosis [15], the reason why Mis18 complex could regulate centromere function only during mitosis is still questionable. Given that Mis18 complex does not function during interphase, we investigated the protein levels of Mis18α and Mis18β during cell cycle. Cells were synchronized using double thymidine block, collected in specific stages of cell cycle depending on release time, and then protein levels of Mis18α and Mis18β were measured from cell extracts. Interestingly, we found that Mis18β level was increased during mitosis, overlapping with phosphorylation of H3S10 (Fig. 1A), while Mis18α protein level remained unchanged during cell cycle (Fig. S1A). We detected increased protein level of Mis18β again when the cells were synchronized in mitosis by treating with nocodazole compared to asynchronous cells (Fig. 1B). Based on these results, we hypothesized that the fluctuation of Mis18β protein level during cell cycle might be due to the regulation of Mis18β stability. To prove this, we first examined the stability of Mis18β by treating cells with cycloheximide (CHX), an inhibitor of protein synthesis, and examined protein turnover rates. Mis18β degraded rapidly for short time after CHX treatment (within 2 h) (Fig. 1C). The stability of Mis18β was significantly increased after blocking proteasome activity by MG132 treatment (Fig. 1D), suggesting that proteasomal degradation is responsible for regulation of Mis18β stability. In addition, we found that ubiquitylation of Mis18β was significantly increased in the cells without mitotic arrest, whereas the ubiquitylation of Mis18β was decreased in mitotic cells (Fig. 1E). Together, these findings suggest that the protein level of Mis18β remained low during interphase through ubiquitylation-dependent proteasomal degradation.

3.2. $SCF^{\beta TrCP}$ is an E3 ligase complex targeting Mis18β

Next, we screened for candidate ubiquitin ligases which are responsible for the regulation of Mis18β stability. Since

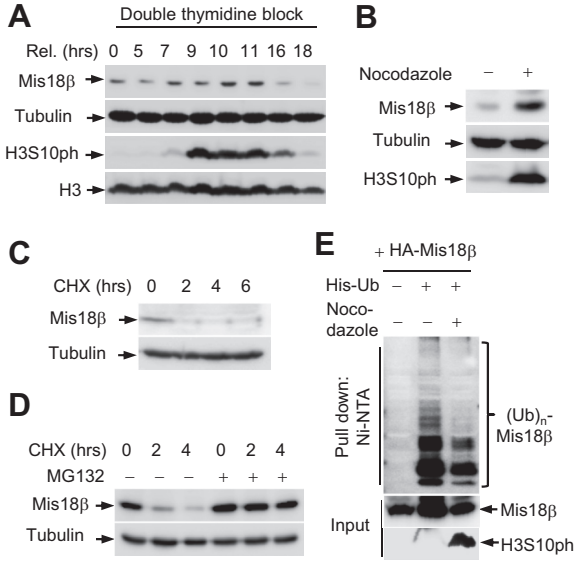

Fig. 1. Cell cycle-dependent, ubiquitin-mediated degradation of Mis18β. (A) HeLa cells stably expressing Flag-Mis18β were synchronized by double thymidine block, released, and harvested at indicated time points. Mitosis was determined by the level of H3S10ph. (B) The protein level of Mis18β was measured from asynchronous or nocodazole-arrested mitotic HeLa cells stably expressing Flag-Mis18β. (C) Cycloheximide (CHX) treated HeLa cells were collected at indicated time points and protein level of Flag-Mis18β was measured. (D) Mis18β stability was measured as in (C) in the absence or presence of proteasome inhibitor, MG132. (E) HeLa cells were transfected with plasmids expressing HA-Mis18β and HisMax-Ubiquitin. After 36 h of transfection, cells were treated with MG132 for 6 h. Ubiquitylated proteins were pulled down with Ni^{2+}-NTA beads and Mis18β was visualized by immunoblotting with anti-Flag antibody. Mitotic arrest was obtained by nocodazole treatment and confirmed by detecting H3S10ph.