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NuSAP is degraded by APC/C–Cdh1 and its overexpression results in mitotic arrest dependent of its microtubules' affinity

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Abstract

Microtubule associated proteins are involved in regulation of microtubule dynamics. Its mutation and dysregulation result in severe consequences such as mitotic block and apoptosis. NuSAP has been reported as a microtubule associated protein, depletion of which by RNAi results in spindle deficiency and cytokinesis failure. However, its role in regulation of cell cycle and how NuSAP protein is controlled during cell cycle progression still remains unclear. Here we show that NuSAP can be ubiquitinated and degraded by APC/C–hCdh1 E3 ligase. Evolutionally conserved KEN box functions as the degron of NuSAP. Overexpression of NuSAP induces mitotic arrest and the microtubule associated domain and nuclear localization are both required for NuSAP to induce mitotic arrest. Furthermore, overexpression of NuSAP results in cells accumulation with microtubule bundling and spindle deficiency. Thus, our results give evidence for the first time that NuSAP protein level is tightly regulated by the APC/C ubiquitin ligase complex and NuSAP induces mitotic arrest dependent of its microtubule affinity.

Keywords: NuSAP; APC/C; Mitotic arrest; Degradation

1. Introduction

The ubiquitin–proteasome system plays important roles in diverse cellular processes, such as cell cycle regulation, signaling transduction, and apoptosis [1,2]. This system requires three enzymes, a ubiquitin activating enzyme (E1), a conjugating enzyme (E2), and a ubiquitin ligase (E3). Ubiquitin is activated by E1; the activated ubiquitin is then transferred to E2 and finally ligated to a substrate via E3. Finally, the polyubiquitinated protein is degraded by the proteasome [3].

The anaphase promoting complex/cyclosome (APC/C), a multisubunit E3 ubiquitin ligase, plays a central role in regulating cell cycle progression from anaphase to G1 by targeting a large group of cell cycle regulators for degradation, including Aurora A, TPX2, Plk1 and Cdc20 [4,5]. Activation of APC/C requires mitotic specific phosphorylation of APC/C subunits and binding of Fizzy family proteins, Cdc20 and Cdh1. Cdc20 directly binds and activates APC/C at the onset of anaphase, whereas Cdh1 replaces Cdc20 at late mitosis until late G1. APC/C–Cdh20 is required for the degradation of the anaphase inhibitors such as securins to trigger sister chromatid separation [6]. APC/C–Cdh1 is responsible for the degradation of Cdc20 to restrict APC/C–Cdc20 activity to a narrow window [7,8]. In contrast, Cdh1 levels remain constant; however, phosphorylation of Cdh1 by Cdc2 inhibits the association of Cdh1 with APC/C, therefore preventing activation of APC/C–Cdh1 until late of anaphase [9,10]. APC/C targets an array of

Abbreviations: APC/C, anaphase promoting complex/cyclosome; MT, Microtubule; NuSAP, nucleolar and spindle-associated protein; RNAi, RNA interference.

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substrates by recognition of either a KEN box [KENxxx (DQEN)] or a destruction box (D box, RxxLxxxxN) in substrate [11]. The D box can be recognized by either APC/C–Cdh1 or APC/C–Cdc20, whereas substrates only containing the KEN box are ubiquitinated only by APC/C–Cdh1 [12]. Ubiquitin and proteasome-dependent degradation is one of key pathways to ensure proper progression of the cell cycle.

Microtubules (MTs) play an important role during mitosis, including chromosome capture, congression, and segregation [13–15]. In order to accomplish cytokinesis successfully, accurate regulation of microtubules dynamics is required. Several reports indicate proper regulation of MTs affinity of microtubule associated proteins is an important task during mitosis, while its dysregulation and mutation often result in mitotic arrest and apoptosis. For example, overexpression of TPX2 variant results in accumulation of mitotic cells with monopolar spindle [16]; overexpression of Stathmin in HeLa cells is associated with collapse of microtubule networks and Golgi fragmentation [17].

Organization of the spindle around chromatin involves the assembly of MTs, their attachment to the chromosome and their organization into a bipolar array. RanGTP plays an important role in the regulation of spindle self-organization. RanGTP is generated at chromatin and activates a series of Ran-targeted spindle regulators such as TPX2, NuMA and NuSAP [18–21]. Nucleolar and spindle-associated protein (NuSAP) was recently identified as a mitotic microtubule associated protein, which showed selective expression and localization during cell cycle [20]). Its mRNA and protein levels peak at the transition of G2 to mitosis and decline after cell division. NuSAP localized in nucleolus in interphase cells, and translocates mainly to spindle during mitosis [20]. Recent reports suggested that NuSAP was a mitotic RanGTPase target that stabilizes and cross-links microtubules [21]. Importin (Imp)α, Impβ and Imp7 bind and inhibit the ability of NuSAP to stabilize and cross-link MTs [21]. During mitosis, NuSAP is dissociated with the importins and immobilized on chromatin to guide MT attachment to the chromosomes and produced high concentrations of MTs in the vicinity of chromatin [22]. Both excessive amount and knockdown of NuSAP lead to disruption of cell division [20,21]. Thus, NuSAP must be tightly controlled during cell cycle progression. However, how NuSAP protein is controlled and the precise role of NuSAP in regulation of cell cycle still remains unclear.

Here we demonstrate that NuSAP is a novel substrate of APC/C–Cdh1 ubiquitin E3 ligase complex. Overexpression of NuSAP results in mitotic arrest in several cell lines dependent of its MTs' affinity. Our results also reveal a p53-independent pathway which is activated to prevent polyploidy in NuSAP-induced mitotic arrest.

2. Materials and methods

2.1. Plasmid construction

Plasmids of NuSAP and its deletion mutants were constructed by PCR. The PCR products were digested with *Sal*I/*Sac*I and inserted in pEGFP-N1 (Clontech). PCR products of full-length NuSAP were digested with *Bam*HI/*Xho*Iand inserted in pcDNA3.1 (Invitrogen). Plasmids which encode hCdh1 and Ub–HA were kindly presented by Drs. Michael Brandeis [23] and Yue Xiong, respectively [24].

2.2. Cell culture and treatments

The human colon adenocarcinoma cell lines HCT116 (*p53*-wild type and *p53*-knockout) were maintained in F12 medium (Hyclone). Human embryonic kidney epithelial cell lines HEK-293, HEK293T and human breast cancer cell line MCF7 were maintained in Dulbecco's Modified Eagle's Medium (Hyclone). All cells were supplemented with 10% fetal bovine serum (FBS; Hyclone), penicillin (50 U/ml), and streptomycin (50 μg/ml) (Hyclone) and grown at 37 °C in a humidified atmosphere containing 5% CO_2. Caffeine (Aldrich) was added to the medium at the concentration of 1 mM or 2 mM 4 h prior to transfection as described previously [25]. COS7 cells were incubated in the medium supplemented with 2 mM thymidine (dissolved in sterile water) for 24 h [26].

2.3. Cell cycle distribution analysis

For each cell line, 2×10^5 cells were seeded per well in 6-well plates. After 18 h of incubation, cells were transfected with pEGFP-N1-NuSAP or pEGFP-N1. Attached cells were harvested by trypsinized treatment and pooled with detached cells from the growth medium. Cells were pelleted by centrifugation at 2000 rpm for 5 min, washed twice with PBS, and resuspended in 300 μl PBS (containing 30 μl FBS) and fixed in 2 ml ice cold 70% ethanol. Samples were kept at −20 °C until analysis by flow cytometry. Cells were centrifuged at 2000 rpm for 5 min and washed with PBS, treated with 0.1 mg/ml RNase A (Pharmacia) for 30 min and stained with 40 μg/ml Propidium Iodide (Sigma). DNA content was analyzed by FACS Calibur (B-D USA) [27].

2.4. Confocal microscopy, immuno-fluorescence analysis

For immuno-staining of endogenous tubulin, HEK-293T cells transfected with NuSAP–GFP were fixed in 4% PFA (paraformaldehyde) for 10 min. Images of endogenous tubulin during mitosis and interphase were primarily made using anti-tubulin antibody in cells that were fixed in 0.1% PBST (containing 0.5% Triton X-100) for 15 min. Further processing included incubating cells in 5% BSA for 10 min before incubations with primary for 3 h at 37 °C and with secondary antibody for 1 h at room temperature. Secondary antibodies were conjugated to TRITC (Molecular Probes, Inc.). Cells were analyzed in PBS when the nucleus was stained with DAPI. Images of fixed cells were acquired on a confocal microscope using LaserSharp software [28].

2.5. Western blot analysis

Cells were washed in PBS and lysed in A™ lysis buffer (50 mM Tris–Cl, (pH 7.5); NaCl, 150 mM; Tween −20, 1% (v/v); NP40, 0.2%; glycerol, 10%) with freshly added protease inhibitor cocktail tablet (1 ml, Roche), 1 mM NaF and 0.1 mg/ml phenylmethylsulphonyl fluoride (PMSF) as described previously [29]. Samples were separated by 12% SDS-PAGE, transferred onto poly-vinylidene fluoride membranes (Millipore) and blotted with anti-p53 (DO-1, Santa Cruz), anti-phospho Histone H3 (Ser10, Cell Signaling Technology), anti-c-Myc (Clontech), anti-GFP (Cell Signaling Technology) or anti-actin (I-19, Santa Cruz). The immuno-stained bands were visualized using Super-Signal (Pierce) Western blotting detection system [29].

2.6. In vivo ubiquitination assay

HEK293T cells in 9-cm plates were transfected with combinations of 1 μg of green fluorescent protein (GFP) expression plasmid, 5 μg of HA–ubiquitin expression plasmid, 1 μg of human NuSAP, and 3 μg of Hcdh1. Thirty-six hours after transfection, cells from each plate were collected and divided into two aliquots. One aliquot (10%) was used for conventional Western blotting to confirm expression and degradation of transfected proteins. The remaining cells (90%) were used for purification of myc-tagged proteins by protein A/G beads. The cell pellet was lysed in cell lysis buffer (6 M guanidinium–HCl, 0.1 M