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The role of nucleophosmin in centrosome duplication

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In higher animal cells, duplication of centrosomes is triggered by CDK2/cyclin E-mediated phosphorylation. Nucleophosmin (NPM)/B23, a multifunctional protein, has recently been identified as one of the substrates of CDK2/cyclin E in centrosome duplication. Centrosome-bound NPM/B23 dissociates from centrosome upon phosphorylation by CDK2/cyclin E, which in turn triggers initiation of centriole duplication. Duplicated centrosomes remain free of NPM/B23 till mitosis. When the nuclear membrane breaks down during mitosis, NPM/B23 re-localizes to centrosomes. Upon cytokinesis, each daughter cell receives one centrosome bound by NPM/B23, which again dissociates from the centrosome upon exposure to CDK2/cyclin E at mid–late G1 phase of the next cell cycle. Thus, NPM/B23 would constitute one of the licensing systems for centrosome duplication, ensuring the coordination of centrosome and DNA duplication, which limiting duplication once per cell cycle.
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Introduction

In higher animal cells, initiation of centrosome duplication occurs concomitantly with initiation of DNA synthesis. Although this coordinated initiation of centrosome and DNA duplication has long been recognized (Vandre and Borisy, 1989; Mazia, 1987), but the mechanism underlying the coordination of these nuclear and cytoplasmic events had been unclear. However, it has recently been shown that CDK2/cyclin E kinase complex, a known inducer of DNA synthesis (Ohtsubo and Roberts, 1993; van den Heuvel and Harlow, 1993), also triggers initiation of centrosome duplication (Hinchcliffe *et al*., 1999; Lacey *et al*., 1999; Matsumoto *et al*., 1999; Tarapore *et al*., 2002). Since CDK2/cyclin E is activated specifically at mid-late G1 phase of the cell cycle (Koff *et al*., 1992; Dulic *et al*., 1992), it is believed that this temporal activation of CDK2/cyclin E is a key event coordinating the initiation of centrosome and DNA duplication (see Hinchcliffe and Sluder in this issue). We have recently identified nucleophosmin (NPM)/B23 as one of the substrates of CDK2/cyclin E in the initiation of centrosome duplication (Okuda *et al*., 2000). In this review, we focus on NPM/B23 as a target for CDK2/cyclin E, and its potential role in the centrosome duplication process.

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NPM/B23 as a multifunctional protein

NPM/B23, also called numatrin (Feuerstein and Mond, 1987a) or NO38 (Schmidt-Zachmann *et al*., 1987), was originally identified as a nucleolar phosphoprotein found at high levels in granular regions of the nucleolus. NPM/B23 has been shown to be associated with preribosomal particles, implicating its role in ribosome biogenesis (Prestayko *et al*., 1974; Spector *et al*., 1984; Yung *et al*., 1985). In support, NPM/B23 can bind nucleic acids (Dumbar *et al*., 1989; Wang *et al*., 1994), and physically interact with maturing preribosomal ribonucleoprotein particles (Prestayko *et al*., 1974; Olson *et al*., 1986; Schmidt-Zachmann *et al*., 1987). In addition, NPM/B23 possesses intrinsic ribonuclease activity (Herrera *et al*., 1995; Savkur and Olson, 1998). NPM/B23 also possesses the ability to shuttle between the nucleus and the cytoplasm (Borer *et al*., 1989), and binds to nuclear localization signal containing peptides (Szebeni *et al*., 1995). These observations suggest that NPM/B23 functions as a shuttle protein in the nuclear import. Indeed, NPM/B23 forms a specific complex with the nucleolar protein p120 (Valdez *et al*., 1994), nucleolin (Li *et al*., 1996), and several viral proteins such as Rex of human T-cell leukemia virus (Adachi *et al*., 1993), Rev (Fankhauser *et al*., 1991) as well as Tat (Li, 1997) proteins of human immunodeficiency virus, and Hepatitis delta virus antigens (Huang *et al*., 2001). NPM/B23 is known to play a role in sub-cellular localization of these proteins. Moreover, NPM/B23 possesses molecular chaperoning activities, including preventing protein aggregation, protecting enzymes during thermal denaturation, and facilitating renaturation of chemically denatured proteins (Szebeni and Olson, 1999).

NPM/B23 in the cell cycle control and in cellular transformation

It has been shown that the level of NPM/B23 is markedly and promptly increased in association with

cellular commitment for mitogenesis (Feuerstein and Mond, 1987b; Feuerstein *et al*., 1988). In agreement with these earlier findings, NPM/B23 was recently identified as a direct target for a transcriptional factor Myc (Zeller *et al*., 2001). NPM/B23 has also been shown to physically interact with RB, a retinoblastoma susceptibility gene product, and synergistically stimulates DNA polymerase α activity *in vitro* (Takemura *et al*., 1994, 1999). These findings strongly implicate NPM/B23 in the control of proper cell cycle progression.

NPM/B23 has also been implicated in cellular transformation. Overexpression of exogenously introduced NPM/B23 in NIH3T3 cells results in malignant transformation (Kondo *et al*., 1997). Moreover, NPM/B23 has been found generally at higher levels in tumor cells than in normal cells (Chan *et al*., 1989). Several findings have been made, which may provide some clues to the mechanisms underlying the oncogenic activity of NPM/B23. For example, NPM/B23 inhibits DNA-binding and transcriptional activity of interferon regulatory factor-1 (IRF-1), a known tumor suppressor protein (Tanaka *et al*., 1994). NPM/B23 has also been shown to be transcriptionally down-regulated during retinoic acid-induced cellular differentiation (Hsu and Yung, 1998) and sodium butyrate-induced apoptosis (Liu and Yung, 1998). Moreover, overexpression of NPM/B23 makes cells resistant to UV-induced growth inhibition and cell death (Higuchi *et al*., 1998; Wu *et al*., 2002). These observations suggest that NPM/B23 is involved in the regulation of cellular differentiation and apoptosis; increased expression of NPM/B23 may have a protective role in differentiation and apoptosis, hence sensitize cells to cellular transformation. NPM/B23 has also been found to be involved in at least three distinct forms of hematologic malignancy. The N-terminal region of NPM/B23, which contains oligomerization domain (Liu and Chan, 1991), is fused to ALK tyrosine kinase in anaplastic lymphoma with t(2;5) (Morris *et al*., 1994), retinoic acid receptor α in acute promyelocytic leukemia with t(5;17) (Redner *et al*., 1996), and MLF1 in myelodyplastic syndrome with t(3;5) (Yoneda *et al*., 1996).

NPM/B23 as a target of CDK2/cyclin E in centrosome duplication

NPM/B23 has recently been found as a constituent of the centrosome, and as one of the targets of CDK2/cyclin E in the initiation of centrosome duplication. This finding was made by the MALDI-TOF mass spectrometric analysis of the isolated centrosomes that were subjected to an *in vitro* kinase reaction with active CDK2/cyclin E (Okuda *et al*., 2000). It was further found that NPM/B23 associates specifically with unduplicated centrosomes, but not with centrosomes after duplication (Figure 1). Disappearance of NPM/B23 from the centrosomes upon duplication is mediated by CDK2/cyclin E-mediated phosphorylation on threonine 199 (Okuda *et al*., 2000; Tokuyama *et al*., 2001). Although physical dissociation of centrosomal NPM/B23 by CDK2/cyclin E-mediated phosphorylation has been observed *in vitro* (Okuda *et al*., 2000), the fate of centrosomal NPM/B23 *in vivo* remains to be shown: since it is known that ubiquitin-dependent proteasome complex is present at the centrosome, and its activity is required for initiation of centrosome duplication (see Jackson in this issue), there remains a possibility that NPM/B23 may be targeted by the protein degradation machinery upon phosphorylation by CDK2/cyclin E.

Phosphorylation of NPM/B23 and subsequent dissociation of centrosomal NPM/B23 from the centrosome is found to be essential for centrosomes to initiate duplication. For instance, microinjection of anti-NPM/B23 monoclonal antibody, which sterically prevents phosphorylation of NPM/B23 by CDK2/cyclin E, strongly inhibited duplication of centrosomes (Okuda *et al*., 2000). Moreover, introduction of NPM/B23 mutants whose phosphorylation site was either deleted (NPMΔ186–239) or substituted with a non-phosphorylatable residue (NPM/S199A) resulted in suppression of centrosome duplication (Okuda *et al*., 2000; Tokuyama *et al*., 2001). These mutants remain at the centrosomes, likely accounting for their dominant negative characteristics. In the cells transfected with these non-phosphorylatable mutants, aberrant mitoses with monopolar spindles were occasionally found, indicating that the transfectants proceeded through the cell cycle to mitosis without duplication of centrosomes. Moreover, random thin-section electron microscopy of the NPMΔ186–239 transfectants showed that the centriole pair was intact with orthogonal configuration, which is typical of a preduplication centriole pair normally observed in early-mid G1 phase, suggesting that the expression of dominant-negative mutant NPM/B23 results in inhibition of centriole separation, the very early step of centrosome duplication (Okuda *et al*., 2000).

NPM/B23 and the centrosome duplication cycle

Meraldi *et al*. (1999) have previously shown that CDK2/cyclin A activity may also play an important role in centrosome duplication. Since cyclin E is intrinsically unstable, cyclin E-dependent CDK2 activity becomes minimal during S phase (Koff *et al*., 1992; Dulic *et al*., 1992). In contrast, the level of cyclin A is low in late G1, but increases during S and G2 phases (Pines and Hunter, 1990; Marraccino *et al*., 1992; Pagano *et al*., 1992; Carbonaro-Hall *et al*., 1993). Thus, during S and G2 phases of the cell cycle, CDK2/cyclin A activity is high. CDK2/cyclin A also phosphorylates NPM/B23 specifically on Thr199 *in vitro* at a similar efficiency with CDK2/cyclin E (Tokuyama *et al*., 2001). Thus, it is possible that the continual presence of active CDK2/cyclin A may be responsible for preventing re-association of any cytoplasmic NPM/B23 to centrosomes during S and G2 phases. Only during mitosis, NPM/B23 was found to re-associate with the centrosomes (or spindle poles)

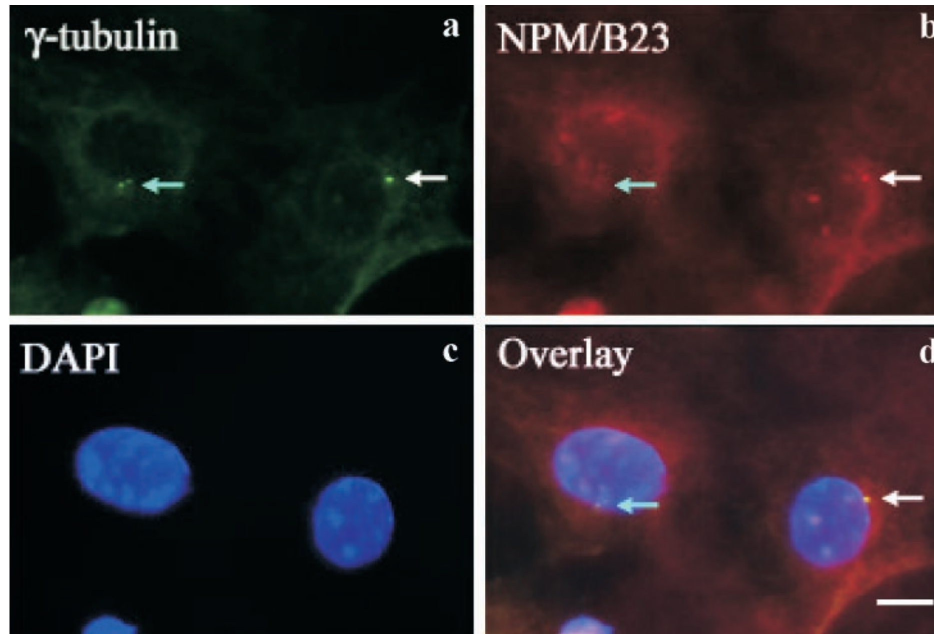

Figure 1 NPM/B23 specifically associates with unduplicated centrosomes. Exponentially growing Swiss 3T3 cells were fixed in methanol : acetone (50 : 50), and co-immunstained with anti-NPM/B23 mouse monoclonal and anti-γ-tubulin rabbit polyclonal antibodies. Cells were also counter-stained with DAPI for visualization of nuclei. (**d**) shows the overlay image of **a**–**c**. The white arrow points to an unduplicated centrosome, while the blue arrow points to duplicated centrosomes. Scale bar; 10 μm

(Zatsepina *et al*., 1999; Okuda *et al*., 2000). This centrosomal re-association of NPM/B23 may be explained by the fact that during mitosis most nuclear proteins, including NPM/B23, disperse throughout the cytoplasm due to the nuclear membrane break-down (Nigg, 1988), and some of NPM/B23 may re-localize to the centrosomes. Interestingly, NPM/B23 has previously been shown to be phosphorylated by CDK1/cyclin B, a mitotic CDK/cyclin complex (Peter *et al*., 1990). It was subsequently found that Thr234 and Thr237 of NPM/B23 was phosphorylated by CDK1/cyclin B, which are different from CDK2/cyclin E (and cyclin A)-mediated phosphorylation sites (Tokuyama *et al*., 2001). Thus, it is of great interest to determine phosphorylation of Thr234 and/or Thr237 by CDK1/cyclin B may play a role in re-association of NPM/B23 with centrosomes during mitosis.

Based on the findings made to date, the following model for the role of NPM/B23 in the regulation of the centrosome duplication cycle can be put forward (Figure 2): centrosome-bound NPM/B23 dissociates from centrosomes (or alternatively becomes degraded) upon phosphorylation by CDK2/cyclin E, which in turn triggers initiation of centrosome duplication. During S and G2 phases, any NPM/B23 present in cytoplasm are prevented for re-association with centrosomes potentially through the activity of CDK2/cyclin A. During mitosis, NPM/B23 re-associates with centrosomes, hence each daughter cell receives one centrosome bound by NPM/B23 upon cytokinesis. In this setting, NPM/B23 would constitute one of licensing systems for centrosome duplication, ensuring the coordination of centrosome and DNA duplication, as well as restricting centrosome duplication to occur once within a single cell cycle.

Future studies

There are several important questions remaining to be answered for the role of NPM/B23 in the regulation of centrosome duplication: (1) Which centrosomal component(s) does NPM/B23 directly interact with? (2) What is the *in vivo* mechanism of disappearance of NPM/B23 from unduplicated centrosomes upon phosphorylation by CDK2/cyclin E? Is it through simple physical dissociation or through proteasomal degradation? (3) Considering the direct involvement of NPM/B23 in the regulation of DNA synthesis, does NPM/B23 play a role in cross-talk between centrosome duplication (cytoplasmic event) and DNA synthesis (nuclear event)? (4) Does the function of NPM/B23 in the regulation of centrosome duplication play any role in its oncogenic activity? The answers to these questions will certainly advance our understanding of the regulation of centrosome duplication in higher animal cells, and unveil its significance in carcinogenesis.

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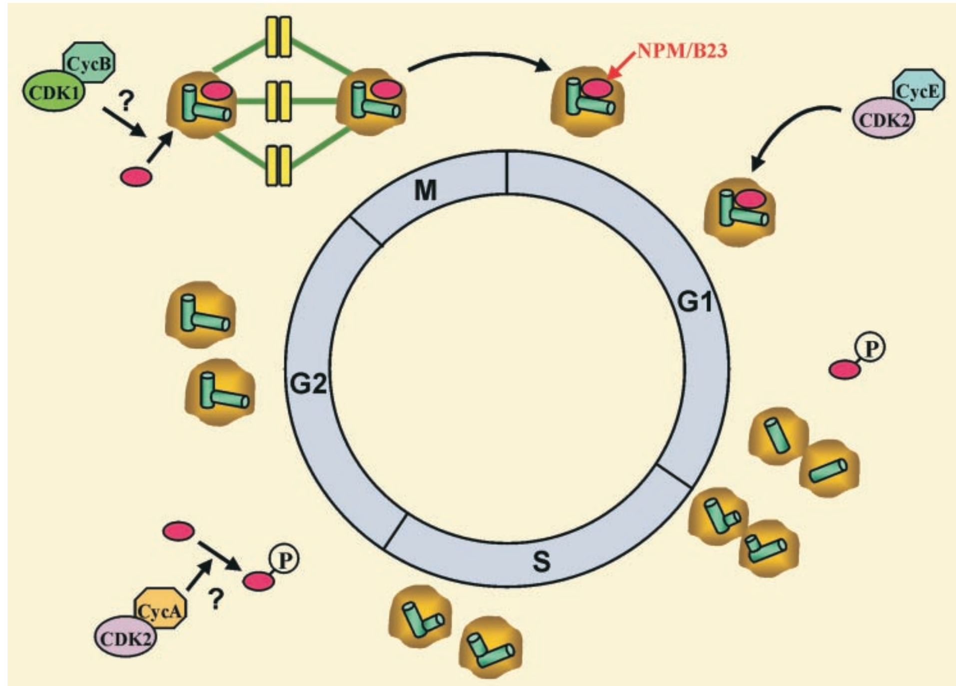

Figure 2 NPM/B23 in the regulation of the centrosome duplication cycle. Centrosome-bound NPM/B23 dissociates from centrosomes upon phosphorylation by CDK2/cyclin E, which in turn triggers initiation of centriole duplication. When the nuclear membrane breaks down during mitosis, NPM/B23 re-localizes to the centrosomes (spindle poles). After mitosis, each daughter cell receives one centrosome bound by NPM/B23, which dissociates from centrosome upon exposure to CDK2/cyclin E in the next cell cycle

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