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Overexpression of the human *MNB/DYRK1A* gene induces formation of multinucleate cells through overduplication of the centrosome

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Abstract

Background: Previously we cloned the human *MNB/DYRK1A* gene from the "Down syndrome critical region" on chromosome 21. This gene encodes a dual specificity protein kinase that catalyzes its autophosphorylation on serine/threonine and tyrosine residues. But, the functions of the *MNB/DYRK1A* gene in cellular processes are unknown.

Results: In this study, we examined HeLa cells transfected with cDNA encoding a green fluorescent protein (GFP)-MNB/DYRK1A fusion protein and found 2 patterns of expression: In one group of transfected cells, GFP-MNB/DYRK1A was localized as dots within the nucleus; and in the other group, it was overexpressed and had accumulated all over the nucleus. In the cells overexpressing GFP-MNB/DYRK1A, multinucleation was clearly observed; whereas in those with the nuclear dots, such aberrant nuclei were not found. Furthermore, in the latter cells, essential processes such as mitosis and cytokinesis occurred normally. Multinucleation was dependent on the kinase activity of MNB/DYRK1A, because it was not observed in cells overexpressing kinase activity-negative mutants, GFP-MNB/DYRK1A (K179R) and GFP-MNB/DYRK1A (Y310F/Y312F). Immunostaining of GFP-MNB/DYRK1A-overexpressing cells with specific antibodies against α- and γ-tubulin revealed that multiple copies of centrosomes and aberrant multipolar spindles were generated in these cells.

Conclusions: These results indicate that overexpression of MNB/DYRK1A induces multinucleation in HeLa cells through overduplication of the centrosome during interphase and production of aberrant spindles and missegregation of chromosomes during mitosis.

Background

Down syndrome (trisomy 21) is the most frequent birth defect and is a major cause of mental retardation and congenital heart disease [1]. Besides the characteristic set of facial and physical features of individuals afflicted with it, this syndrome is associated with defects of the immune and endocrine systems, an increased rate of leukemia, and early onset of Alzheimer disease [1]. Although little is known about the mechanism by which trisomy 21 interferes with normal development, the increased dosage of the chromosomal elements clearly implies altered levels of gene expression as a causative factor.

In most cases, patients with Down syndrome show trisomy of chromosome 21. Studies of cases with partial trisomy of chromosome 21 have suggested that the region around locus D21S55 is particularly important in the etiology of the syndrome [2–4]. This subchromosomal region is called the "Down syndrome critical region." Earlier we performed exon trapping experiments using a series of cosmid clones isolated from this chromosomal region, and identified the genomic structure and cDNA sequence of the human *MNB/DYRK1A* gene in this region [5–7].

The human *MNB/DYRK1A* gene is a human homolog of *Drosophila minibrain* and rat *DYRK* genes. Mutant flies with a reduced expression of *minibrain* have a reduced number of neurons in distinct areas of the adult brain; and this gene is therefore required for a distinct neuroblast proliferation during postembryonic neurogenesis [8]. A previous report on the rat DYRK protein showed that the *DYRK* gene encodes a dual specificity kinase that catalyzes its autophosphorylation on both serine/threonine and tyrosine residues [9–11]. The human *MNB/DYRK1A* gene has been suggested to be a strong candidate gene for learning defects associated with Down syndrome, because transgenic mice carrying a 180-kb YAC contig containing the human *MNB/DYRK1A* gene showed defects in learning and memory [12,13]. But, the roles of the *MNB/DYRK1A* gene in cellular processes are far from established.

To clarify the physiological role of MNB/DYRK1A, in the present study we transfected HeLa cells with cDNA encoding a GFP-MNB/DYRK1A fusion protein. The subcellular localization of the transfected-MNB/DYRK1A depended on its expression level. In the case of a low level, the protein was concentrated at specific loci in the nucleus; but in the case of a high level, it was located all over the nucleus. This high-level expression of MNB/DYRK1A protein induced the overproduction of the centrosome and led to multinucleation in HeLa cells. These results indicate that MNB/DYRK1A may play a specific function in coordinating nuclear division with other cell-cycle progression events.

Results

MNB/DYRK1A protein is expressed at high levels in various organs including the central nervous system during the embryonic period, but its level gradually decreases with postnatal growth and is extremely low in the adult [7]. This finding may indicate a significant role of this protein in the proliferation and differentiation of cells during development. To examine the physiological role of MNB/DYRK1A, we transiently transfected HeLa cells with cDNA encoding a GFP-MNB/DYRK1A fusion protein and determined its subcellular localization. The fluorescent fusion protein was found inside the nucleus of HeLa cells and showed a speckled pattern, as reported previously (Fig. 1A, panel C) [11]. We also found other cells with GFP-MNB/DYRK1A in a diffuse pattern all over the nucleus, in which cells multiple nuclei were often observed (Fig. 1A, panel D). Such a speckled pattern or multinucleation was not observed if cDNA encoding GFP or GFP-NLS, a fusion protein of GFP and bipartite nuclear localization sequence corresponding to amino acids 104–133 in MNB/DYRK1A, was used for the transfection. GFP was mainly detected in the cytoplasm, and GFP-NLS was localized in the nucleus, where it accumulated in nucleoli (Fig. 1A, panels A and B). These subcellular localization patterns were observed independently of the expression level of GFP and GFP-NLS. We next expressed MNB/DYRK1A protein without the GFP tag in HeLa cells and found cells with nuclear dots (Fig. 1B, panel C) and multiple nuclei (Fig. 1B, panel D). Thus, it appears that the speckled distribution of GFP-MNB/DYRK1A in the nucleus of HeLa cells and the emergence of multinucleate cells are not artifacts caused by high-level expression of the GFP fusion protein.

Next, we performed detailed analysis of the multinucleate cells by confocal laser scanning microscopy to exclude the possibility that expression of the GFP-MNB/DYRK1A fusion protein produced nuclei surrounded by a continuous and polymorphous nuclear membrane. Serial optical section image data for the cells strongly expressing MNB/DYRK1A were obtained at 0.4-μm intervals (Fig. 2). Observation of all of the focal planes of cells with 3 nuclei showed each nucleus to be independent, i.e., to have its own nuclear membrane, indicating that MNB/DYRK1A indeed induced multinucleation if its expression level was high.

We previously prepared a polyclonal antibody (anti-MNB antibody) immunospecific for the MNB/DYRK1A protein, one that recognized 2 major bands and 1 minor band with apparent molecular masses of about 90 kDa when samples from various rat tissues were analyzed [7]. These

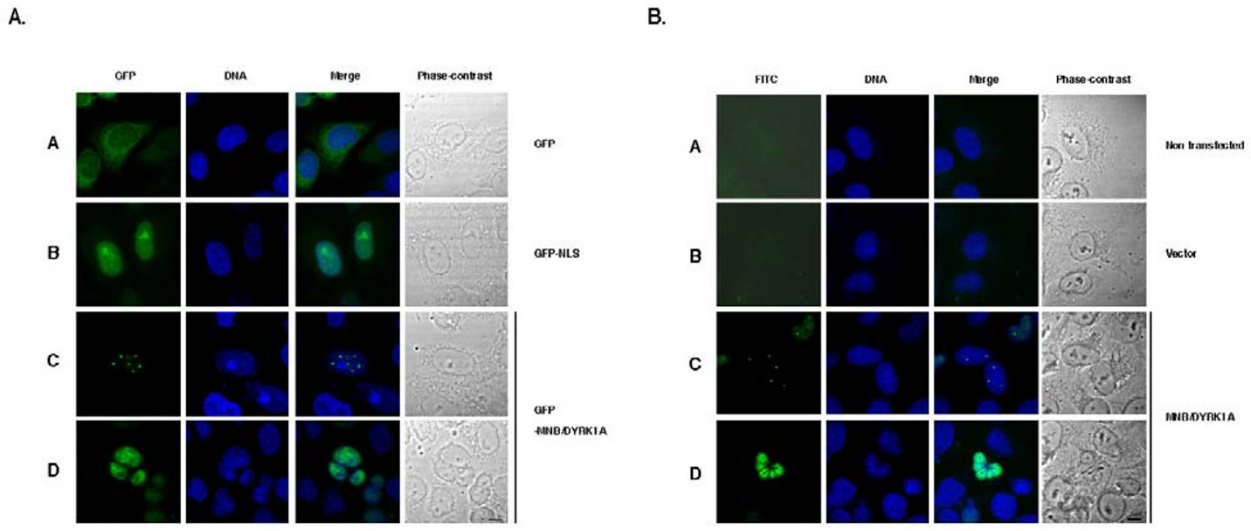

Figure 1

Subcellular localization of GFP-MNB/DYRK1A (A) and FLAG-tagged MNB/DYRK1A (B) in HeLa cells. HeLa cells were transfected with constructs encoding GFP, GFP-NLS, GFP-MNB/DYRK1A, or FLAG epitope-tagged MNB/DYRK1A as described in "Materials and Methods." The cells were then fixed, stained with 1 μM TOTO-3 for the counterstaining of DNA, and observed by confocal laser scanning microscopy. GFP or FITC and DNA are displayed in green and blue, respectively. Merged images are also shown in the third photo from the left. Phase-contrast images are shown at the right. The data are representative of those of 5 independent experiments. Scale bar, 10 μm.

3 MNB/DYRK1A proteins were also detected in nontransfected HeLa cells by immunoblot analysis using this anti-MNB antibody (data not shown), but nontransfected HeLa cells were stained only weakly with this antibody (Fig. 3, panel A). To determine the expression level of GFP-MNB/DYRK1A protein in multinucleate cells, we stained the cells with the anti-MNB antibody after transfection with GFP-MNB/DYRK1A cDNA. When cells with the dotted distribution of GFP-MNB/DYRK1A were immunostained with this antibody, a similar dotted pattern was observed (Fig. 3, panel B). On the other hand, cells with the diffuse distribution of the GFP-MNB/DYRK1A fusion protein all over the nuclei were stained strongly with the anti-MNB antibody (Fig. 3, panels C and D). This strong staining was not observed when control immunostaining was performed without the addition of anti-MNB antibody as the primary antibody (Fig. 3, panel E). These results show that the GFP-MNB/DYRK1A protein level was much higher in cells with the diffuse distribution of this protein than in the nontransfected control cells or transfected cells having the dotted distribution of GFP-MNB/DYRK1A.

To examine the relationship between the MNB/DYRK1A overexpression and multinucleation, we determined the percentage of mutinucleate cells in MNB/DYRK1A-overexpressing cells at 24 hours or 48 hours after transfection with GFP-MNB/DYRK1A (Fig. 4A). The % of multinucleate cells was very low in the control HeLa cells without the transfection, and it did not change in cells subjected to transfection treatment without the vector. When cDNA encoding GFP or GFP-NLS was used to transfect the cells, the % of multinucleate cells was also very low; and it did not change whether cells possessed the GFP signal or not. On the other hand, about 30% and 40% of the GFP-MNB/DYRK1A-overexpressing cells possessed multinuclei at 24 hours and 48 hours, respectively, after the transfection. The percentage for cells with no fluorescent signal or dotted fluorescent signal of GFP-MNB/DYRK1A was about 2% or 3%. We next determined the % of multinucleate cells after transfection treatment with cDNA encoding kinase activity-negative mutants (Fig. 4B). The % of multinucleate cells in GFP-MNB/DYRK1A (K179R)- or GFP-MNB/DYRK1A (Y310F/Y312F)-overexpressing cell population was very low, as compared with the % in the wild-

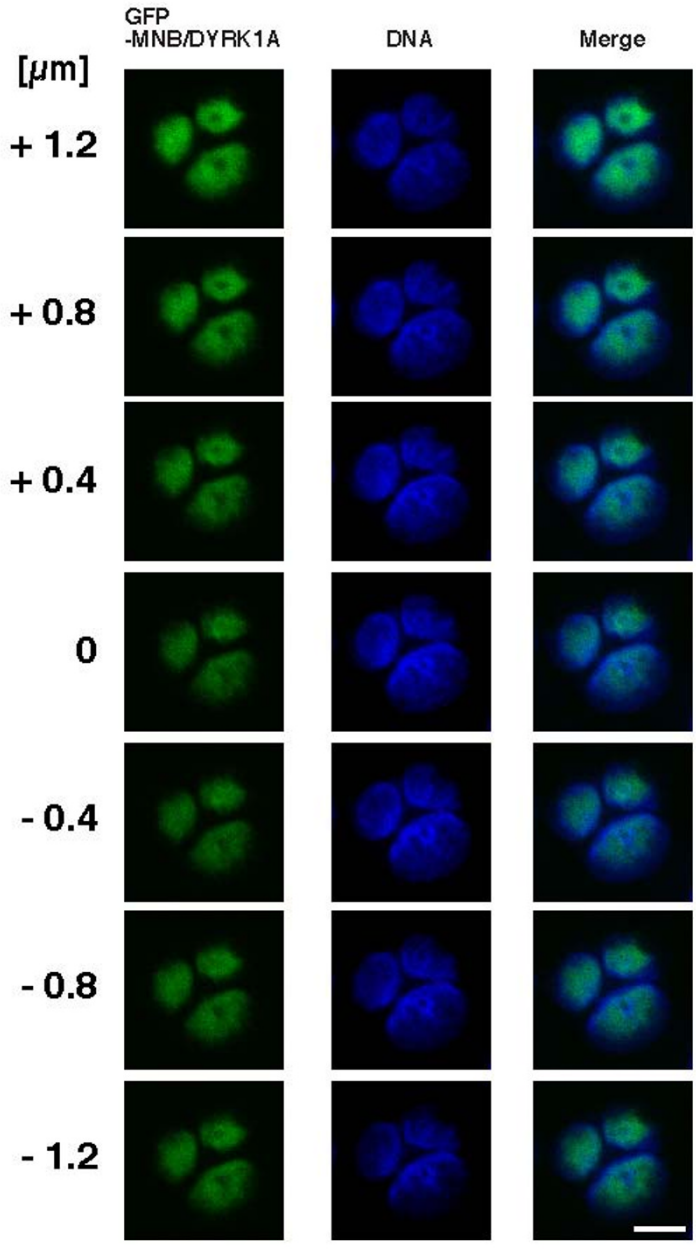

Figure 2
Appearance of multiple nuclei in GFP-MNB/DYRK1A-overexpressing cells. HeLa cells transfected with the plasmid encoding GFP-MNB/DYRK1A were incubated for 48 hours and then fixed as described in "Materials and Methods." Fixed cells were stained with 1 μM TOTO-3 for the counterstaining of DNA and observed by confocal laser scanning microscopy. Images were collected at 0.4-μm Z axis intervals. GFP-MNB/DYRK1A (left) and DNA (middle) are displayed in green and blue, respectively. Merged images are shown in the right panel. Scale bar, 10 μm.

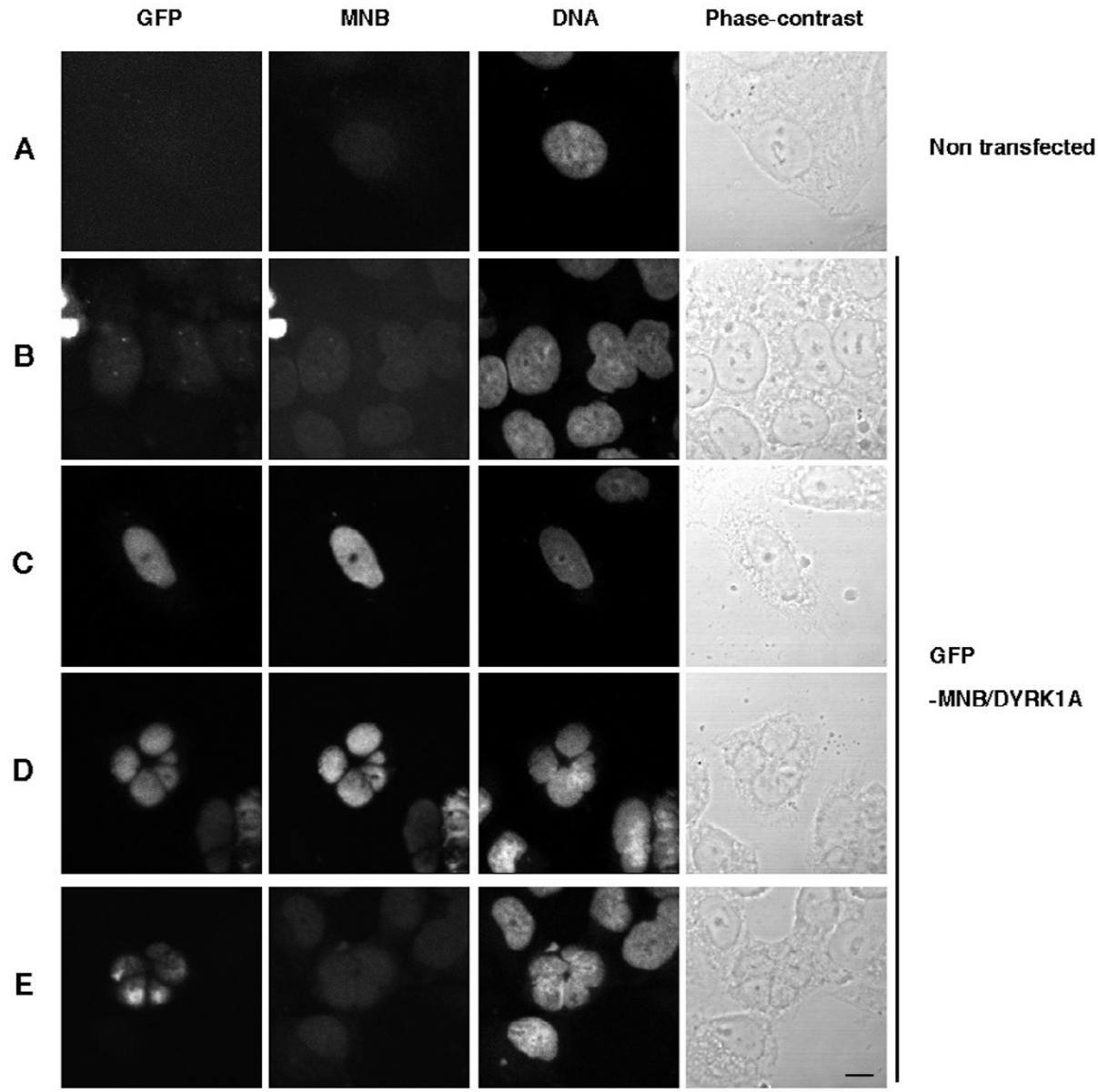

Figure 3
High-level expression of GFP-MNB/DYRK1A in multinucleate cells. HeLa cells were transfected with cDNA encoding GFP-MNB/DYRK1A. At 48 hours after transfection, the cells were fixed, stained with anti-MNB antibody and TOTO-3, and observed with a confocal laser scanning microscope as described in "Materials and Methods." Scale bar, 10 μm.

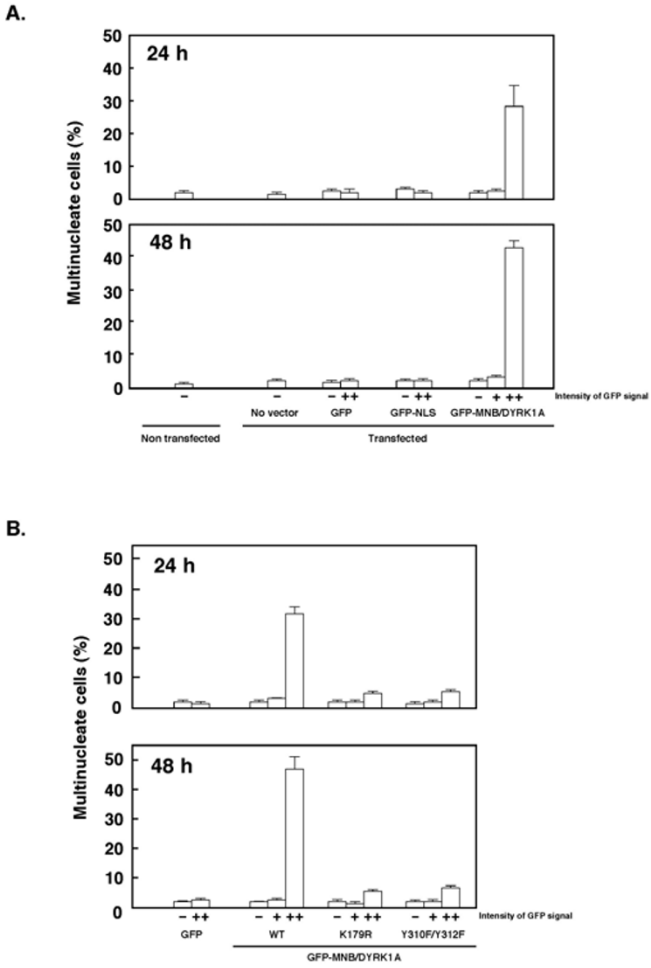

Figure 4

Overexpression of MNB/DYRK1A increases the number of multinucleate cells. HeLa cells were transfected with constructs encoding GFP, GFP-NLS, GFP-MNB/DYRK1A (WT), GFP-MNB/DYRK1A (K179R), or GFP-MNB/DYRK1A (Y310F/Y312F), as described in "Materials and Methods." The cells were then fixed at 24 hours (Upper panel) or 48 hours (Lower panel) and stained with 1 μM TOTO-3. The percentage of multinucleate cells was calculated by dividing the number of cells with more than 2 nuclei by the number of total cells (a total of 200 cells were examined for determining each percentage). In cells transfected with no vector, GFP cDNA, or GFP-NLS cDNA, '-' denotes cells with no fluorescent signal; '++', cells with a fluorescence signal, the level of which was comparable to that in cells with diffuse distribution of GFP-MNB/DYRK1A all over the nuclei. In cells transfected with cDNA encoding GFP-MNB/DYRK1A, GFP-MNB/DYRK1A (K179R), or GFP-MNB/DYRK1A (Y310F/Y312F), '-' denotes cells with no fluorescent signal; '+', cells with a dotted distribution of fluorescence; '++', cells with a diffuse distribution of fluorescence. The data shown are means ± S.E. of 3 independent experiments. (A) The percentages of cells expressing GFP, GFP-NLS, and GFP-MNB/DYRK1A at 24 hours after transfection were 12.24 ± 0.19%, 10.08 ± 0.37%, and 7.76 ± 0.64%, respectively (dotted distribution, 4.21 ± 0.40%; diffuse distribution, 3.55 ± 0.36%); and at 48 hours, 14.55 ± 0.21%, 11.74 ± 0.29%, and 9.34 ± 0.51%, respectively (dotted distribution, 5.42 ± 0.41%; diffuse distribution, 3.92 ± 0.15%). (B) The percentages of cells expressing GFP, GFP-MNB/DYRK1A, GFP-MNB/DYRK1A (K179R), and GFP-MNB/DYRK1A (Y310F/Y312F) at 24 hours after transfection were 13.8 ± 0.56%, 8.48 ± 0.79% (dotted distribution, 5.33 ± 0.40%; diffuse distribution, 3.15 ± 0.45%), 12.18 ± 0.55% (dotted distribution, 7.15 ± 0.80%; diffuse distribution, 5.03 ± 0.65%), and 10.43 ± 0.60% (dotted distribution, 6.10 ± 0.49%; diffuse distribution, 4.33 ± 0.38%), respectively; and at 48 hours, 15.34 ± 0.57%, 10.10 ± 0.52% (dotted distribution, 6.51 ± 0.63%; diffuse distribution, 3.59 ± 0.21%), 14.44 ± 0.30% (dotted distribution, 8.72 ± 0.76%; diffuse distribution, 5.55 ± 0.40%), and 12.07 ± 0.52% (dotted distribution, 7.45 ± 0.77%; diffuse distribution, 4.63 ± 0.54%), respectively.

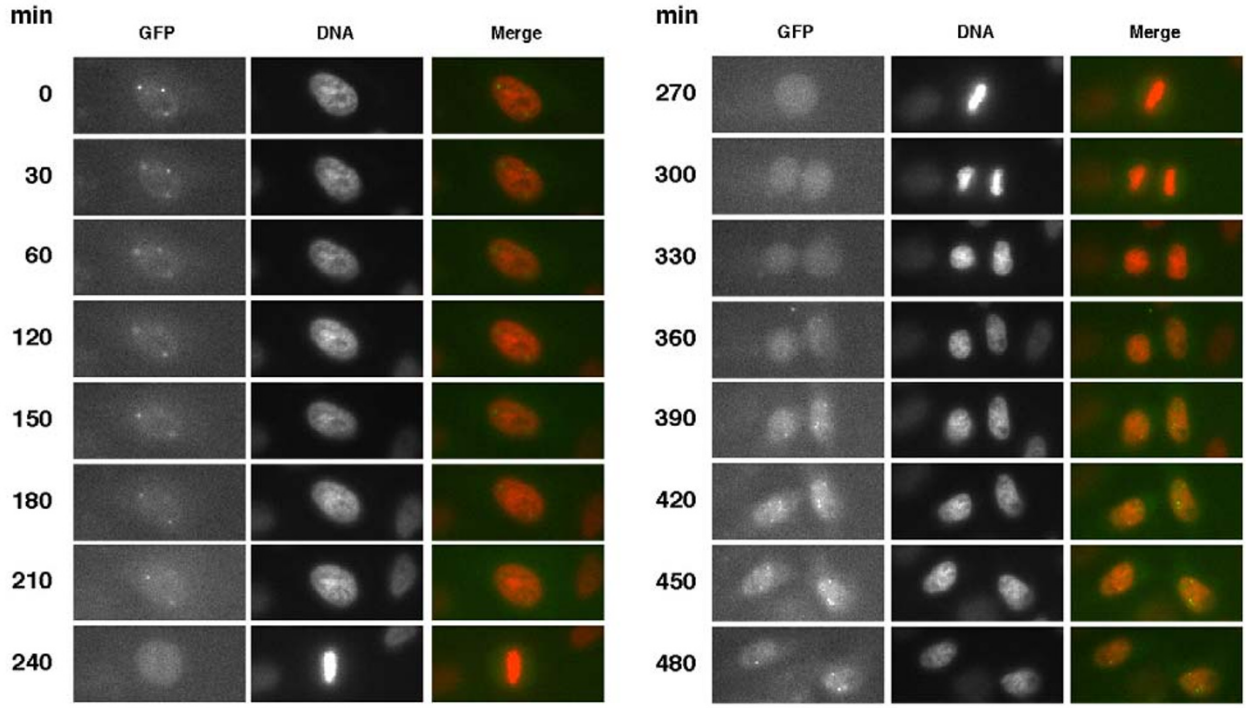

Figure 5
Subcellular localization of GFP-MNB/DYRK1A in a living cell during the cell cycle. HeLa cells transfected with the plasmid encoding GFP-MNB/DYRK1A were incubated for 24 hours, stained with 100 ng/ml Hoechst33342 for the counter-staining of DNA, and then observed with a fluorescence microscope. In the merged images (right), GFP-MNB/DYRK1A and DNA are displayed in green and red, respectively. The numbers on the left side of each image represent the time after the start of microscopic observation.

type GFP-MNB/DYRK1A-overexpressing cell population, 31% (at 24 hours) and 47% (at 48 hours). These data indicate that the kinase activity of MNB/DYRK1A was essential for the appearance of multinucleation.

Next, to investigate the subcellular localization of MNB/DYRK1A protein during the cell cycle, especially during mitosis, we transiently expressed GFP-MNB/DYRK1A in HeLa cells and chased interphase cells with a speckled fluorescent pattern in a time-lapse manner (Fig. 5). At 240 minutes when the nuclear envelope disappeared, GFP-MNB/DYRK1A fluorescence gave a diffuse pattern and was located in the cytoplasm with relative exclusion from the condensed chromatin. After reformation of the nuclear envelope at telophase (see 360 minutes), GFP-MNB/DYRK1A again exhibited a speckled pattern. Thus, MNB/DYRK1A was concentrated at specific loci in the nucleus during interphase, whereas during mitosis it was located all over the cells and not condensed with either chromosome, mitotic spindle, or spindle pole. Fig. 5 also shows that the interphase cells with a speckled pattern in their nucleus progressed into M phase and produced 2 daughter cells. Further, both the condensation and subsequent segregation of chromosomes occurred in these cells as properly as in non-transfected control cells. On the other hand, chromosome missegregation was observed in HeLa cells with overexpressed MNB/DYRK1A, as will be described later (Fig. 6A, panel d).

The enhanced level of MNB/DYRK1A protein induced the emergence of cells with multiple nuclei. This observation may be important, because aberrant overexpression of

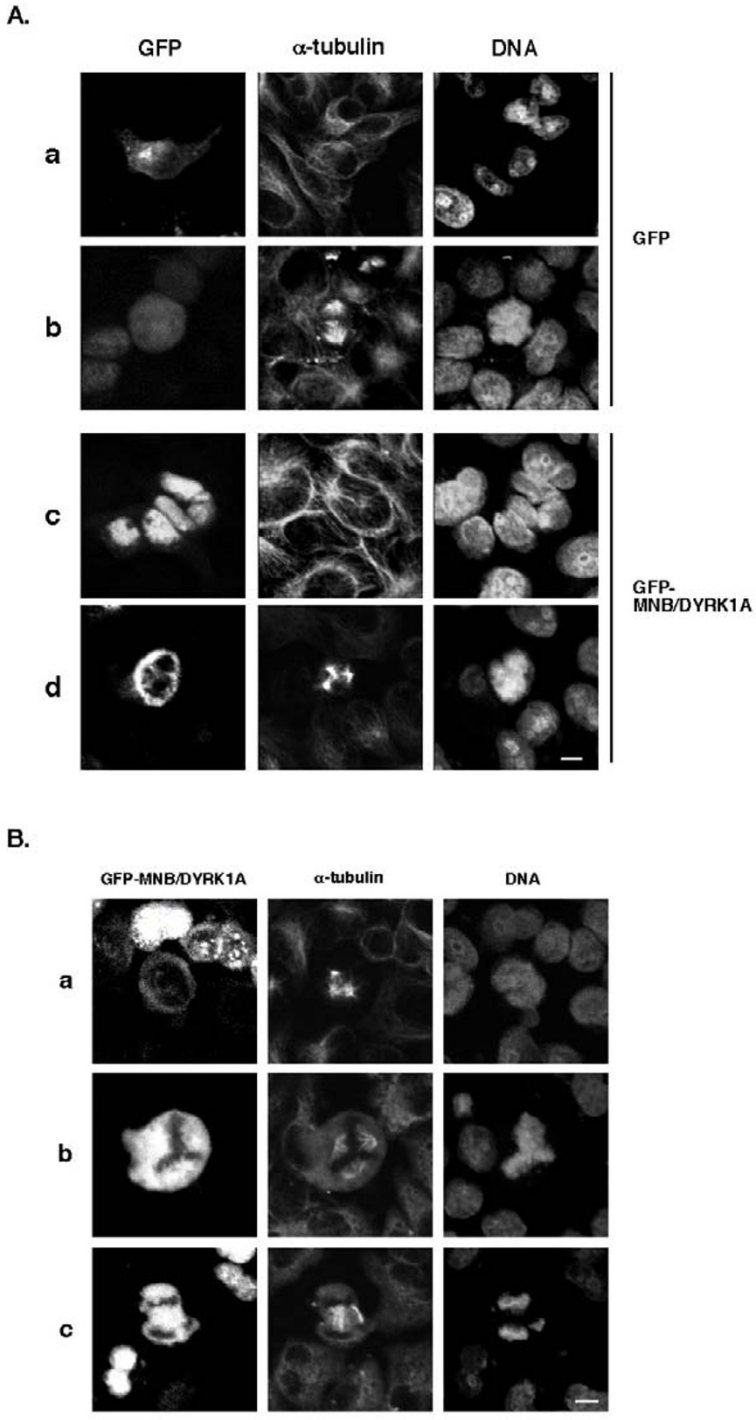

Figure 6
Microtubule structures in GFP-MNB/DYRK1A-overexpressing cells. HeLa cells were transfected with constructs encoding GFP or GFP-MNB/DYRK1A. After incubation for 36–48 hours, the cells were fixed, stained with anti-α-tubulin antibody and TOTO-3, and observed with a confocal laser scanning microscope as described in "Materials and Methods." (A) a and c, interphase; b and d, M phase. (B) a, prometaphase; b, metaphase; c, late-anaphase. The data are representative of those of 5 independent experiments. Scale bar, 10 μm.

MNB/DYRK1A protein is thought to contribute to the characteristic features of Down syndrome. Although multinucleation can be produced via various mechanisms, defects in the mitotic machinery may play a major role in this nuclear abnormality. The mitotic machinery accurately separates and distributes chromosomes into each daughter cell. This accurate segregation is achieved by mitotic spindles composed of microtubules. So, we determined the effect of over-expressed GFP-MNB/DYRK1A on microtubule structures by immunostaining cells with an antibody specific for α-tubulin, a main constituent of microtubules (Fig. 6). When interphase cells were observed after the transfection with GFP, α-tubulin was detected in the cytoplasm, especially in the nuclear periphery (Fig. 6A, panel a). In a multinucleate cell, α-tubulin showed a localization similar to that in the GFP-transfected cell, but it was localized around a mass of nuclei rather than around each nucleus (Fig. 6A, panel c). At the M phase of the cell cycle, typical mitosis with a bipolar mitotic spindle was observed in the GFP-transfected cells (Fig. 6A, panel b), whereas aberrant mitotic spindles were noted in cells expressing a high level of GFP-MNB/DYRK1A (Fig. 6A, panel d). Further analysis of mitotic cells overexpressing GFP-MNB/DYRK1A revealed that tripolar spindles appeared in prometaphase cells (Fig. 6B, panel a) and that multiple mitotic spindle poles and abnormal chromosome condensation appeared in metaphase cells and late-anaphase cells (Fig. 6B, panels b and c).

The centrosome is the major microtubule-organizing center in eukaryotic cells and features prominently in mitosis, where it is required for the establishment of spindle bipolarity, spindle microtubule assembly, and balanced segregation of chromosomes. So, the aberrant mitotic spindles seen in MNB/DYRK1A-overexpressing cells may have resulted from the generation of multiple centrosomes. So next we visualized these structures by immunostaining cells with an antibody specific for γ-tubulin, a well-characterized component of centrosomes in all phases of the cell cycle (Fig. 7). HeLa cells transfected with the GFP construct, which was used as a control, contained 1 or 2 centrosomes juxtaposed to the nucleus at interphase (Fig. 7, panel A). At M phase, they contained 2 centrosomes, between which the chromosomes were aligned (Fig. 7, panel B). In contrast, MNB/DYRK1A-overexpressing cells contained more than 2 centrosomes at interphase (Fig. 7, panels C and D) and also at M phase (Fig. 7, panel E), thus indicating centrosome overduplication. Since overduplication of the centrosome was not detected in the cells in which MNB/DYRK1A was localized as nuclear dots within the nucleus (data not shown), such overduplication must have resulted from MNB/DYRK1A overexpression. Further, the presence of cells with centrosome amplification but not with multinucleation indicates that a high level of MNB/DYRK1A induces the overduplication of the centrosome in interphase, subsequently producing multiple spindle poles leading to the multinucleation.

We next quantified the number of cells with an abnormal number of centrosomes at 24 hours and 48 hours after the transfection with the cDNA encoding the GFP-MNB/DYRK1A fusion protein. Cells with 3 or more centrosomes were observed predominantly in the case of GFP-MNB/DYRK1A transfection (Fig. 8). The percentage of cells with 2 centrosomes was also higher in this cell population, than in that transfected with GFP or GFP-NLS. Since centrosome amplification was not observed even in cells that expressed GFP or GFP-NLS at a high level, the overproduction of centrosomes is concluded to have occurred through the overexpression of MNB/DYRK1A protein.

Discussion

The transfection experiments with cDNA encoding GFP-MNB/DYRK1A showed that the subcellular distribution pattern of GFP-MNB/DYRK1A protein depended on its intracellular expression level. When the expression level of transfected MNB/DYRK1A cDNA was low, MNB/DYRK1A protein was localized with a speckled pattern in the nucleus. A similar subnuclear localization of MNB/DYRK1A has been observed in both COS-7 cells and HEK293 cells transfected with the GFP fusion protein of MNB/DYRK1A [10,11]. Furthermore, we found that MNB/DYKR1A without a GFP tag became localized with a speckled pattern in the nucleus. These findings indicate that the speckled distribution of GFP-MNB/DYRK1A in the nucleus is not an artifact. Thus, MNB/DYRK1A probably localizes to subnuclear domains in the nucleus; and its association with this distinct subnuclear structure may be critical for some specific function of MNB/DYRK1A, although we couldn't detect this subnuclear localization of endogenous MNB/DYRK1A by using our specific antibody against MNB/DYRK1A.

Similar speckled patterns of subnuclear localization have been shown for other proteins [14–18]. One of them is the transcription factor forkhead in rhabdomysarcoma (FKHR), and this protein is known to co-localize and interact with MNB/DYRK1A [19]. We found that GFP-MNB/DYRK1A co-immunoprecipitated with FKHR by immunoprecipitation using anti-FKHR antibody (manuscript in preparation), indicating that FKHR binds to GFP-MNB/DYRK1A in HeLa cells. FKHR transcription factors mediate cell-cycle regulation of a variety of cell lines, dependent on the cell-cycle inhibitor $p27^{kip\,1}$. They also play a role in the control of gene expression by insulin, as well as in the regulation of apoptosis mediated by survival factors [20–22]. These signals trigger the phosphorylation

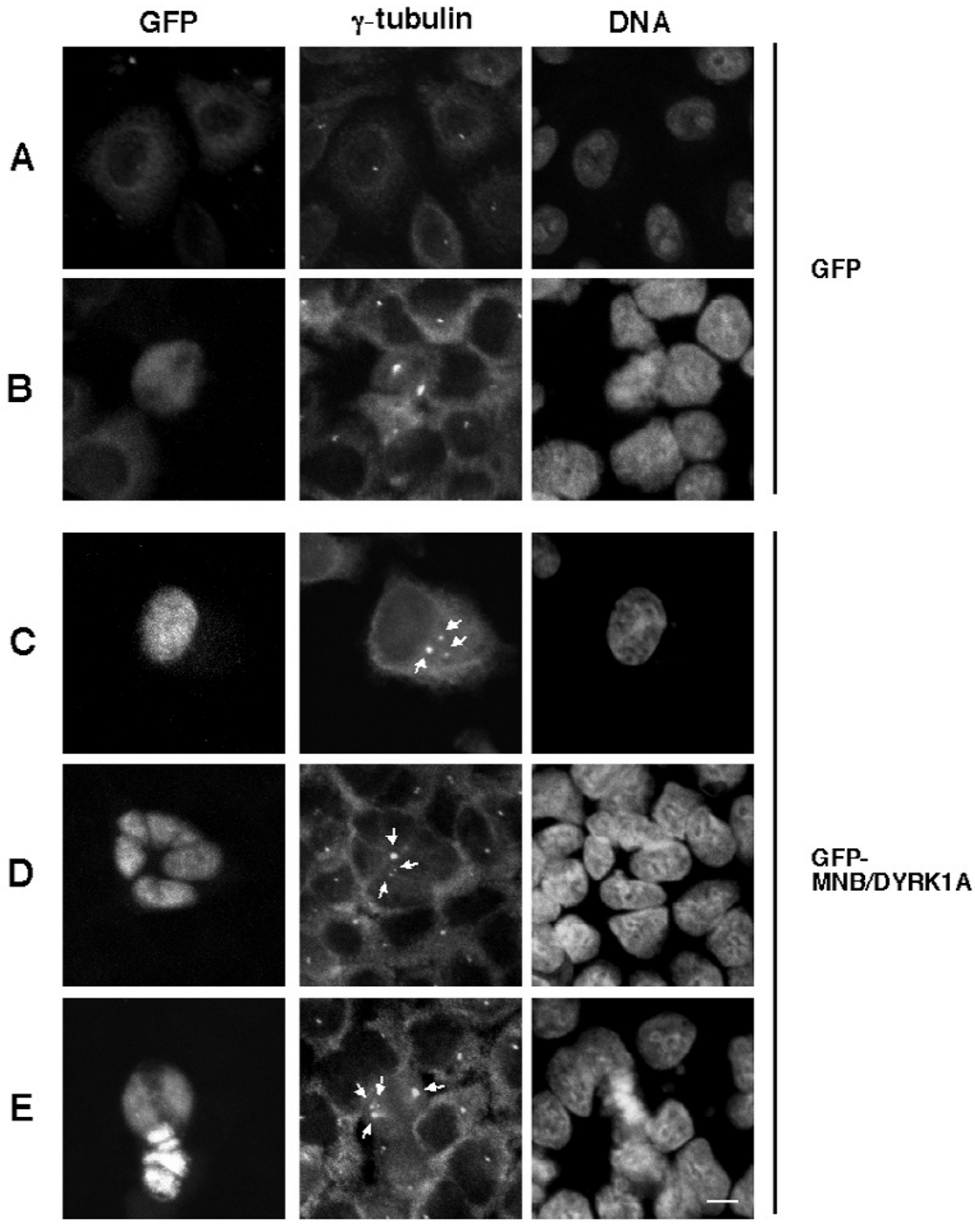

Figure 7
Overproduction of centrosomes in GFP-MNB/DYRK1A-overexpressing cells. HeLa cells were transfected with constructs encoding GFP or GFP-MNB/DYRK1A and then fixed as described in the legend of Fig. 6. Fixed cells were stained with anti-γ-tubulin antibody and TOTO-3 as described in "Materials and Methods." The stained cells were observed with a confocal laser scanning microscope. Arrows in panels C, D, and E point to centrosomes. A, C and D, interphase; B and E, M phase. The data are representative of those of 5 independent experiments. Scale bar, 10 μm.

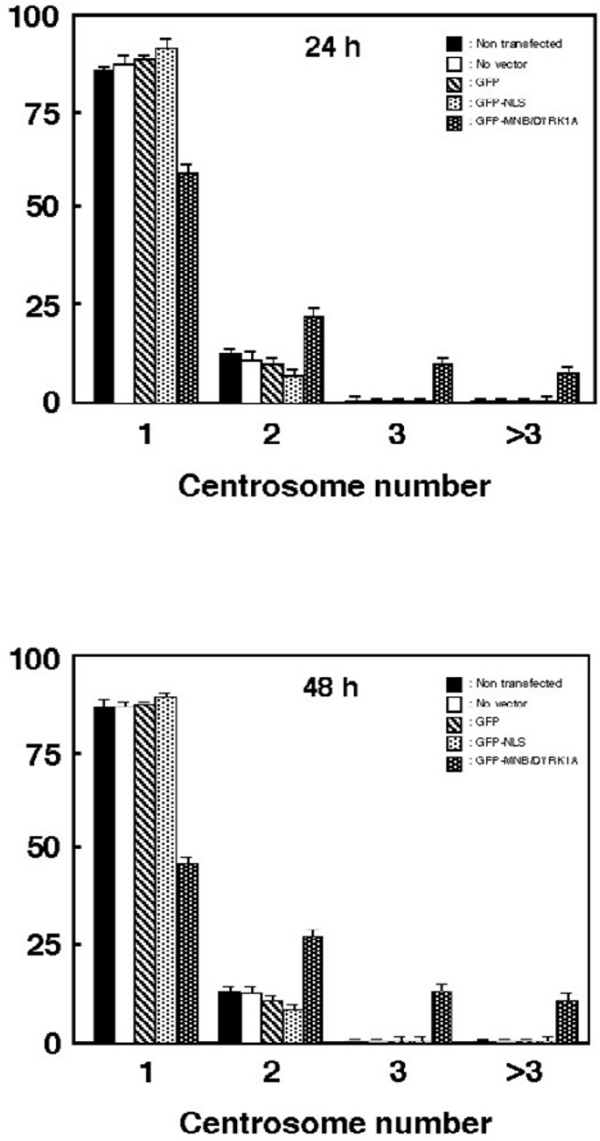

Figure 8

Centrosome number in GFP-MNB/DYRK1A-overexpressing cells. HeLa cells were transfected with constructs encoding GFP, GFP-NLS, or MNB/DYRK1A as described in "Materials and Methods." The cells were then fixed, stained with anti-γ-tubulin antibody and TOTO-3, and observed by confocal laser scanning microscopy at the indicated time points. Each cell population (%) was determined by counting the centrosome number in each cell for a total of 200 cells. Paired centrosomes, which could be distinguished under microscopic observation, were scored as 2 centrosomes. The centrosome number was counted for non transfected cells (normal cells and those treated with LipofectAMINE but without a vector), for cells with fluorescence signal (GFP and GFP-NLS), and for cells with a diffuse distribution of fluorescence within their nuclei (GFP-MNB/DYRK1A). The data shown are means ± S.E. of 3 independent experiments.

of FKHR at 3 residues (Thr24, Ser253 and Ser319) catalyzed by protein kinase B through a phosphoinositide-3-kinase-dependent pathway. MNB/DYRK1A phosphorylates Ser329 residue on FKHR in vivo [19], but upstream kinases that can phosphorylate and activate MNB/DYRK1A are still unknown. The phosphorylation of these residues including Ser329 on FKHR has been reported to reduce the proportion of FKHR present within the nuclei and to decrease the ability of FKHR to stimulate gene transactivation [19–22]. MNB/DYRK1A in a discrete subnuclear structure may, therefore, play a role in the control of cell-cycle progression or apoptosis by regulating the nuclear level of FKHR.

HeLa cells with a speckled pattern in their nucleus progressed into M phase and produced 2 daughter cells, and both the condensation and subsequent segregation of chromosomes occurred in these cells as properly as in non-transfected control cells. On the other hand, in HeLa cells expressing a high level of MNB/DYRK1A, multinucleation was observed. Immunostaining with antibody specifically recognizing γ-tubulin revealed that the multinucleation had resulted from overduplication of the centrosome. Balczon and co-workers [23] reported that CHO cells arrested at the G1/S boundary of the cell cycle by treatment with hydroxyurea underwent multiple rounds of centrosome replication in the complete absence of DNA synthesis and cell division. Thus, one possible explanation for the overduplication of the centrosome, which was seen in this study, is that the overexpression of MNB/DYRK1A influences cell-cycle progression, possibly by affecting the nuclear level of FKHR. For cell division to occur properly, the centrosome must be duplicated once during each cell cycle; and thus in normal cells, the centrosome duplication cycle is tightly regulated. Failure of the normal cycle of this duplication would result either in cell-cycle arrest before the onset of mitosis or in the formation of an aberrant monopolar or multipolar spindle [24–27]. It is possible that the overexpression of MNB/DYRK1A induces the overduplication of the centrosome prior to the next mitosis, subsequently producing multiple spindle poles leading to the multinucleation.

MNB/DYRK1A is a dual specificity protein kinase whose activity depends on the phosphorylation of tyrosines in its activation loop [28,29]. Outside this catalytic domain, the sequence comprises a bipartite nuclear translocation signal (amino acids 105–139). A previous report showed that the nuclear translocation of MNB/DYRK1A is mediated by this signal sequence but that its characteristic subnuclear localization depends on additional N-terminal elements [11]. Thus, the protein kinase activity of MNB/DYRK1A is not required for its subnuclear localization. We have also obtained data leading to the same conclusion: when cDNA encoding kinase activity-negative mutants, GFP-MNB/DYRK1A (K179R) and GFP-MNB/DYRK1A (Y310F/Y312F), were used to transfect HeLa cells, speckled signals were detected in the nucleus (data not shown). On the other hand, cells with multinuclei were not observed if the kinase activity-negative mutants were overexpressed. It thus appears that the kinase activity of MNB/DYRK1A is essential for the formation of multinucleation, but not for its speckled subnuclear formation with FKHR.

Centrosome overduplication and multi-polar mitotic spindles were also observed in some tumor cells after γ-irradiation [30,31]. Since mitotic cell death was predominantly observed in these irradiated cells, cells expressing a higher level of MNB/DYRK1A than that in normal cells may die in a similar fashion. A possibility that mitotic cell death or apoptosis may occur in MNB/DYRK1A-overexpressing cells is worth studying, because brain development is markedly affected in Down syndrome patients and the number of neurons is reduced in their brain [32,33].

Conclusions

In this study, we found that overexpression of MNB/DYRK1A induced overduplication of the centrosome during interphase, resulting in aberrant spindles and missegregation of chromosomes during mitosis and subsequent multinucleation. A major goal of Down syndrome research is to correlate dosage imbalance of specific genes from human chromosome 21 with various clinical aspects of the syndrome. Our experimental system using overexpression of MNB/DYRK1A is a very useful model for studying the effect of gene dosage in Down syndrome *in vitro*. Further studies on the molecular mechanism underlying centrosome dysregulation by overexpression of MNB/DYRK1A should provide more important insights into the role of this protein kinase in Down syndrome.

Methods

Materials

The following materials were purchased from the sources indicated: Mouse antibodies against α-tubulin, γ-tubulin, and FLAG epitope from Sigma Chemical Co. (St. Louis, MO); Cy3-conjugated anti-mouse IgG antibody and Cy3-conjugated anti-rabbit IgG antibody from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA); fluorescein isothiocyanate-conjugated anti-mouse IgG antibody from Leinco Technologies, Inc. (St. Louis, MO); Hoechst33342 and TOTO-3 from Molecular Probes Inc. (Eugene, OR); LipofectAMINE reagent and Opti-MEM I from Invitrogen Co. (Carlsbad, CA). All other chemicals were commercial products of reagent grade.

Cell Culture

HeLa cells were maintained at 37°C in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's (DME) medium supplemented with 10% fetal calf serum and 10 μg/ml kanamycin.

Plasmid Construction

A full-length MNB/DYRK1A cDNA was isolated from a human fetal brain cDNA library as described previously [5]. For the construction of the GFP-MNB/DYRK1A expression vector, a carboxy terminal FLAG sequence was added by PCR to full-length MNB/DYRK1A cDNA by using specific primers (MNB2069F: 5'-caatcaggcctaccagaatcgccca-3', and MNB cFLAGR: 5'-ccgctcgagtctagatcacttgtcatcgtcgtccttgtagtccgagctagctacaggactct-3'), and it was subcloned into the pEGFPC2 expression vector (Clontech, San Diego, CA) at the HindIII and XhoI sites. For the construction of expression vectors for kinase activity-negative mutants (GFP-MNB/DYRK1A-K179R and GFP-MNB/DYRK1A-Y310F/Y312F), site-directed mutagenesis was performed by use of the following primers: MNB590 (KR) F, 5'-atgggttgccattagaataataaag-3', and MNB590 (KR) R, 5'-ctttattattctaatggcaacccat-3', and MNB988 (2YF) F, 5'-agaggatattccagtttattcagag-3', and MNB988 (2YF) R, 5'-ctctgaataaactggaatatcctct-3'. The PCR products were then subcloned into the GFP-MNB/DYRK1A expression vector to generate the GFP-MNB/DYRK1A (K179R) and GFP-MNB/DYRK1A (Y310F/Y312F) expression vectors. For the construction of a GFP-tagged NLS expression vector, the following oligonucleotides were synthesized: MNB381S, 5'-gaagatctcgaaaaagaagcgaagacaccaacagggccagggagacgattctagtcataagaaggaacggaagagctcaagcttcgaattccg-3'; and MNB381AS, cggaattcgaagcttgagctcttccgttccttcttatgactagaatcgtctccctggcccctgttggtgtcttcgcttctttttcgagatcttc-3'. After having been annealed and digested with BglII and EcoRI, the resulting BglII and EcoRI-codigested DNA fragment was subcloned into the BglII and EcoRI sites of the pEGFPC2 expression vector to generate the GFP-NLS expression vector. For the construction of a FLAG epitope-tagged expression vector for wild-type MNB/DYRK1A, GFP-MNB/DYRK1A was digested with HindIII and ApaI sites of the pcDNA3.1 vector (Invitrogen, Carlsbad, CA) to generate the pcDNA-MNB/DYRK1A. All constructs obtained were confirmed by nucleotide sequencing with an ABI377 DNA Sequencer (Applied Biosystems, Foster, CA).

Immunostaining of cells for confocal laser scanning microscopic observation

Transient transfection with constructs encoding GFP-MNB/DYRK1A and FLAG epitope-tagged MNB/DYRK1A were carried out by using LipofectAMINE reagent as recommended by the manufacturer. Briefly, HeLa cells were seeded onto cover slips in 35-mm dishes at least more than 24 hours before transfection. The plasmid DNA was mixed with LipofectAMINE reagent in serum-free Opti-MEM, incubated at room temperature for 30 minutes, and then added to the seeded cells. The total amount of transfected plasmid DNA was 1.0 μg per dish. At 3 hours after the addition of the plasmid DNA, the transfection mixture was replaced with DME medium supplemented with 10% fetal calf serum. The cells were then incubated in a CO_2 incubator for 24–48 hours, washed twice with ice-cold phosphate-buffered saline (PBS), and then fixed with methanol for 5 minutes at -20°C. The fixed cells were washed 3 times with PBS and were subsequently incubated for 2 hours with anti-α-tubulin antibody (1:200), anti-γ-tubulin antibody (1:200), anti-FLAG antibody (1:200), or anti-MNB antibody (1:50) [7]. They were then incubated for 1 hour with 1 μM TOTO-3 and goat Cy3-conjugated anti-mouse IgG antibody (1:100), donkey Cy3-conjugated anti-rabbit IgG antibody (1:100), or goat fluorescein isothiocyanate (FITC)-conjugated anti-mouse IgG antibody (1:100) at room temperature. The stained cells were observed with a confocal laser scanning microscope (MRC1024, Bio-Rad). A total of 200 cells were examined for centrosome number and for percentage of multinucleate cells in each of 3 independent experiments.

Microscopic observation of MNB/DYRK1A in living cells during the cell cycle

The procedure for preparation of fluorescently-stained living cells for microscopic observation was described previously [34,35]. Briefly, HeLa cells were plated on a 35-mm glass-bottom culture dish (MatTek Corp., Ashland, MA) and cultured for 1 day in a CO_2 incubator in 2 ml of DME medium supplemented with 10% fetal calf serum. The cells were then transiently transfected with the plasmid DNA encoding the GFP-MNB/DYRK1A fusion gene by using LipofectAMINE reagent. At 24 hours after the transfection, the cells were stained with 100 ng/ml of Hoechst33342 for 5–30 minutes and then washed 3 times with DME medium supplemented with 10% calf serum. The Hoechst33342-stained cells were cultured in a phenol red-free DME medium supplemented with 10% fetal bovine serum in a CO_2 incubator for at least 30 minutes and used for microscopic observation. This medium also contained 80 μg/ml of kanamycin sulfate and Hepes buffer (pH 7.4) at a final 20 mM concentration.

Author's contribution

EF carried out the construction of the expression vectors and immunocytochemical studies, and drafted the manuscript. TH and YH participated in the observation of dynamics of chromosomes and MNB/DYRK1A in living cells during the cell cycle. JK and TH participated in the design of the study. FI and NS conceived the study, raised

the funding for the project, and participated in its design and coordination.

All authors read and approved the final manuscript.

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