FACTORS GOVERNING THE PATTERN OF SPINDLE MICROTUBULE REGROWTH AFTER TUBULIN DEPOLYMERIZATION

© *G. A. Pavlova*,[1, 2, #] *Yu. A. Galimova*,[1, #] *Yu. V. Popova*,[1, 3] *A. F. Munzarova*,[1, 4] *A. V. Razuvaeva*,[1, 4] *A. L. Alekseeva*,[1] *M. B. Berkaeva*,[1] *A. V. Pindyurin*,[1, 3, 4] *M. P. Somma*,[5] *M. Gatti*,[1, 5] *F. Renda*[5, *]

[1] *Institute of Molecular and Cellular Biology SB RAS, Novosibirsk, 630090, Russia,*
[2] *Kazan Federal University, Kazan, 420008, Russia,*
[3] *Institute of Cytology and Genetics SB RAS, Novosibirsk, 630090, Russia,*
[4] *Novosibirsk State University, Novosibirsk, 630090, Russia, and*
[5] *IBPM CNR and Department of Biology and Biotechnology, Sapienza University of Rome, Rome, 00185, Italy;*
[#] *These authors contributed equally to this work*
* *e-mail: fioranna.renda@uniroma1.it*

We analyzed the pattern of spindle microtubule (MT) regrowth after cold- or colcemid-induced MT depolymerization in *Drosophila* S2 cells. Cold-induced MT disassembly at low temperature (–2 °C) destroyed kinetochore-driven MT regrowth without affecting astral MT formation. Conversely, colcemid-induced MT depolymerization strongly impaired centrosome-dependent MT nucleation, allowing kinetochore-driven MT regrowth. Collectively, these results indicate that the kinetochore- and the centrosome-mediated MT assembly pathways exploit molecular mechanisms that are at least in part different.

Key words: mitosis, microtubules, spindle assembly, cold treatment, colcemid, *Drosophila.*

Abbreviations: MT — microtubule, PCM — pericentriolar material, γ-TuRCs — γ-tubulin ring complexes.

The spindle is a microtubule (MT)-based, highly dynamic molecular machine that mediates chromosome segregation during mitosis and meiosis. Spindle assembly requires extensive polymerization of MTs and their organization into a fusiform structure through the action of MT-associated molecular motors. In most animal cells, the spindle MTs originate from both the centrosomes and the chromosomes/kinetochores. In addition, it has been shown that MTs can emanate from preexisting MTs within the spindle. In all cases MTs are nucleated by the γ-tubulin ring complexes (γ-TuRCs), which are either embedded into the centrosome, free in the cytoplasm near the chromosomes, or bound to the sides of preexisting MTs via the augmin complex (reviewed by Duncan, Wakefield, 2011; Gatti et al., 2012).

Systems lacking centrosomes, such as higher plants and animal oocytes, can form functional spindles exploiting MTs nucleated near the chromosomes. Chromosome-driven MT formation occurs also in systems with centrosomes. Examination of cells undergoing mitosis with unreplicated genomes, in which kinetochores and the bulk of chromatin are well separated, showed that kinetochores can drive MT growth more efficiently than chromatin, suggesting that kinetochores play a dominant role in chromosome-driven MT assembly (O'Connell et al., 2009). Live imaging of mitosis suggested that the plus ends of the MTs nucleated near the chromosomes are captured by the kinetochores and continue to polymerize there, forming kinetochore fibers (Maiato et al., 2004). These kinetochore-driven k-fibers are then captured by the astral MTs and integrated into a bipolar spindle (Maiato et al., 2004). The mechanism underlying kinetochore-driven MT growth involves MT nucleating/stabilizing factors such as TPX2, localized RanGTP, and augmin-mediated MT growth (Tulu et al., 2006; Goshima et al., 2008; Torosantucci et al., 2008; Bucciarelli et al., 2009; Mishra et al., 2010; Duncan, Wakefield, 2011; Gatti et al., 2012).

One of the approaches to investigate the mechanisms of kinetochore-driven MT growth and their relationships with those governing centrosome-dependent astral MT formation is the analysis of MT regrowth after spindle MT depolymerization. MTs can be depolymerized using antimitotic drugs or cold-exposure. Antimitotic drugs such as colcemid or nocodazole block MT polymerization by reversibly binding soluble tubulin (Jordan, Wilson, 1999), and have been largely used for analyzing spindle reassembly. For example, a colcemid-based assay showed that augmin-depleted *Drosophila* S2 cells are defective in regrowth of kinetochore-associated MT bundles (Goshima et al., 2008). Moreover, the analysis of mammalian cells treated with nocodazole showed that kinetochore-driven MT regrowth specifically requires RanGTP and the Ran-dependent spindle assembly factor TPX2 (Tulu et al., 2006; Torosantucci et al., 2008).

When exposed to cold (0 °C or less), spindle MTs disassemble and quickly reassemble when cells are brought back to physiological temperature. An analysis of MT regrowth after cold exposure showed that augmin depletion suppresses MT regrowth from chromosomes/kinetochores in both em-

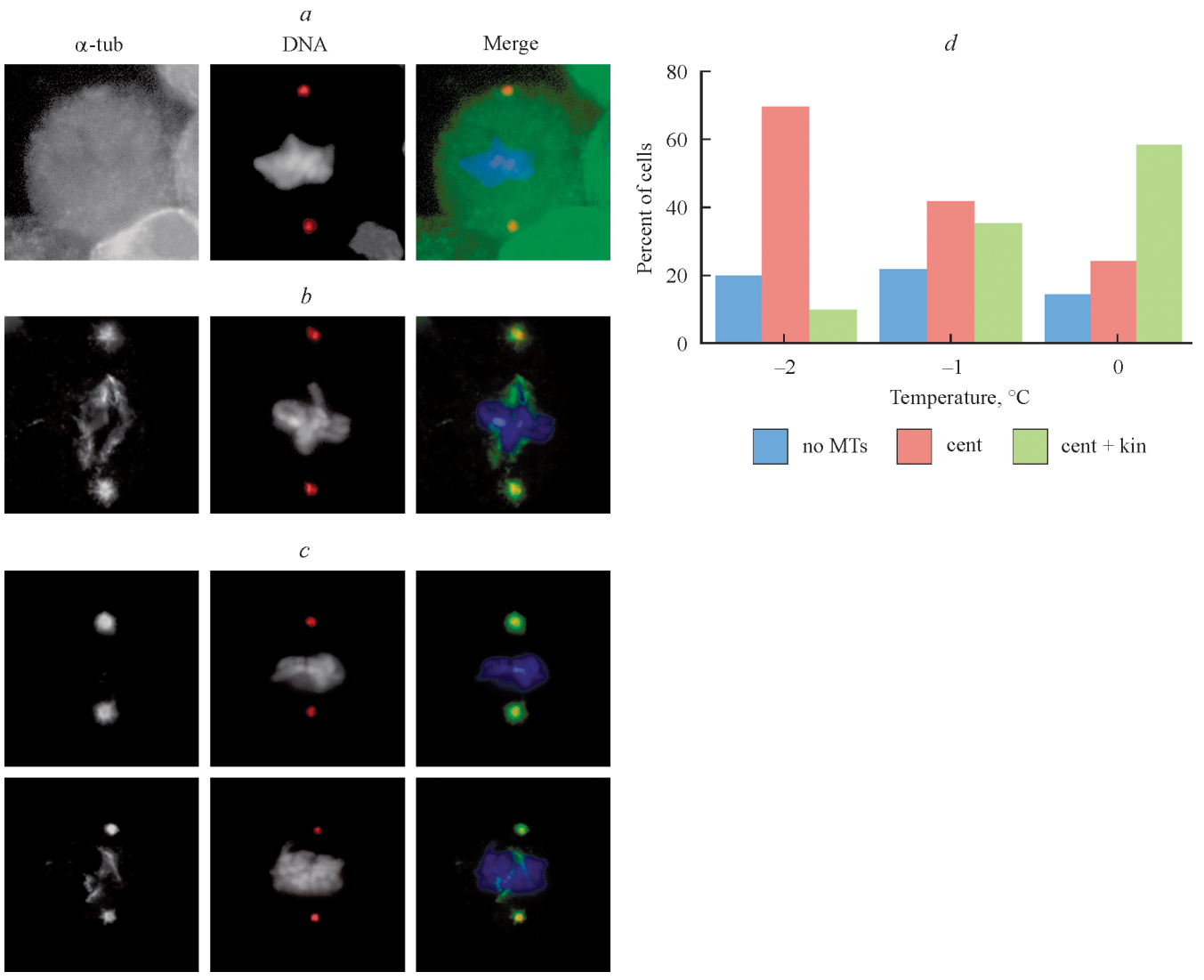

Fig. 1. MT regrowth after cold exposure.

a — metaphase with depolymerized spindle MTs; *b* — MT regrowth from a metaphase exposed to 0 °C and then returned at 22 °C for 30 s; *c* — MT regrowth from cells exposed to –2 °C and then returned at 22 °C for 30 s; cells were exposed for 3 h to the indicated temperature; they were then fixed and stained 30 s after their return to 22 °C. *Red colour* — DSpd2 staining. *d* — frequencies of cells showing no MT regrowth (no MTs), regrowth from centrosomes only (cent) or regrowth from both centrosomes and kinetochores (cent + kin).

bryonic and S2 *Drosophila* cells with little or no effect on astral MT formation (Bucciarelli et al., 2009; Hayward et al., 2014). The same assay revealed that Misato (Mst) and the Mst-interacting TCP-1 tubulin-folding complex are primarily required for chromosome-driven MT regrowth in *Drosophila* brain cells (Mottier-Pavie et al., 2011; Palumbo et al., 2015). The MT regrowth assay after cold-induced MT depolymerization has been also employed in mammalian cells; in agreement with the nocodazole-based assay, it has been shown that RanGTP accumulation at kinetochores is essential for spindle MT regrowth (Torosantucci et al., 2008).

Although both drug- and cold-induced MT depolymerization allows the analysis of spindle MT regrowth, the results obtained with these two depolymerization methods are not identical. Studies performed in mammalian cells have shown that the first regrowing MTs after nocodazole-induced depolymerization are those emanating from the kinetochores (Tulu et al., 2006; Torosantucci et al., 2008). In contrast, when MTs are depolymerized by cold exposure, the centrosome-driven astral MTs are the first to regrow (Torosantucci et al., 2008). These findings prompted us to analyze the effects of the MT depolymerization procedure on MT regrowth in *Drosophila* S2 cells. We found that the depolymerization method affects the MT regrowth pattern and the spindle reassembly process.

Material and Methods

The *Drosophila* S2 cells used here have been described previously (Somma et al., 2008). These cells were originally grown in Shields and Sang M3 medium (Sigma, USA) supplemented with 20 % heat-inactivated fetal bovine serum (FBS, Gibco, USA). However, since 2009, they were grown in Schneider's medium (Sigma, USA) supplemented with 10 % FBS. For the work described here, the cells have been readapted to the M3 medium with 20 % FBS; the experiments have been performed two months after re-adaptation to the M3 medium.

Colcemid (10 μg/ml solution; Sigma, USA) was added to medium at the final concentration of 3.3 μg/ml. After 3 h in-

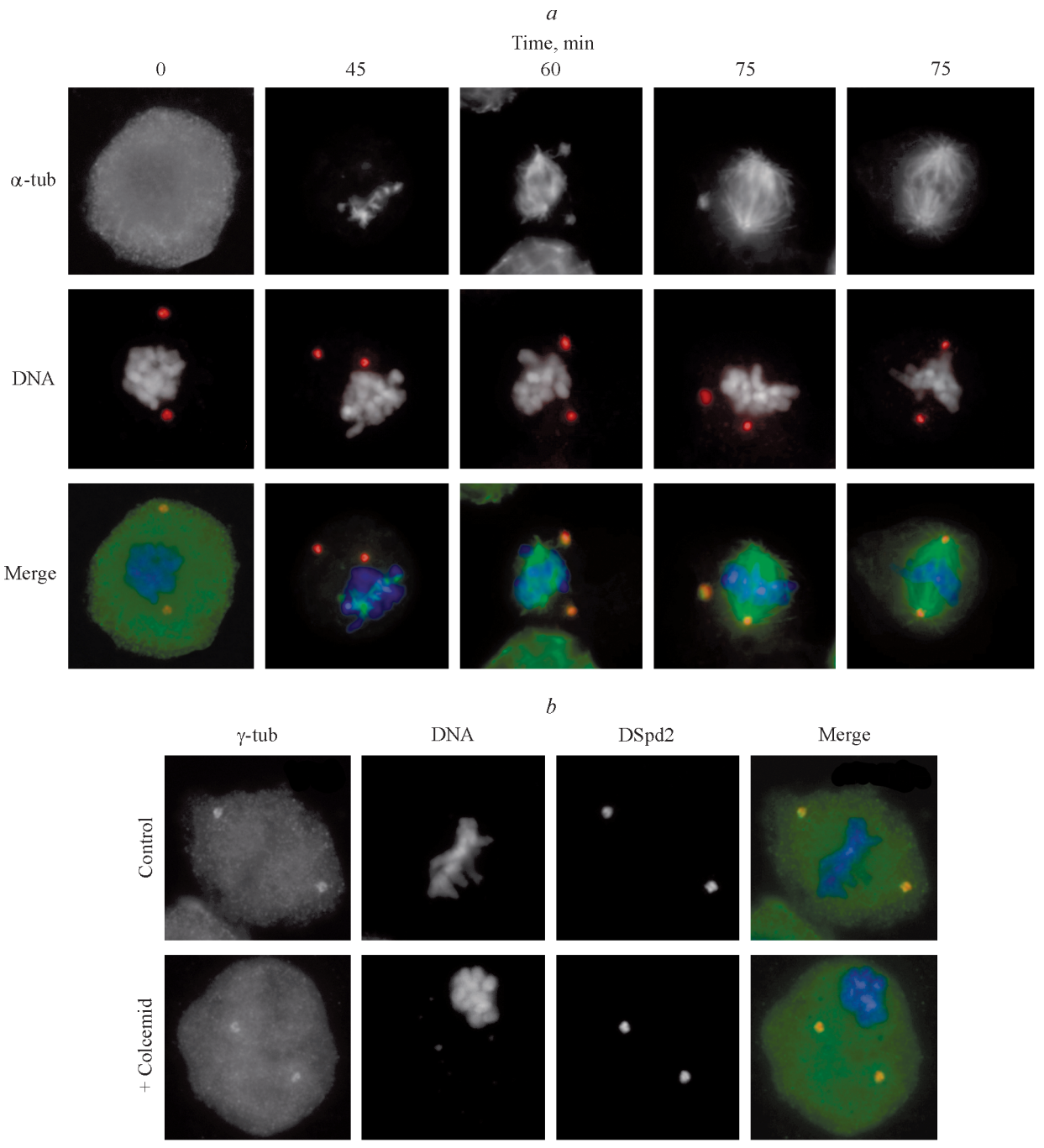

Fig. 2. MT regrowth after colcemid treatment.

a — MT regrowth from cells fixed 0, 45, 60 and 75 min after colcemid removal; note the metaphases with the centrosomes detached from the spindle poles; *red colour* — DSpd2 staining. *b* — control and colcemid-treated (+ colcemid) cells exhibit comparable centrosome structures as revealed by γ-tubulin (γ-tub) or DSpd2 staining.

cubation, colcemid was removed by washing the cells three times in drug-free medium. For the analysis of MT regrowth after cold treatment, cells were returned to 22 °C for 30 s and then fixed. In all cases, fixation and immunostaining were perfomed according to (Somma et al., 2008), using the following antibodies and dilutions. Primary antibodies: mouse anti-α-tubulin (Sigma T6199, 1 : 200), mouse anti-γ-tubulin (Sigma T6557, 1 : 100) and rabbit anti-DSpd2 (Giansanti et al., 2008, 1 : 4000). Secondary antibodies: goat anti-mouse-FITC (Sigma F8264, 1 : 20) and goat anti-rabbit-Alexa-568 (Invitrogen A11036, 1 : 300).

Results and Discussion

The temperature used for MT depolymerization affects the pattern of MT regrowth. To investigate the pattern of MT regrowth after cold-induced depolymerization, S2 cells have been incubated for 3 h at 3 temperatures: 0, –1 and –2 °C. Incubation at different temperatures resulted in different frequencies of prometaphase/metaphase cells with completely depolymerized spindles (Fig. 1, *a*); at 0, –1 and –2 °C the frequencies of fully depolymerized cells were 72, 75 and 90 %, respectively. The analysis of pro-

metaphase/metaphase cells returned to 22 °C for 30 s, and then fixed and stained for both α-tubulin and DSpd2, revealed that the pattern of MT regrowth depends on the depolymerization temperature. Most cells (60 %) incubated at 0 °C displayed both nascent kinetochore-associated MT bundles and short centrosome-driven astral MTs (Fig. 1, *b*, *d*). In cells incubated at –1 °C, regrowth from both kinetochores and centrosomes was reduced to 37 % of the cells (Fig. 1, *d*). Finally, in cells incubated at –2 °C, MTs were mostly regrowing from the centrosomes (70 %); in the few cells (8 %) in which MT regrowth was from both kinetochores and centrosomes, the nascent kinetochore-associated MT bundles were thinner than those observed in cells incubated at 0 °C (Fig. 1, *c*, *d* and data not shown). It should be noted that the regrowing asters observed in cells incubated at –2 °C are much larger than those observed in cells incubated at 0 °C and often even larger than those observed in untreated prometaphase/metaphase cells. As already suggested, this finding might reflect availability of an increased pool of unpolymerized tubulin, due to the inhibition of MT regrowth from kinetochores (Tulu et al., 2006; Bucciarelli et al., 2009; Hayward et al., 2014). Collectively, these results indicate that the capability of kinetochores to drive MT growth is severely compromised by exposures to low temperatures (–2 °C). In contrast, exposure of cells at –2 °C does not affect MT nucleation from the centrosomes.

Colcemid treatment disrupts MT regrowth from centrosomes but not from kinetochores. 3 h of colcemid treatment resulted in a high degree of spindle MT depolymerization. In 95 % of the prometaphase/metaphase cells, the spindle was completely depolymerized (Fig. 2, *a*). To follow MT regrowth, after colcemid removal cells were fixed after 45, 60 or 75 min, and then immunostained for α-tubulin and DSpd2. At 45 min after colcemid removal, regrowing MTs were only observed at kinetochores but not around centrosomes. At the 60 min regrowth time, the cells displayed kinetochore-driven MT bundles, which at the 75 min time converged at the spindle poles giving rise to bipolar spindles (Fig. 2, *a*). Notably, we rarely observed clear MT regrowth from the centrosomes; most cells showed anastral spindles not associated with the centrosomes; only in a few cases, the centrosomes were found at one or both spindle poles (Fig. 2, *a*). Thus, MT depolymerization with colcemid disrupts the ability of prometaphase/metaphase cells to reform MTs from the centrosomes but not from the kinetochores. To understand why colcemid treatment inhibits MT nucleation from centrosomes, we asked whether this drug causes pericentriolar material (PCM) fragmentation, as observed in mammalian cells treated with low concentrations of either colcemid or nocodazole (Sellitto, Kuriyama, 1988; Jordan et al., 1992). Immunostaining of colcemid-treated cells with antibodies against DSpd2 or γ-tubulin showed that the PCM remains compact, even if the centrosomes have lost their MT nucleating ability (Fig. 2, *b*).

Conclusions. Our results provide novel methodological tools for the dissection of the chromosome- and the centrosome-dependent pathways of MT formation during spindle assembly. Using RNAi, it is possible to deplete S2 cells of virtually any protein involved in mitotic division (Goshima et al., 2007; Somma et al., 2008). Employing a suitable combination of the MT depolymerization methods described here, we can now determine whether the RNAi-mediated depletion of a given protein differentially affects kinetochore- and/or centrosome-driven MT regrowth. We unexpectedly found that MT depolymerization at –2 °C and colcemid treatment specifically impair regrowth from kinetochores and centrosomes, respectively. These results are intriguing and suggest that the cold and colcemid treatments selectively inactivate different kinetochore- and centrosome-associated determinants of MT regrowth. However, the nature of these determinants is currently unknown and their definition will be the aim of our future studies, which will include ultrastructural analysis of spindle MT regrowth.

The work at the Institute of Molecular and Cellular Biology SB RAS was supported by a grant from the Ministry of Education and Science of the Russian Federation (14.Z50.31.0005) to MG.

References

Bucciarelli E., *Pellacani C.*, *Naim V.*, *Palena A.*, *Gatti M.*, *Somma M. P. 2009. Drosophila* Dgt6 interacts with Ndc80, Msps/XMAP215, and gamma-tubulin to promote kinetochore-driven MT formation. Curr. Biol. 19 : 1839—1845.

Duncan T., *Wakefield J. G. 2011.* 50 ways to build a spindle: the complexity of microtubule generation during mitosis. Chromosome Res. 19 : 321—333.

Gatti M., *Bucciarelli E.*, *Lattao R.*, *Pellacani C.*, *Mottier-Pavie V.*, *Giansanti M. G.*, *Somma M. P.*, *Bonaccorsi S. 2012.*The relative roles of centrosomal and kinetochore-driven microtubules in *Drosophila* spindle formation. Exp. Cell Res. 318 : 1375—1380.

Giansanti M. G., *Bucciarelli E.*, *Bonaccorsi S.*, *Gatti M. 2008. Drosophila* SPD-2 is an essential centriole component required for PCM recruitment and astral-microtubule nucleation. Curr. Biol. 18 : 303—309.

Goshima G., *Mayer M.*, *Zhang N.*, *Stuurman N.*, *Vale R. D. 2008.* Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle. J. Cell Biol. 181 : 421—429.

Goshima G., *Wollman R.*, *Goodwin S. S.*, *Zhang N.*, *Scholey J. M.*, *Vale R. D.*, *Stuurman N. 2007.* Genes required for mitotic spindle assembly in *Drosophila* S2 cells. Science. 316 : 417—421.

Hayward D., *Metz J.*, *Pellacani C.*, *Wakefield J. G. 2014*. Synergy between multiple microtubule-generating pathways confers robustness to centrosome-driven mitotic spindle formation. Dev. Cell. 28 : 81—93.

Jordan M. A., *Thrower D.*, *Wilson L. 1992.* Effects of vinblastine, podophyllotoxin and nocodazole on mitotic spindles. Implications for the role of microtubule dynamics in mitosis. J. Cell Sci. 102 : 401—416.

Jordan M. A., *Wilson L. 1999*. The use and action of drugs in analyzing mitosis. Methods Cell Biol. 61 : 267—295.

Maiato H., *Rieder C. L.*, *Khodjakov A. 2004.* Kinetochore-driven formation of kinetochore fibers contributes to spindle assembly during animal mitosis. J. Cell Biol. 167 : 831—840.

Mishra R. K., *Chakraborty P.*, *Arnaoutov A.*, *Fontoura B. M.*, *Dasso M. 2010.* The Nup107-160 complex and gamma-TuRC regulate microtubule polymerization at kinetochores. Nat. Cell Biol. 12 : 164—169.

Mottier-Pavie V., *Cenci G.*, *Verni F.*, *Gatti M.*, *Bonaccorsi S. 2011.* Phenotypic analysis of *misato* function reveals roles of noncentrosomal microtubules in *Drosophila* spindle formation. J. Cell Sci. 124 : 706—717.

O'Connell C. B., *Loncarek J.*, *Kaláb P.*, *Khodjakov A. 2009.* Relative contributions of chromatin and kinetochores to mitotic spindle assembly. J. Cell Biol. 187 : 43—51.

Palumbo V., *Pellacani C.*, *Heesom K. J.*, *Rogala K. B.*, *Deane C. M.*, *Mottier-Pavie V.*, *Gatti M.*, *Bonaccorsi S.*, *Wakefield J. G. 2015.* Misato controls mitotic microtubule generation by stabilizing the TCP-1 tubulin chaperone complex. Curr. Biol. 25 : 1777—1783.

Sellitto C., *Kuriyama R. 1988*. Distribution of pericentriolar material in multipolar spindles induced by colcemid treatment in Chinese hamster ovary cells. J. Cell Sci. 89 : 57—65.

Somma M. P., *Ceprani F.*, *Bucciarelli E.*, *Naim V.*, *De Arcangelis V.*, *Piergentili R.*, *Palena A.*, *Ciapponi L.*, *Giansanti M. G.*, *Pellacani C.*, *Petrucci R.*, *Cenci G.*, *Verni F.*, *Fasulo B.*, *Goldberg M. L.*, *Di Cunto F.*, *Gatti M. 2008.* Identification of *Drosophila* mitotic genes by combining co-expression analysis and RNA interference. PLoS Genet. 4 : e1000126.

Torosantucci L., *De Luca M.*, *Guarguaglini G.*, *Lavia P.*, *Degrassi F. 2008.* Localized RanGTP accumulation promotes microtubule nucleation at kinetochores in somatic mammalian cells. Mol. Biol. Cell. 19 : 1873—1882.

Tulu U. S., *Fagerstrom C.*, *Ferenz N. P.*, *Wadsworth P. 2006.* Molecular requirements for kinetochore-associated microtubule formation in mammalian cells. Curr. Biol. 16 : 536—541.

Received 1 XII 2015

ФАКТОРЫ, ОБУСЛОВЛИВАЮЩИЕ ХАРАКТЕР ПОВТОРНОГО РОСТА МИКРОТРУБОЧЕК ВЕРЕТЕНА ДЕЛЕНИЯ ПОСЛЕ ДЕПОЛИМЕРИЗАЦИИ ТУБУЛИНА

Г. А. Павлова,[1, 2, #] *Ю. А. Галимова*,[1, #] *Ю. В. Попова*,[1, 3] *А. Ф. Мунзарова*,[1, 4] *А. В. Разуваева*,[1, 4] *А. Л. Алексеева*,[1] *М. Б. Беркаева*,[1] *А. В. Пиндюрин*,[1, 3, 4] *М. П. Сомма*,[5] *М. Гатти*,[1, 5] *Ф. Ренда*[5, *]

[1] Институт молекулярной и клеточной биологии СО РАН, Новосибирск, 630090, Россия,
[2] Казанский (Приволжский) федеральный университет, Казань, 420008, Россия,
[3] Институт цитологии и генетики СО РАН, Новосибирск, 630090, Россия,
[4] Новосибирский государственный университет, Новосибирск, 630090, Россия,
[5] Институт молекулярной биологии и патологии Национального исследовательского совета и Отделение биологии и биотехнологии, Римский университет Ла Сапиенса, Рим, 00185, Италия;
[#] Эти авторы внесли равный вклад в работу
* электронный адрес: fioranna.renda@uniroma1.it

Мы проанализировали характер повторного роста микротрубочек (МТ) веретена деления после их деполимеризации, вызванной обработкой холодом или колцемидом в клетках S2 дрозофилы. Разборка МТ, вызванная низкой температурой (–2 °C), приводила к нарушению повторного роста МТ от кинетохоров, но не влияла на повторное формирование астральных МТ. Напротив, деполимеризация МТ, вызванная колцемидом, существенно нарушала нуклеацию МТ на центросомах, но позволяла повторный рост МТ от кинетохоров. В совокупности эти результаты указывают на то, что в кинетохор- и центросомзависимые пути сборки МТ вовлечены, по крайней мере частично, разные молекулярные механизмы.

Ключевые слова: митоз, микротрубочки, сборка веретена, холодовая обработка, колцемид, *Drosophila.*