Excessive Expression of the Plant Kinesin TBK5 Converts Cortical and Perinuclear Microtubules into a Radial Array Emanating From a Single Focus

Yuhei Goto 1, 2 and Tetsuhiro Asada 1, *

1 Department of Biological Science, Graduate School of Science, Osaka University, Toyonaka, Osaka, 560-0043 Japan

TBK5 is a plant-specific kinesin constantly expressed in tobacco BY-2 cells. An analysis of the distribution of green fluorescent protein-tagged TBK5 (GFP–TBK5) transiently expressed in BY-2 protoplasts revealed that TBK5 could associate with microtubules in vivo. GFP–TBK5 often assembled to form a single particle when accumulated in cells. The particle was located in close proximity to the nucleus, and its formation was accompanied by the development of a radial array of microtubules emanating from it and the loss of cortical microtubules. Microtubule depolymerization by treatment with propyzamide inhibited particle formation and stimulated the formation of dispersed aggregates of GFP–TBK5. Through expression of different TBK5 mutants as GFP fusions, the motor domain, two separated coiled-coil domains and the C-terminal domain of TBK5 were identified as the domains playing essential roles in particle formation. Mutants with putatively non-motile motor domains or lacking the C-terminal domain were localized to cortical and perinuclear microtubules, whereas those lacking either of the coiled-coil domains were preferentially distributed around the nucleus and along perinuclear microtubules. Further, the deletion of one of the coiled-coil domains or the C-terminal domain was sufficient to inhibit the propyzamide-induced formation of dispersed aggregates, whereas the mutation in the motor domain was not. These results led us to propose a model in which the particle is formed through the microtubule-based movement of GFP–TBK5 toward the nucleus and subsequent microtubule-independent aggregation based on coiled-coil interactions. The dramatic microtubule rearrangement would be explained if GFP–TBK5 relocated and gathered newly formed microtubules and/or microtubule-nucleating units.

Keywords: Cortical microtubules — Kinesin — Microtubule — Microtubule-organizing center — Tobacco BY-2 cell.

Introduction

The microtubule cytoskeleton in eukaryotic cells forms the mitotic spindle and other cell type-specific arrays required for motility, morphogenesis and division of cells. The formation of microtubules in the cytoplasm relies on microtubule-nucleating units to prepare microtubule seeds for promoting tubulin polymerization (Hyman and Karsenti 1998, Job et al. 2003). The formation of focused microtubule arrays, seen in many animal cell types that contain centrosomes, is based on the localization of microtubule-nucleating units to the compact organelles that function as microtubule-organizing centers (Lüders and Stearns 2007). Land plant cells lack centrosomes or their equivalents, and contain dispersed microtubule-nucleating units to generate diverse microtubule arrays with characteristically non-focused or parallel nature (Vaughn and Harper 1998, Wasteneys 2002, Schmit 2002, Chan et al. 2003, Murata et al. 2005, Lüders and Stearns 2007). One of the major problems in understanding the mechanism of microtubule organization in land plant cells lies in regulating the distribution of microtubule-nucleating sites (Mazia 1984, Lamberti 1995). Theoretically, modification of this regulation could result in concentration of microtubule-nucleating sites to generate an unusually focused array. Even when induced artificially, such a phenomenon could provide a useful system for dissecting the unknown mechanism of microtubule organization in land plant cells.

The kinesin superfamily of eukaryotic microtubule motors includes some subfamilies whose members were initially identified in fungal and animal cells as key players in the formation of the bipolar spindle (Vale 2003, Lawrence et al. 2004) and then identified from plants or cultured plant cells (Mitsui et al. 1993, Asada et al. 1997, Oppenheimer et al. 1997, Reddy et al. 1997, Matsui et al. 2001, Preuss et al. 2003, Preuss et al. 2004, Ambrose et al. 2005). Interestingly, one of these subfamilies, kinesin-14,
is far more diverse in plants than in other eukaryotes (Richardson et al. 2006) and includes plant-specific variants such as TBK5 (Matsui et al. 2001). TBK5 was identified from tobacco BY-2 suspension culture cells, where its mRNA was constantly present at a high level (Matsui et al. 2001). A recent comprehensive analysis of genomic sequences by Richardson et al. (2006) allows identification of its counterparts not only in Arabidopsis but also in Oryza and Populus. In addition to the closely related motor domain, TBK5 and other kinesin-14s share a similar neck domain. Because of this similarity, TBK5 has been predicted to be a minus end-directed motor (Matsui et al. 2001). The unique structural features that distinguish TBK5 polypeptide from other characterized kinesin-14s are the presence of two coiled-coiled regions separated by a centrally located motor domain and a positively charged C-terminal region. The actual activities, subcellular localization, in vivo function and role of this kinesin remain to be elucidated.

In this study, we analyzed the distribution of green fluorescent protein (GFP)-tagged TBK5 transiently expressed in tobacco BY-2 protoplasts. This analysis, which originally aimed at obtaining information about the subcellular localization of TBK5, demonstrated that the expressed GFP-tagged TBK5 proteins often assembled to form a single particle, with this generating an unusual radial array of microtubules emanating from a focus. Here we describe the detection and partial characterization of this artificial phenomenon.

Results and Discussion

Protoplasts of tobacco BY-2 cells were transformed with plasmids, which were designed to express the full-length TBK5 transiently as GFP fusions under the control of the cauliflower mosaic virus 35S promoter. Upon plasmid introduction, approximately half of the GFP-positive cells contained a fibrous pattern of green fluorescence after being cultured for 15–25 h (Fig. 1a). Tubulin immunostaining confirmed that this pattern corresponded to that of cortical and perinuclear microtubules in cells (Fig. 2a, left). The other half of the GFP-positive cells contained a single green fluorescent particle that had either an aster-like (Fig. 1b) or a globular shape (Fig. 1c). This particle was always located in close proximity to the nucleus. Distributions of the expressed N- and C-terminal GFP fusions were indistinguishable, suggesting that the GFP tag did not interfere with the original action of TBK5.

Labeling of the cells by green fluorescence appeared initially marked by a cytosolic green fluorescence in which fibrous patterns were visible. The cells containing fluorescent particles were detected 12 h after plasmid introduction, and the ratio of those cells increased in a consistent manner with time (Fig. 1d). Therefore, expressed fusion proteins were initially distributed diffusely in the cytoplasm and along microtubules before pooling to generate the fluorescent particles. Cells marked by the aster-shaped particle were rarely observed, especially in the initial and terminal phases of transient expression. Absence of the fluorescent particle in cells during the early stage of transient expression suggests that the induction of particle formation requires the presence of a high level of GFP–TBK5. Since the changes were not reversed at the late stage of transient expression, where the expression level was probably reduced, the particles would have probably remained static. Rather than at the beginning of the expression, the fluorescent particles were always larger toward the end of transient expression (data not shown), where dead cells with protruding cytoplasm and fluorescent particles were occasionally observed.

Indirect immunofluorescence using anti-α-tubulin antibodies revealed that non-transformed control cells typically contained plasma membrane-associated cortical microtubules, and those distributed around the nucleus and in cytoplasm between the nucleus and plasma membrane (Fig. 2b). In cells with a relatively small GFP–TBK5 particle, the number of cortical microtubules was reduced, and microtubules were arranged in an aster-shaped array that sharply focused on the particle (Fig. 2a, center). In cells with a larger GFP–TBK5 particle, while cortical microtubules were depleted, microtubules arranged in a radial array appeared to emanate from the particle (Fig. 2a, right). Co-localization of GFP–TBK5 and microtubules was observed only on the particle.

Cytochalasin E, an inhibitor of actin filament assembly (Kobayashi and Hakuno 2003), did not affect particle formation (Fig. 1e). However, propyzamide, an inhibitor of microtubule assembly (Akashi et al., 1988), not only inhibited particle formation but also stimulated the formation of dispersed aggregates of GFP–TBK5 (Fig. 1e, f). Indirect immunofluorescence using anti-α-tubulin antibodies confirmed that propyzamide treatment abolished microtubules in cells, and revealed that the dispersed aggregates of GFP–TBK5 contained tubulin (Fig. 2d). In cells cultured for 20 min after the termination of propyzamide treatment, microtubules formed around the dispersed GFP–TBK5 aggregates (Fig. 2c). In the same cells, recovery of cortical microtubule was less detectable (Fig. 2c). Propyzamide treatments caused fragmentation of the previously formed particles, while dispersed aggregates tended to coalesce after the termination of propyzamide treatment (data not shown).

The inhibition of particle formation by propyzamide indicated that intact microtubules were required for
Fig. 1 Distribution of transiently expressed GFP–TBK5 fusion proteins in tobacco BY-2 cells. (a) Fibrous patterns of fluorescence observed in the periphery (left) and center (right) of cells 16 h after transformation. Cells with an aster-shaped fluorescent particle (b) or a globular fluorescent particle (c) were observed. The photographs were prepared by superimposing the images of differential interference contrast microscopy and those of fluorescence microscopy (inserts show only the fluorescence image). N, nucleus (d) A time course of changes in the percentage of each green fluorescent pattern (bar) and GFP–TBK5-expressing cells (line). (e) Effects of propyzamide on the formation of GFP–TBK5 particles. Transformed cells were cultured for 8 h in normal conditions, and further cultured for 4 h in the presence of 1% DMSO, 1% DMSO plus 100 μM cytochalasin E or 1% DMSO plus 100 μM propyzamide. (f) Dispersed aggregates of GFP–TBK5 generated by the propyzamide treatment. Scale bar = 5 μm.
concentrating expressed GFP–TBK5 into single particles, while the propyzamide-stimulated formation of dispersed aggregates suggested that some mechanisms existed through which GFP–TBK5 can locally and efficiently be gathered without the involvement of intact microtubules. To understand the mechanism by which GFP–TBK5 proteins assembled to form a particle or aggregates, we prepared six different mutant TBK5 proteins (Fig. 3b). TBK5ΔN lacks a small N-terminal domain, whereas TBK5ΔC lacks the C-terminal domain with a calculated pI of 10.74. TBK5T182N is a putative non-motile, rigor-type mutant that has a point mutation within an ATP-binding consensus motif located in the motor domain (Nakata and Hirokawa 1995). TBK5ΔNC lacks the coiled-coil domain located on the N-terminal side of the motor domain, whereas TBK5ΔCC is deficient of the other coiled-coil domain. TBK5C consists of the basic C-terminal domain.

The distribution of GFP–TBK5ΔN was indistinguishable from that of GFP–TBK5 (Fig. 3c). Expression of GFP–TBK5T182N yielded fibrous patterns of green fluorescence (Fig. 3d), without the formation of fluorescent particles (Fig. 3c). When GFP–TBK5T182N-expressing cells were treated with propyzamide, however, these cells generated dispersed aggregates of the fluorescent protein (Fig. 3c, e). Thus, the TBK5 motor domain might actually exhibit ATP hydrolysis-dependent activity, with this being essential for particle formation, but not for local aggregation.

The expression of GFP–TBK5ΔNC and GFP–TBK5ΔCC resulted in fluorescent labeling preferentially distributed around the nucleus and along the perinuclear microtubules (Fig. 3d) without yielding green fluorescent particles (Fig. 3c). Propyzamide treatment of these cells did not induce formation of dispersed aggregates (Fig. 3c). Thus, coiled-coil interactions on both sides of the motor domain seem to be the essential events for particle formation and local microtubule-independent aggregation. Propyzamide-treated GFP–TBK5ΔNC- and GFP–TBK5ΔCC-expressing cells devoid of any bright fluorescent dots were marked with cytosolic fluorescence, when observed under conventional fluorescent microscopy (data not shown). Observations of the propyzamide-treated cells with a microscope equipped with an image deconvolution system, however, clearly visualized that fluorescent fibers were located around the nucleus (Fig. 3e). This observation suggests that association of the fusion protein increases the stability of microtubules.

TBK5 was predicted to be a minus-end directed motor, because its motor and neck domains resemble those of other kinesin-14s (Case et al. 1997, Endow and Waligora 1998, Endow and Higuchi 2000, Matsui et al. 2001). These data can be combined with results of the expression of GFP–TBK5T182N, GFP–TBK5ΔNC and GFP–TBK5ΔCC to

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Fig. 2 Microtubule reorganization associated with the formation and growth of the GFP–TBK5 particle. (a) Distributions of microtubules (white) and GFP–TBK5 (green) are indicated accordingly. Cells with fibrous microtubule patterns (left), a relatively small particle (center) and a larger particle (right) of GFP–TBK5 were stained with anti-α-tubulin antibodies. (b) Microtubule arrangement in a non-transformed cell that had been cultured overnight after cell wall digestion. (c) Microtubule regeneration from the GFP–TBK5 aggregates. Transformed cells were cultured for 4 h in the presence of 100 μM propyzamide, and further incubated for 20 min without propyzamide. Immunostaining with anti-α-tubulin antibodies visualized microtubules (red), which extended from the GFP–TBK5 aggregates. (d) Detection of tubulins in GFP–TBK5 aggregates. Transformed cells were cultured for 4 h in the presence of 100 μM propyzamide and analyzed by indirect immunofluorescence using anti-α-tubulin antibodies. Scale bar = 10 μm.
Fig. 3  Distribution of various GFP-tagged TBK5 mutants transiently expressed in BY-2 protoplasts. Schematic representations of the primary structures of TBK5 (a) and its mutants fused with GFP (b). (c) The ability of each fusion protein to form a particle, aggregates or to yield strong perinuclear labeling. (d) Typical distributions of GFP–TBK5T182N, GFP–TBK5ΔNC, GFP–TBK5ΔCC and TBK5ΔC–GFP. N, nucleus. (e) The fluorescent patterns in propyzamide-treated GFP–TBK5T182N-, GFP–TBK5ΔNC- and GFP–TBK5ΔCC-expressing cells. (f) Preferential distribution of stably expressed GFP–TBK5C on the phragmoplast. Densely packed fluorescent fibers, probably corresponding to phragmoplast microtubules, were observed in the region between two separated groups of chromosomes. Scale bar = 10 μm.
furnish the following model of particle formation (Fig. 4). Non-cortical microtubules were arranged in a polarized array with their minus ends located on the nuclear membrane. Because of this microtubule arrangement with TBK5 movement directed to the minus end, GFP–TBK5 accumulated in the perinuclear region (Fig. 4, Step 1). This process was followed by microtubule-independent aggregation, where accumulated GFP–TBK5 proteins assembled on the basis of coiled-coil interactions to form a particle that served as a microtubule-organizing center (Fig. 4, Step 2). The expressed GFP–TBK5 may co-assemble with endogenous TBK5. If the aggregation first occurred locally at different sites, multiple GFP–TBK5 particles and microtubule-radiating foci would appear. However, since the formation of multiple particles and microtubule-radiating foci was never detected in our assays, aggregation might have first generated only a single perinuclear particle that subsequently served as a focus for accumulation of fusion proteins through further aggregation. It remains to be determined whether or not the particle formation can be induced only at the onset of the G1 phase (Kumagai et al. 2004) and/or during the G2 phase (Katsuta et al. 1990) when many microtubules are present in the region between the nucleus and cell periphery.

Expression of TBK5ΔC–GFP yielded fibrous patterns of green fluorescence (Fig. 3d) without inducing formation of fluorescent particles (Fig. 3c), and propyzamide treatment did not result in the formation of dispersed aggregates of fluorescent proteins (Fig. 3c, e). The results from experiments with propyzamide indicated that the C-terminal domain played an essential role in aggregating GFP–TBK5. The same domain may also be required for TBK5 mobility because, unlike GFP–TBK5ΔC and GFP–TBK5ΔCC, TBK5ΔC–GFP was not preferentially distributed around the nucleus (Fig. 3d). Therefore, in the case of TBK5ΔC–GFP, the failure in particle formation may be due to both poor mobility and deficient local aggregation (Fig. 4). The apparent microtubule association of TBK5ΔC–GFP possibly involves an interaction between TBK5ΔC and endogenous TBK5, although these data would also be explained if TBK5ΔC itself binds directly to the microtubules.

To understand the function of the C-terminal domain, we analyzed TBK5C (basic C-terminal region) fused with GFP. Transient expression of GFP–TBK5C resulted in clear accumulation of green fluorescence in dividing cells in which the nuclear envelope has broken down, revealed that the GFP–TBK5C was localized on the phragmoplast (Fig. 3f). Thus, the C-terminal domain may be able to associate with microtubules in vivo. If the binding of tubulins or microtubule fragments to this C-terminal domain induces the aggregation process, the inability of TBK5ΔC–GFP to form aggregates and particles would then be obvious. In addition, the fact that...
propyzamide treatment stimulates the formation of dispersed aggregates (Fig. 1e, f) would also be explained, because propyzamide would have increased the cytosolic concentration of tubulins and/or microtubule fragments.

Propyzamide-treated TBK5AC-GFP-expressing cells devoid of any bright fluorescent dots (Fig. 3c) contained cytosolic fluorescence when observed under conventional fluorescent microscopy (data not shown). However, observations using a microscope equipped with an image deconvolution system occasionally visualized thin and short fibers at the cell cortex (Fig. 3e). This debris probably corresponded to the remnants of cortical microtubules. Thus, association of TBK5ΔC-GFP may increase the stability of microtubules.

Although different microtubule-associated proteins have been analyzed in plant cells with GFP fusions, particle formation or remodeling of overall microtubule organization in patterns similar to those observed in this study have not been reported to date. It seems worth exploring the possible mechanism of the microtubule rearrangement induced by GFP-TBK5 expression, since this endeavor might offer an insight on the essential mechanism for land plant cells to generate and maintain their non-focused or parallel microtubule arrays. In animal cells, formation of radial microtubule arrays involves the action of a minus end-directed microtubule motor (cytoplasmic dynein) in transporting and gathering centrosomal components (Young et al. 2000) or membrane-bound organelles responsible for microtubule nucleation (Vorobjev et al. 2001). This knowledge led us to hypothesize that the microtubule rearrangements we observed was caused by co-assembly of the GFP-TBK5 fusion proteins with endogenous microtubule-nucleating units. The finding that dispersed aggregates of GFP-TBK5 were able to regenerate microtubule asters (Fig. 2c, d) is consistent with the hypothesized co-assembly of GFP-TBK5 and microtubule-nucleating units. However, the observed microtubule regeneration would not necessarily indicate that microtubule-nucleating units were collected as aggregates, since the same microtubule regeneration would be expected if the aggregates contained microtubule seeds as they were formed in the presence of propyzamide, and since the aggregates did in fact contain tubulins after propyzamide treatment (Fig. 2d). In addition, our immunofluorescence assays occasionally failed to detect the expected vigorous microtubule regeneration from the aggregates (data not shown). Therefore, at this stage, we can merely hypothesize that the microtubule rearrangement associated with the particle formation is a result of accumulation of active microtubule-nucleating units without strong support.

In further exploring how microtubule rearrangements occurred in GFP-TBK5-expressing cells, we note that microtubule nucleation in non-dividing tobacco BY-2 cells depends on placement of microtubule-nucleating units on existing cortical microtubules and that it usually yields branches of new microtubules extending from the pre-existing microtubules (Murata et al. 2005). If there is a system that could continuously collect the microtubule seeds or branches to form a small domain, this domain would function as a dominant microtubule-organizing center, since the collected microtubules could either elongate to yield radiating microtubules directly or provide new sites for microtubule nucleation. In fact, it is highly possible that the GFP-TBK5 fusion proteins would co-assemble with microtubules, since anti-α-tubulin antibodies recognized the particle (Fig. 2a, right) and dispersed aggregates (Fig. 2d). Therefore, it seems rational to hypothesize that the observed microtubule rearrangement is based on the actions of accumulated GFP-TBK5 for relocating and gathering microtubule seeds or branches in a manner either together with or separately from microtubule-nucleating units. For microtubule seeds or branches to be relocated by the expressed GFP-TBK5 fusion proteins through the proposed microtubule-based mechanism (Fig. 4), GFP-TBK5 would have to select a component of the microtubule-nucleating units and/or new microtubules as a cargo in order to transport them along the pre-existing microtubules. The finding that GFP-TBK5C expression in dividing cells yielded labeling of the phragmoplast supports the previous hypothesis that TBK5 binds acidic tubulins at the extremely basic C-terminal domain possibly to transport microtubules against neighboring microtubules (Matsui et al. 2001).

The finding that the GFP-TBK5 fusion proteins co-localized with microtubules in the early stages of transient expression leads us to speculate that endogenous TBK5 is distributed along microtubules in non-transformed tobacco BY-2 cells. Determining the subcellular localization of endogenous TBK5 and discriminating the functional similarities/differences between endogenous TBK5 in non-transformed cells and accumulated GFP-TBK5 fusion proteins in transformed cells may provide a clue to understanding the molecular mechanisms that regulate the distribution and arrangement of microtubules in land plant cells. As illustrated by a previous study on NACK1, a tobacco kinesin (Nishihama et al. 2001), inducible expression of mutant proteins as a dominant-negative inhibitor is useful for identifying the processes involving the corresponding endogenous proteins. Mutant TBK5 proteins created in the present study (i.e. TBK5T182N, TBK5ΔNC, TBK5ΔCC and TBK5ΔC) are candidates for expressing the dominant-negative inhibitor of TBK5, since their actions and distributions were found to be distinct from those of full-length TBK5. Beside analyses using RNA interference technology or arabidopsis insertion lines with disrupted TBK5 ortholog genes, analysis in which
any of the four TBK5 mutants are expressed in a regulated fashion is expected to be useful for elucidating the function and role of endogenous TBK5 proteins in non-transformed cells.

Materials and Methods

Cell culture and transformation

Tobacco BY-2 cells (Nicotiana tabacum L. cv. Bright Yellow 2) were maintained using standard procedures (Nagata et al. 1982). Protoplasts of 3-d-old subcultured BY-2 cells were transformed using a PEG-mediated method (Maas et al. 1995). Transformation efficiency estimated from the ratio of GFP-positive cells was about the same for each experiment, although not always determined. Transformed protoplasts were suspended in MS medium supplemented with 0.5 M mannitol, spread on 1% agarose, and incubated at 28°C in the dark. Stable BY-2 transformants were prepared by the Agrobacterium-mediated method using the LBA4404 strain.

Preparation of plasmids

All plasmids for transient expression were prepared by inserting a DNA fragment that encoded TBK5 or its variants into the 5′ or 3′ end of the GFP-coding region of the CaMV35S-SGFP(S65T)-nos3 plasmid (Niwa et al. 1999). DNA encoding the mutant TBK5 protein with a point mutation or without one of the coiled-coil domains was prepared by recombinant PCR. In preparing a binary vector construct that expresses GFP–TBK5, the DNA cassette designed to overexpress GFP–TBK5 was inserted into an Xba site of pPZP112 (Hajdukiewicz et al. 1994). GFP–TBK5, GFP–TBK5T182N, GFP–TBK5N, GFP–TBK5/C1, GFP–TBK5C, GFP–TBK5ΔC and GFP–TBK5ΔC contained a linker peptide (GSGKDPSM) between the TBK5 and C-terminally located GFP sequences. Constructs were verified by DNA sequencing exploiting the use of a dye deoxy-terminator cycle sequencing kit [Applied Biosystems (ABI), Tokyo, Japan] with an ABI model-310 sequencing system. Details of plasmid construction and DNA constructs used in this study are available upon request.

Treatments with cytochalasin E or propyzamide

For experiments examining the effects of cytochalasin E and propyzamide on particle formation, transformed cells were first cultured for 8 h in normal conditions, and then subjected to a further 4 h culture with 1% dimethylsulfoxide (DMSO), 1% DMSO plus 100 μM cytochalasin E or 1% DMSO plus 100 μM propyzamide. For experiments examining the effects of propyzamide on the distribution of GFP-tagged mutant TBK5 proteins, transformed cells were first cultured under normal conditions until the appearance of GFP-positive cells, and then cultured in the presence of 1% DMSO plus 100 μM propyzamide for >2 h before observation.

Fluorescence microscopy

Cells were observed by using a conventional fluorescence microscope (Olympus BH-2, Tokyo, Japan) or a microscopy system equipped with an image deconvolution system (Applied Precision DeltaVision, Washington, DC, USA). Conventional microscopy was used to observe living GFP-positive cells (Fig. 1b, c, f and results summarized in Fig. 3c). The DeltaVision microscopy system was used to observe living GFP-positive cells (Figs. 1a, 3d, e, f) and immunostained cells (Fig. 2). Figs. 1a, 2c, and 3d, e were produced with the deconvolution program of the DeltaVision microscopy system; other photographs are raw images. For immunostaining, cells were suspended in a fixation medium (50 mM PIPES, KOH, pH 7.0, 5 mM EGTA, 2 mM MgSO₄, 4% formaldehyde, 0.5M mannitol) for 45 min, and then in a fixation medium containing 0.05% Triton X-100 for 15 min. Fixed cells were washed three times with a washing buffer (50 mM PIPES, KOH, pH 7.0, 5 mM EGTA, 2 mM MgSO₄, 0.5 M mannitol). After two washes with PBS containing 0.02% Tween-20, and incubation with PBS containing 586-labeled goat anti-mouse IgG (Molecular probes) for 1 h. Stained cells were washed with PBS containing 0.02% Tween-20 before observation.

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