**GAK, a regulator of clathrin-mediated membrane traffic, also controls centrosome integrity and chromosome congression**

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**Summary**

Cyclin G-associated kinase (GAK) is an association partner of clathrin heavy chain (CHC) and is essential for clathrin-mediated membrane trafficking. Here, we report two novel functions of GAK: maintenance of proper centrosome maturation and of mitotic chromosome congression. Indeed, GAK knockdown by siRNA caused cell-cycle arrest at metaphase, which indicates that GAK is required for proper mitotic progression. We found that this impaired mitotic progression was due to activation of the spindle-assembly checkpoint, which senses protruded, misaligned or abnormally condensed chromosomes in GAK-siRNA-treated cells. GAK knockdown also caused multi-aster formation, which was due to abnormal fragmentation of pericentriolar material, but not of the centrioles. Moreover, GAK and CHC cooperated in the same pathway and interacted in mitosis to regulate the formation of a functional spindle. Taken together, we conclude that GAK and clathrin function cooperatively not only in endocytosis, but also in mitotic progression.

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Key words: Centrosome, Clathrin, Mitosis, Ptk1, Tubulin, GAK

**Introduction**

Chromosome instability is a key defect of malignant cancer cells, which are primarily the result of uncontrolled mitosis caused by abnormal spindle formation (multi-aster or mono-aster), aberrant centrosome number, chromosome missegregation and/or failure of cytokinesis (Chi and Jeang, 2007; Fukasawa, 2008; Barr and Gruenberg, 2008). Recent reports indicate that endocytic proteins are also involved in mitotic events. For example, GM130, a Golgi-complex-associated protein, regulates centrosome morphology (Kodani and Süttérlin, 2008), and dynamin, a GTPase enzyme that is active in the early stages of endocytosis, is involved in centrosome separation and cytokinesis (Thompson et al., 2004). Clathrin, a major player in receptor-mediated endocytosis during interphase, but not during mitosis when no endocytosis occurs, is targeted to the mitotic spindle and functions in microtubule stability at the onset of mitosis (Royle et al., 2005). Depletion of clathrin by RNA interference (RNAi) causes abnormal chromosome segregation and activates spindle-assembly checkpoint (SAC)-mediated prometaphase-metaphase arrest. Thus, regulators of clathrin are also expected to play essential roles in mitotic progression.

One of the proteins that regulates endocytosis cooperatively with clathrin is cyclin G-associated kinase (GAK), a serine/threonine kinase first identified as an association partner of cyclin G (Kanaoka et al., 1997). In addition to having a kinase domain, GAK is highly homologous with neuron-specific auxilin, which plays a pivotal role in clathrin-dependent trafficking in neural cells. As expected from this structural similarity, the ubiquitously expressed GAK protein localizes to the trans-Golgi network (TGN) and is an essential cofactor for Hsc70-dependent uncoating of clathrin-coated vesicles in many non-neural cells (Korolchuk and Banting, 2002; Kametaka et al., 2007). Indeed, clathrin-mediated endocytosis is partially blocked in GAK-knockdown cells, and the J domain of GAK was found to be important for this event (Zhang et al., 2005). Furthermore, GAK is transiently recruited to clathrin puncta, and this recruitment is dependent on the PTEN-like domain of GAK (Lee et al., 2006). Moreover, GAK phosphorylates Thr156 of the AP-2 μ2 subunit, which is important for its endocytotic activity (Zhang et al., 2005; Olusanya et al., 2001). Thus, the functions of GAK in endocytosis are well known (Eisenberg and Greene, 2007). However, considering the subcellular colocalization of both GAK and clathrin, and the association between these molecules, it is expected that GAK also functions in mitosis. This expectation is supported by a recent report showing that GAK and clathrin heavy chain (CHC) localize to both the cytoplasm and nucleus, and that almost all nuclear CHC signals colocalize with GAK, suggesting an important function for GAK in the nucleus (Sato et al., 2009).

In the present study, we provide evidence that GAK regulates mitotic progression by demonstrating that siRNA-mediated GAK knockdown caused metaphase arrest and multipolar spindles. We present two novel functions of GAK as a regulator of mitosis: first, GAK maintains centrosome structure, and second, GAK functions cooperatively with clathrin, not only during endocytosis, but also during mitotic progression.

**Results**

GAK is required for mitotic progression

To investigate the putative novel function of GAK beyond its role in membrane trafficking, we generated two GAK siRNA constructs,
designed Ki5 (#525) (Zhang et al., 2005) and Ki9 (#1309), based on their location in the mRNA sequence of GAK. When HeLa S3 cells were transfected with Ki5 or Ki9, GAK protein expression decreased (Fig. 1A). Transfection with GL2 control siRNA, which knocks down firefly luciferase mRNA, had no effect on GAK protein levels. We also performed reverse-transcriptase (RT)-PCR analysis to determine whether expression of the GAK homologues, AP-2 associated kinase (AAK1) and tensin, was affected by Ki5 and Ki9. As expected, although both Ki5 and Ki9 downregulated GAK mRNA expression compared with the GL2 control (Fig. 1B, lanes 2 and 3), AAK1 and tensin mRNA levels were unaltered by transfection with GL2, Ki5 or Ki9 (Fig. 1B). This result confirmed that Ki5 and Ki9 specifically targeted GAK mRNA.

Notably, GAK knockdown that was induced by Ki5 or Ki9 (Ki5/Ki9) caused transfected cells to adopt a round shape, reminiscent of cells at the mitotic (M) phase of the cell cycle (Fig. 1C). This was not observed in the GL2 control cells (Fig. 1C). To determine whether the Ki5/Ki9-treated cells were actually undergoing mitosis, we stained their chromosomes and spindles with Hoechst-33258 dye and anti-α-tubulin antibody, respectively. Indeed, Ki5/Ki9-treated cells had condensed chromosomes and formed mitotic spindles (Fig. 1C). We quantified these observations and found that there were significantly more mitotic cells in the GAK-knockdown cell populations than in the GL2 control (Fig. 1D). Importantly, the point at which the cells were arrested was strongly restricted to prometaphase-metaphase (Fig. 1C,D). Because Ki9 altered the percentage population of prometaphase-metaphase cells more efficiently than Ki5 (Fig. 1D), we used the Ki9 construct to deplete GAK protein in all subsequent experiments. We found that Ki9 siRNA also increased the levels of cyclin B1 (CCNB1) and the phosphorylation of histone H3 at Ser10 (Fig. 1E). Moreover, fluorescence-activated cell sorting (FACS) analysis also indicated that Ki9 treatment caused an increased proportion of G2-M cells (supplementary material Fig. S1A), which confirmed that the
GAK-knockdown cells are mostly mitotic. These data suggest that GAK is required for proper mitotic progression.

To further investigate the mitotic defects of GAK-knockdown cells, we monitored the round mitotic cells for a 2-hour period (mitosis and cytokinesis are usually completed within 1 hour). In the control GL2 cells, 27 of 32 round mitotic cells completed cytokinesis within 1 hour (representative images of a typical cell are shown in the top panels of supplementary material Fig. S1B). In this cell, the metaphase plate formed 11 minutes 57 seconds (11:57 minutes) after nuclear-envelope breakdown, after which the cells quickly entered cytokinesis; chromosome separation and cleavage-furrow formation were observed at 41:57 and 47:57 minutes, respectively. By contrast, only one of 44 GAK-knockdown cells entered cytokinesis, and none displayed chromosome separation or cleavage-furrow formation during the 2-hour observation period (middle and bottom panels of supplementary material Fig. S1B). Notably, the GAK-knockdown cells showed chromosome movement without separation. These observations of live cells provide further evidence that GAK is required for proper mitotic progression.

GAK depletion activates the spindle assembly checkpoint

Because Ki9-treated cells were rarely in anaphase or telophase, we speculated that SAC was activated in these cells. To examine this, we stained cells with an antibody against BubR1, an important component of SAC (Sudakin et al., 2001; Tang et al., 2001), and found that anti-BubR1 antibody signals were observed from prophase to prometaphase in both GL2 and GAK-knockdown cells (data not shown). However, whereas GL2 cells lost the BubR1 signal after successful chromosome alignment during metaphase (top panels in Fig. 2A), Ki9-treated cells retained the BubR1 signal, probably because the misaligned chromosomes maintained SAC in an activated state (arrowhead in Fig. 2A); this phenotype is similar to that of CHC-knockdown cells (Royle et al., 2005). We found the...
localization of other kinetochore-related markers, such as CENP-A, Aurora-B and survivin, to be normal in Ki9-treated cells (supplementary material Fig. S2A,B).

In order to confirm that the prevention of mitotic progression in GAK-knockdown cells is due to retained SAC activation, we depleted GAK (Ki9) and/or BubR1 (si-BR1) by siRNA. If the metaphase arrest of GAK-knockdown cells is due to SAC activation, knockdown of BubR1 would allow these cells to continue through mitosis. Indeed, when GAK alone was depleted (lane 2 of Fig. 2B), a portion of the BubR1 proteins in Ki9-treated cells migrated more slowly through a polyacrylamide gel [see arrowhead in anti (α)-BubR1 panel of Fig. 2B] than proteins extracted from GL2 cells (lane 1 in α-BubR1 panel of Fig. 2B); this implies that at least a portion of the BubR1 proteins remained phosphorylated in GAK-knockdown cells. Because activated BubR1 is phosphorylated (Chan et al., 1999), this observation indicates that GAK depletion caused the activation of BubR1. Similarly, whereas cells transfected with GL2 control alone or together with BubR1 showed low levels of the mitotic proteins cyclin B1 and securin (lanes 1 and 3 of α-cyclin B1 and α-securin panels in Fig. 2B), which are known to be degraded in anaphase (Musacchio and Salmon, 2007), high levels of both of these proteins were observed in Ki9-treated cells (lane 2, Fig. 2B). By contrast, when both GAK and BubR1 were knocked down, low levels of cyclin B1 and securin were detected (lane 4, Fig. 2B).

When we measured the proliferation rate of the single-knockdown cells over 3 days, we found that Ki9-treated cells grew very slowly, whereas GL2 control and BubR1-knockdown cells grew at normal rates (Fig. 2C). However, when both GAK and BubR1 were knocked down, the proliferation rate returned to the normal level (Fig. 2C, turquoise curve). To confirm that this recovery was due to release from mitotic arrest, we determined the mitotic indices of the different cell types and examined the levels of relevant mitotic proteins. As expected, Ki9-treated cells harboured increased mitotic indices due to arrest in metaphase (arrow in Fig. 2D), whereas GL2 control and BubR1-knockdown cells showed normal mitotic indices. By contrast, the percentage of prometaphase-metaphase cells after GAK and BubR1 double knockdown was almost normal (arrowhead in Fig. 2D). These phenotypes were also confirmed by FACS analysis (supplementary material Fig. S1A).

Because the SAC was activated in Ki9-treated cells, we next observed the metaphase plates and found them to be abnormal in many of these cells (Fig. 2E). Three kinds of defects were detected in the metaphase chromosomes, namely, chromosomes were protruded, misaligned or abnormally condensed. As shown in Fig. 2F, these abnormal cell types occurred at almost equal frequencies in Ki9-treated cells, whereas these abnormalities were rarely observed in GL2-treated cells. Because these phenotypes were also observed in Ki5-treated cells, these abnormalities were not off-target effects of the Ki9 siRNA construct. Taken together, these results suggest that the impaired mitotic progression resulting from GAK knockdown is due to SAC activation associated with defective chromosome congression and alignment.

GAK knockdown causes multi-aster formation

When we immunostained Ki9-treated cells with anti-γ-tubulin antibody to detect centrosomes and anti-α-tubulin antibody to detect spindles, we found that many of the these cells harboured additional asters; i.e. they carried more than two centrosomes (or γ-tubulin foci) from which spindles (composed of α-tubulin) extended radially (Fig. 3A). More than 50% of the Ki9-treated mitotic cells had more than two γ-tubulin foci, whereas less than 5% of the GL2-treated cells in mitosis displayed this abnormality (Fig. 3B). Multipolar spindles can arise either from over-replication of the centrosomes or from defects in cytokinesis. To establish the primary cause of this defect, we determined in which stage of the cell cycle an abnormal number of γ-tubulin foci first appeared. For this purpose, we chemically fixed Ki9-treated cells at various time points (Fig. 3C), immunostained the cells with anti-γ-tubulin antibody and counted the percentage of cells harbouring more than two γ-tubulin signals in interphase (39, 42 and 45 hours) and mitosis (48 hours). We found that most Ki9- and GL2-treated cells had only one or two γ-tubulin signals during interphase (Fig. 3C), indicating that GAK depletion did not trigger abnormal centrosome amplification or cytokinesis, and that Ki9-treated cells entered mitosis with two γ-tubulin signals, as is normal. By contrast, more than 50% of Ki9-treated cells had additional asters 48 hours after Ki9 treatment, when cells are expected to be at the M phase of the cell cycle; only about 5% of GL2-treated cells harboured such multi-asters (rightmost bars, Fig. 3C).

We next measured the intensity of the integrated γ-tubulin signal, because if the centrosome was fragmented, the signal intensity of Ki9 cells might have become weaker than that of the control cells. Indeed, when GL2- or Ki9-treated cells were probed with γ-tubulin 48 hours after siRNA treatment, we found that the integrated γ-tubulin intensity of Ki9-treated cells carrying more than two γ-tubulin foci was much lower than that of GL2-treated cells (Fig. 3A,D). Moreover, the integrated γ-tubulin intensity of Ki9-treated cells having only two foci was also slightly lower than that of GL2-treated cells (supplementary material Fig. S3A), which indicates that centrosome maturation was abnormal. Because the centrosome was composed of two centrioles surrounded by pericentriolar material (PCM), we next examined whether multiple centrioles were also observed in Ki9-treated cells during mitosis. We found that additional centrioles were rarely observed in Ki9-treated cells harbouring extra γ-tubulin foci; this suggests that the PCM, but not the centrioles, was fragmented (supplementary material Fig. S3B,C).

Because centrosomes are subjected to microtubule-dependent pulling and pushing forces from various directions during mitosis, we surmised that these forces caused the fragmentation of PCM in Ki9-treated cells. To explore this possibility, we determined the number of γ-tubulin foci in the presence of Taxol (paclitaxel; a tubulin-depolymerization inhibitor) using immunofluorescence microscopy. When microtubule dynamics in Ki9-treated cells were perturbed and the forces generated by the mitotic spindle towards the centrosome were attenuated by Taxol, PCM fragmentation was blocked and the number of γ-tubulin foci was normal (i.e. two were present) (Fig. 3E). These results indicate that microtubule-mediated forces caused PCM fragmentation in Ki9-treated cells.

GAK and CHC function cooperatively in mitotic progression

Next, we examined the reasons for the other phenotypes of Ki9-treated cells, i.e. misaligned and abnormally condensed chromosomes (Fig. 2E,F). It is reported that phosphorylation of CENP-A (phosphorylated on Ser7; pS7) by Aurora-A and Aurora-B is important for proper chromosome alignment (Kunitoku et al., 2003). Because Aurora-A and Aurora-B are expressed normally in Ki9-treated cells (data not shown), we examined the phosphorylation state of CENP-A. Western blot analysis indicated that the amount of phosphorylated CENP-A was higher in Ki9-treated cells than in GL2-treated cells (supplementary material Fig. S4A); namely, phosphorylation of CENP-A was normal in Ki9-treated cells. This
was confirmed by indirect immunofluorescence using CENP-A pS7 (supplementary material Fig. S4B).

CHC is known to be an essential factor for mitotic progression, and CHC knockdown produces an abnormal metaphase plate with misaligned and abnormally condensed chromosomes (Royle et al., 2005). Because these phenotypes are similar to those of GAK-knockdown cells, we surmised that CHC and GAK act closely together in the pathway that leads to mitotic progression. Because the percentage reduction of prometaphase-metaphase cells after CHC knockdown was not as aberrant as that of GAK-knockdown cells (compare Fig. 1 with Fig. 4G), we speculated that GAK functions epistatically upstream of CHC. To test this, we first examined the localization of CHC in GAK-knockdown cells and found that CHC completely colocalized with α-tubulin in control cells during metaphase (Fig. 4A, upper panels). This result is consistent with a previous report (Royle et al., 2005). By contrast, CHC diffused away from the mitotic spindle into the cytoplasm in GAK-knockdown cells (Fig. 4A, lower panels), whereas GAK localization was not affected even when CHC was knocked down (supplementary material Fig. S5). This abnormality was confirmed by determining the frequency of cells showing such diffusion (Fig. 4B).

Where does GAK localize during mitosis? To answer this, we established a HeLa S3 cell line that constitutively expresses pEGFP-GAK (Fig. 4D), which showed that GAK colocalizes with CHC at the mitotic spindle (Fig. 4C; supplementary material Fig. S6). Moreover, this GAK localization was not affected in CHC-depleted cells (supplementary material Fig. S5). We next performed immunoprecipitation to confirm associations between GAK and CHC during mitosis. Indeed, pEGFP-GAK associated with CHC at interphase, which is consistent with our previous report that GAK associates with CHC during interphase (Sato et al., 2009). Here, we also found that GAK associated with CHC during mitosis (Fig. 4E). These observations suggest that GAK and CHC cooperate in the same pathway to regulate the formation of a functional spindle.

If this interpretation is correct, the GAK and CHC double-knockdown cells would have a similar percentage of prometaphase-metaphase cells as the GAK single-knockdown cells. To test this, we depleted the cells of GAK and CHC proteins (Fig. 4F, arrow and arrowhead in upper two rows) and examined the protein levels of cyclin B1 and Plk1, another M-phase marker (Petronczki et al., 2008). As expected, we found that the cyclin-B1 level was almost equal between GAK and CHC double-depleted cells and Ki9-treated cells (Fig. 4Fii). Moreover, cells depleted of GAK alone or together
with CHC showed similar mitotic indices (Fig. 4G). From these results, we conclude that GAK and CHC act cooperatively in the same pathway to regulate proper spindle assembly: GAK seems to function upstream of CHC.

**Discussion**

We showed here that knockdown of GAK results in mitotic arrest during metaphase, as does knockdown of CHC (Royle et al., 2005). Although GAK-knockdown or -knockout experiments have already been performed in several laboratories (Zhang et al., 2005; Lee et al., 2008), the mitotic arrest caused by GAK depletion has not been described. This is partly because endocytotic experiments were performed before mitotic arrest was observed in these studies, or because vector-based small hairpin RNA was employed after antibiotic selection. In the latter case, it is surmised that some unknown factors complemented the GAK functions during the selection of transfected cells. Why, then, is the percentage of prometaphase-metaphase cells in the Ki9-treated cell population higher than that in CHC-knockdown cells? Interestingly, CHC-knockdown cells showed increased Plk1 protein levels compared with those in GAK-knockdown cells despite having lower mitotic indices than those of GAK-knockdown cells (Fig. 4Fi, lane 3; and Fig. 4Fii). Thus, one possible explanation is that Plk1 is involved in SAC signalling at the kinetochore and that its depletion causes defects in mitotic structure.

Fig. 4. GAK functions upstream of CHC.
(A) Immunofluorescence analysis probed with anti-α-tubulin and anti-CHC antibodies indicates that CHC was mislocalized in GAK-depleted cells. (B) Frequency of cells in which CHC and α-tubulin signals failed to colocalize completely. The bar graph represents the average value of three independent experiments. More than 50 GL2-treated cells and more than 100 Ki9-treated cells were scored in each experiment. (C) Localization of pEGFP-GAK during mitosis. GL2 or Ki9 were introduced into HeLa S3 cells that constitutively expressed pEGFP-GAK. Then, cells were subjected to immunofluorescence using anti-GFP and anti-γ-tubulin antibodies. (D) Ki9, but not GL2, treatment abolished the pEGFP-GAK band from HeLa S3 cells constitutively expressing pEGFP-GAK. The western blot was probed with anti-GFP antibody. Anti-α-tubulin antibody was also used as a loading control. (E) Association of pEGFP-GAK with CHC not only at interphase, but also during mitosis. To collect mitotic cells, pEGFP-GAK-expressing cells were treated with Taxol for 15 hours. Then, cells were collected and western blot analysis was performed for whole-cell extract (WCE) and immunoprecipitant (IP). Asyn., asynchronized cells; M, mitotic cells; Vec., vector control. (Fi) Western blot analysis showing the successful depletion of CHC and GAK proteins. Arrow, arrowhead and asterisk indicate the bands for GAK, CHC and an uncharacterized protein, respectively. (Fii) The bar graph represents the relative intensity of the denoted band, which was calculated by comparing its intensity with that of the loading control (α-tubulin). (G) Depletion of CHC in GAK-knockdown cells did not alter the percentage of prometaphase-metaphase cells. Each percentage value of mitotic cells was scored by means of an immunofluorescence assay. To identify the mitotic cells, α-tubulin and chromosomes were stained. The bar graph represents the average value of three independent experiments; in each experiment, more than 500 cells were scored. The errors bars in B and G show the standard deviation. Scale bars: 10 μm.
and SAC-mediated prometaphase-metaphase arrest. Indeed, the Plk1 protein level at the kinetochore is reduced in Ki9-treated cells and the protein level at the kinetochore is reduced in Ki9-treated cells and the centrosomal signals (α-tubulin and Plk1) were observed. These signals are less intense than those in untreated control cells. Moreover, aneuploidy and cancer.

Materials and Methods

Cell cultures
HeLa S3 cells were maintained in Dulbecco’s modified Eagle’s medium (DMEM) with 5% foetal bovine serum (FBS; HyClone, Logan, UT), 100 U/ml penicillin and 100 μg/ml streptomycin.

Indirect immunofluorescence analyses
HeLa S3 cells were fixed by sequential incubations at room temperature in 3.7% formaldehyde in PBS, 0.1% Triton X-100 in PBS, and 0.05% Tween-20 in PBS, each for 10 minutes. For centrifugation, cells were fixed at –20°C in methanol for 10 minutes and washed with PBS–. Then, they were incubated with primary antibody for 3 hours at room temperature, followed by incubation with Alexa-Fluor-594 and –488 (Molecular Probes, Eugene, OR)-conjugated antibody and mouse immunoglobulin G in TBST (100 mM Tris-Cl, pH 7.5, 150 mM NaCl, 0.05% Tween-20) containing 5% FBS. DNA was stained with Hoechst 33258 (Sigma). The stained cells were observed with the confocal laser scanning microscope LSM510 (Zeiss) or a BX51 microscope (Olympus).

RT-PCR
Total RNA was extracted from siRNA-treated HeLaS3 cells using the AGPC (acid guanidinium-phenol-chloroform) method (Fujii et al., 2002) or an RNeasy kit (Qiagen). PCR was performed with the following primer pairs: GAK: forward, 5'-GTAATGATCTCCAGCAGAAGG-3'; reverse, 5'-TTATGGCCTGTCCTTATCGAG-3'; GAPDH: forward, 5'-ATACACAACGCTGTCCTATCGAG-3'; reverse, 5'-GCTGTAGCCGCTCCTAACCG-3'; AAK1: forward, 5'-CATGTCATTCCCAGCAGAAGG-3'; reverse, 5'-GCTGTAGCCGCTCCTAACCG-3'; tensin: forward, 5'-CGATCCCGCTCCCTCAAGGACCAGCCGAG-3'; reverse, 5'-CGAAGTTTGGCTCATTGGGGCAGG-3'; phospho-histone H3 (Upstate-Millipore, Bedford, MA); BubR1, CENP-A, and phospho-histone H3 (Santa Cruz Biotechnology, Santa Cruz, CA); Aurora-A, survivin and histone H3 (Cell Signaling Technology, Danvers, MA); and GAPDH (monoclonal from BD Transduction Laboratory and polyclonal from Abcam); and GFP (monoclonal from MBL and polyclonal from Sawady Technologies, Tokyo, Japan). Hoechst 33258 (Sigma, St Louis, MO) and TO-3 (Invitrogen, San Diego, CA) were used to stain DNA.

siRNA and live-cell imaging
siRNA duplexes against GAK, BubR1, and CHC were chemically synthesized (Dharmacon Research, Lafayette, CO; Gene Design, Osaka, Japan). The GAK siRNA duplexes were designated as K5 (Zhang et al., 2005) and K9 after their location in the GAK mRNA sequence. The sequence of the siRNA duplexes was as follows: K5: 5'-GGUUGAACAAUUCGUUCAUdTdT-3' for sense, 5'-GAAGCACAAGUUCUCAACGdTdT-3' for antisense; K9: 5'-GATGGTGGGTGTTCTCAGGdTdT-3' for sense, 5'-GGUUGAACAAUUCGUUCAUdTdT-3' for antisense; CL2: 5'-GUACCGGAAGAUAUCUGAdTdT-3' for sense, 5'-GGUUGAACAAUUCGUUCAUdTdT-3' for antisense; and BubR1: 5'-GGAAGCAGCCAGAAGGAAAdTdT-3' for sense, 5'-GGUUGAACAAUUCGUUCAUdTdT-3' for antisense. Knockdown of G2L2, GAPDH, and BubR1 was carried out in glass-bottom dish or tissue-culture dish using Oligofectamine 2000 according to the manufacturer’s protocol (Invitrogen). We used MetaMorph software (Photometrics UK, Buckinghamshire, UK) to control the camera and prepare the time-lapse images.

Immunoprecipitation and western blot analysis
Cells expressing GFP-tagged vector and GAK were lysed in lysis buffer (50 mM Tris-HCl, pH 7.5, 250 mM NaCl, 0.1% Nonidet P-40) supplemented with protease inhibitors (Sigma). After clarifying the extract by centrifugation at 10,000 g for 5 minutes, aliquots of the supernatant were pre-cleared using protein-A-Sepharose alone. The pre-cleared lysates were then subsequently mixed with anti-GFP polyclonal antibody, and then the immune complexes were harvested by addition of 50% protein-A-Sepharose slurry (Amersham Pharmacia Biotech, Piscataway, NJ) and washed six times with lysis buffer. Th, immunoprecipitated samples were subjected to 10% SDS-polyacrylamide-gel electrophoresis (SDS-PAGE). For western blotting, total cellular proteins and immunoprecipitates resolved on gels were transferred to nitrocellulose filters and probed with the relevant antibodies. Immunoreactive protein bands were visualized using Renaissance chemiluminescence reagents (DuPont NEN, Boston, MA).

Establishment of a pEGFP-GAK stable clone
HeLa S3 cells were transfected with the pEGFP-GAPDH vector and pEGFP-vector as described previously (Sato et al., 2009) using Lipofectamine2000 according to the manufacturer’s instructions (Invitrogen, Carlsbad, CA). After 2 days, the medium was replaced by a new medium containing G418 (62.5 μg/ml). After G418 selection, surviving colonies were checked under fluorescence microscopy and western blotting, and GFP-positive colonies were isolated.

Measurement of signal intensity
The centrosomal signals (γ-tubulin and Plk1) were observed and their intensities were measured using MetaView software. Plk1 signal intensity at the kinetochore was also measured by MetaView software. The brightest three dots were selected per cell and their intensities were measured. Three patterns were categorized, depending on signal intensity.

Statistical analysis
Significant differences were determined using the Mann-Whitney U-test (Fig. 3) and supplementary material Fig. S3). The data are expressed as means ± s.e. P<0.01 was considered to be statistically significant.

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