LAPSER1/LZTS2: a pluripotent tumor suppressor linked to the inhibition of katanin-mediated microtubule severing

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Human chromosome region 10q23–24 is one of the most frequently found regions that show loss of heterozygosity in prostate cancers. A candidate tumor suppressor LAPSER1/LZTS2 (LAPSER1) is located in 10q24.3 that has been reported to be deleted as frequently as the neighboring PTEN locus. We previously reported that LAPSER1 binds p80 katanin, a subunit of the katanin heterodimer. In this report, we show that the LAPSER1 C terminal domain inhibits katanin-mediated microtubule severing in vitro and we detected this inhibition at centrosomes by tracing the nucleated de novo, severed, and transported microtubules in cells. This functional association is also supported by the intracellular localization. Centrosomal localization of LAPSER1 was independent of microtubules and was preferential to mother centrioles. In primary cultured neurons, LAPSER1 also colocalizes with p80 katanin. LAPSER1 alters cell proliferation by regulating cytokinesis. As subcellular mechanisms that underlie the tumor suppressive activity, exogenous LAPSER1 expression inhibited central spindle formation by abrogating microtubule transportation and a similar mode of inhibition was found in axogenesis. Katanin knockdown and dominant negative inhibitor of katanin provided similar phenotypes. Prophase LAPSER1 inhibited centrosomal γ-tubulin accumulation, which resulted in retardation of mitotic entry. Furthermore, interphase inhibition of katanin by LAPSER1 expression resulted in prevention of cell motility that was accompanied by the increased acetylated microtubules. LAPSER1 knockdown increased cell migration that was inhibited by the expression of ninein, a microtubule release inhibitor. These results indicate that microtubule severing at centrosomes is a novel tumor-associated molecular subcircuit in cells, in which LAPSER1 is a regulator.

INTRODUCTION

We have previously shown that LAPSER1/LZTS2 (LAPSER1) shares centrosomal and midbody localization with the microtubule-severing protein katanin and directly binds its p80 subunit (1,2). However, the effects of this binding upon katanin activity and the mechanisms of tumor suppression that arise from the LAPSER1/katanin interaction remain to be revealed. Katanin is composed of a catalytic p60 subunit (p60 katanin) and also a regulatory p80 subunit (p80 katanin) that is responsible for centrosomal targeting and enhancement of the severing activity by p60 (3,4). The p80 katanin is not required for the severing activity of p60 katanin, and ratios of the p80 katanin to the p60 katanin vary quite dramatically in different cell types (5). It has been shown that the p80 katanin has one domain called procon80 that increases the severing properties of p60 katanin and another domain called WD40 that targets to centrosomes and decreases the severing properties of p60 katanin (4).

Katanin severs microtubules, and microtubule severing plays various roles in a cell-type-specific manner. In the proliferating cells, katanin contributes to mitosis by severing the microtubules at the mitotic spindle poles and increasing the number of minus ends. This results in γ-tubulin accumulation at mitotic centrosomes (6). A recent study has shown that the katanin located at the spindle poles also elicits severing activity to shorten the metaphase spindles (7). In the neuronal cells, katanin contributes to neurite outgrowth. It is thought in...
this instance that the microtubules in nascent neurons are severed in the cell body and are thereby translocated into axons to form microtubule bundles (8).

It has been hypothesized that the mechanisms operating during mitosis also underlie neurite formation (9). According to this theory, the apparently different phenotypes induced by alterations of katanin activity during mitosis or neurite outgrowth are given an explanation from the basic aspects of microtubule systems using the common mechanisms of microtubule severing. The apparent differences are thus thought to be due to the differences in the destinations of the microtubules after severing.

In mitotic cells, microtubule severing on the spindles is thought to contribute to spindle reorganization. In contrast, severed microtubules in neurons are released from certain sites in order to operate at different locations within the same neurons. This same scenario may be applied to proliferating cells during interphase, although the degree of release appears to be smaller than that in neurons (10). In this case, severed microtubules are released and scattered throughout the cytoplasm. In addition, microtubule release is also involved in the regulation of cell migration (11). All of these findings suggest that when LAPSER1 alters the activity of katanin, phenotypic changes at the cellular level can be expected to manifest in diverse biological contexts.

In our current report, we show that katanin inhibition by LAPSER1 causes alterations in microtubule systems and that these contribute to its tumor suppressive properties.

RESULTS
Localization of LAPSER1 to centrosomes
We previously reported that a subpopulation of LAPSER1 localized to the centrosomes during all phases of the cell cycle, and that LAPSER1 and p80 katanin co-localized in mitotic centrosomes and midbodies. In addition, a subpopulation of katanin localizes to centrosomes independently of microtubules during all phases of the cell cycle (12). We therefore examined the centrosomal localization of LAPSER1 with respect to microtubules and centrioles.

Saos-2 cells were treated with the microtubule-disrupting agent nocodazole and co-stained for LAPSER1 and for α- or γ-tubulin. Nocodazole treatment completely disrupted the filamentous network of cytoplasmic microtubules (Fig. 1A-1) and the remaining α-tubulin signals showed centrosomal localization as reported previously (13) and overlapped with LAPSER1 signals. We confirmed that LAPSER1 co-localizes with the centrosomal marker protein γ-tubulin in nocodazole-treated cells (Fig. 1A-2). These results indicate that LAPSER1 is an integral centrosomal component that is independent of microtubules, further suggesting an interaction between LAPSER1 and katanin at the centrosomes.

We next evaluated the centriolar localization of LAPSER1. It has been reported previously that the staining of serum-starved rodent fibroblasts for acetylated-tubulin discriminates the mother centriole that has primary cilia from the daughter centriole that lacks these structures (14). In our current analyses, after starving NIH3T3 cells or 3Y1 cells for 24 h, we found a preferential localization of LAPSER1 at the centrioles with primary cilia (Fig. 1B) (3Y1 cells; data not shown). The mother centriole has been shown to have specific morphological characteristics, such as appendages required for microtubule anchoring and to be located at the focus of the microtubule array (15). It is hypothesized that microtubules severed in the vicinity of both mother and daughter centrioles are tethered to the mother centriole. Maybe the LAPSER1 uses the mother centriole as a place to be anchored but its function is relevant to the entire centrosome.

Analysis of LAPSER1 localization in neurons
The functions of katanin have been well characterized previously in neuronal cells (5,16,17). Thus, an analysis of LAPSER1 in neurons would be informative with regards to its interaction with katanin. We first addressed whether endogenous LAPSER1 is expressed in primary cultured rat cortical neurons. As shown in Figure 2A, LAPSER1 was detectable in lysates from neurons that had been cultured for 3 days in vitro (3DIV). Given that an earlier report has demonstrated p80 katanin expression in the cortex (5), we next stained for both molecules.

Approximately 80% of neurons at 3DIV showed mature morphologies with extended axons, axonal branches and dendrites. Approximately 20% of these cells showed nascent neuronal properties with large growth cones at the tips of growing axons. In these cells, p80 katanin was far more enriched in cell bodies. It was also evenly distributed within the processes and slightly enriched in growth cones. LAPSER1 was also enriched in cell bodies and slightly enriched in growth cones. Both molecules were colocalized in cell bodies and growth cones (Fig. 2B). In mature neurons, p80 katanin was more enriched in cell bodies and proximal regions of dendrites, evenly distributed within the axon, slightly enriched in branch points but not enriched in axonal branches and slightly increased levels were observed in the tips of branches. The localization of p80 katanin was consistent with previous findings (5). LAPSER1 was enriched in the cell bodies, and showed weak localization in the branch points and at the tips of axonal branches. Both proteins showed co-localization in the cell bodies, branch points and at the tips of axonal branches (Fig. 2C). The association of LAPSER1 and p80 katanin detected in neurons strengthens the evidence that this interaction is a common event underlying the biological roles of the microtubules.

LAPSER1-C terminus inhibits katanin activity in vitro
To address whether the association of p80 katanin with LAPSER1 affects microtubule severing activity, we expressed and purified tagged protein products including His (histidine)-tagged-p60 katanin, GST-tagged-procon80 domain of p80 and GST-LAPSER1 C-terminus. We used procon80 instead of p80 since full-length human p80 katanin has been reported to be unstable in Sf9 cells (4). Procon80 includes the amino acid sequence responsible for binding to LAPSER1 (2). As shown in Figure 3A, we confirmed the expression of both katanin constructs in insect cells and a LAPSER1 construct in bacteria. We then performed a DAPI-based in vitro microtubule depolymerization assay as described previously (4). The fluorescent signals corresponding to pre-formed microtubules were

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relatively constant in the absence of additional proteins, or in the presence of purified p60 and procon80 without ATP (Fig. 3B). The addition of p60 and procon80 with ATP was found to decrease the microtubule fluorescence indicating depolymerization (4). The addition of the GST-LAPSER1 C-terminus fusion product, but not GST alone, significantly inhibited microtubule depolymerization. This inhibition was not observed when we tested the effects of GST-LAPSER1 C-terminus on microtubule severing by p60 katanin in the absence of procon80 (Supplementary Material, Fig. S1). We furthermore confirmed the severing pattern and the effects of GST-LAPSER1 C-terminus on microtubule depolymerization by imaging (Fig. 3C). These results suggest that the LAPSER1-C terminal region inhibits katanin activity via the p80 subunit.

Pharmacological analyses at cellular levels: during anaphase/cytokinesis

We previously showed that the forced expression of LAPSER1 induces binucleation through defects in central spindle formation during cytokinesis (2). We observed a centrosomal localization of endogenous p80 katanin and exogenous LAPSER1 in Saos-2 cells with central spindle defects in our current experiments (Fig. S2). To elucidate the mechanisms of this central spindle malformation, we employed established pharmacological analyses (10,16,18). Since this method was also shown to be applicable to rat fibroblast-like cells (10), we used stable LAPSER1-flag expressing Rat 1 cells we previously established (2). Rat 1 cells stably expressing LAPSER1-flag were treated with 10 μM nocodazole for 6 h to depolymerize most of the microtubule polymers. Afterwards, the drug was removed for 3 min to permit a burst of synchronized microtubule assembly from the centrosome (Fig. 4A). To prevent further microtubule assembly, 50 nM vinblastine was added. Under these conditions, microtubules are rapidly released from the centrosome and conveyed to the cell periphery in a dynein-dependent manner (18). Thus, this regime is useful for studying the severing activity in the centrosome and outward transport of microtubules from the centrosome.

Figure 1. Immunolocalization of LAPSER1 in interphase cells. (A) After microtubules were depolymerized with 10 μM nocodazole for 6 h, Saos-2 cells were fixed and stained with anti-LAPSER1 (green) and (A1) anti-α-Tubulin (red) or (A2) anti-γ-Tubulin (red). DNA (blue). LAPSER1 signals co-localized with centrosomal signals (red) even after microtubules were completely de-polymerized. Bars, 10 μm. (B) NIH3T3 cells were serum starved, fixed and immunostained with anti-acetylated-tubulin to label centrioles, primary cilia and anti-LAPSER1 (green), which is preferentially localized to the maternal centriole associated with the primary cilium. DNA (blue). Bars, 5 μm in the left panels and 1 μm in the right panels.
Sub-mitotic phases are defined by cell morphology of differential-interference-contrast (DIC) images, centrosomal separation and the state of the chromosomes (DNA staining), which were supported by the mitotic marker [phospho-histoneH3 (S10)] staining (Fig. S3). We focused on cells during anaphase/cytokinesis, which we defined as two separating daughter cells with a cytoplasmic bridge between them. In the control cells, the microtubules were scattered throughout the cytoplasm from the centrosome, and in ~30% of these cells a subpopulation of microtubules could be seen funneling into the cytosolic bridge. However, in LAPSER1-expressing cells, a majority of the microtubules remained clustered within a discrete region around the centrosome, and only 5% of the cells showed microtubules transported into the cytosolic bridge (Fig. 4).

The central spindle is considered to comprise anti-parallel, non-kinetochore microtubules that are released from the centrosome during the onset of anaphase/cytokinesis (19). In this regard, our current results suggest that katanin harbors the activity that releases microtubules during the onset of anaphase/cytokinesis and that LAPSER1 expression impairs this
release via katanin inhibition. As described below, these possibilities can account for the no central spindle phenotype.

Responses of microtubule bundles to altered LAPSER1 or katanin expression

We previously revealed central spindle defects in Saos-2 cells infected with adeno-LAPSER1-flag virus (2). Rat 1 cells also showed significant defects of central spindle formation in response to stable LAPSER1 expression (Fig. 5A), which indicates that LAPSER1 inhibits microtubule release from the centrosome during anaphase/cytokinesis. We also observed central spindle defects in p80 katanin knockdown Rat 1 cells that were accompanied by the absence of discernable midbody matrix formation in DIC images, whereas control cells had few such defects and a dense midbody matrix (Fig. 5B and C). Furthermore, the expression in HeLa cells of mutant p60 katanin (GFP-p60 P loop KA), which is an established dominant negative, resulted in decreased acetylated-tubulin signals on the central spindles, although this was observed only in cells with centrosome-focused GFP signals (Supplementary Material, Fig. S4-A). Some population of GFP-p60 P loop KA expressing cells showed spindle pole localization of GFP signals (4). Consistent with this, we observed herein that ~10% of the cells in anaphase/cytokinesis showed centrosomal localization of this mutant. Similar effects of a functional knockdown of katanin on the central spindle have also been reported during oocyte meiosis in Caenorhabditis elegans (7). These findings indicate that katanin severs a subset of microtubules on the centrosomes during anaphase/cytokinesis, which then migrate to the center of the midbody to form the central spindle. This explains the central spindle formation defects resulting from the functional inhibition of katanin by

Figure 4. Inhibited release and translocation of microtubules from the centrosomes to the midbody in stably LAPSER1-flag expressing Rat 1 cells during cytokinesis. (A) Nocodazole-treated cells showed few microtubules (after nocodazole treatment). Cells recovered for 3 min showed a small population of microtubules emanating from the centrosome (after recovery). After 30 min exposure to vinblastine, 30% of the microtubules were transported into the midbody in control cells (mock), whereas significantly less transport was evident in cells expressing LAPSER1-flag (LAPSER1-flag). Upper panels: merged images of microtubules (red), γ-tubulin (green) and DNA (blue). Lower panels: differential-interference-contrast (DIC) images of corresponding upper panels. Bars, 10 μm. (B) Percentages of cells with nucleation of microtubules (MT) (after recovery) and transport of microtubules into the midbody (after vinblastine) as shown in (A).
LAPSER1, p80 katanin knockdown and dominant negative katanin inhibitor, i.e. LAPSER1 inhibits microtubule bundle formation by interfering with the transport of component materials.

A similar phenomenon has been reported whereby the disruption of katanin function prevents axonal outgrowth via the inhibition of the transportation of microtubules from the cell body to the axon (17). The activity of microtubule severing by katanin which generates transportable short microtubules in the cell body plays a critical role to supply microtubules to the axon where they form bundles in nascent primary neurons (8). Furthermore, in both axons and central spindles, each microtubule within the bundle is oriented with its plus end directed away from the cell body. 

This prompted us to test the effects of LAPSER1 on neuritogenesis in primary cultured neurons. One day after plating, when most neurons had few and short neurites (shorter than the diameter of a neuronal cell body), LAPSER1-flag expressing neurons (yellow cell body) or LAPSER1-flag expressing neurons (data not shown) failed to extend their neurites (left lower panel). Quantification of neurite outgrowth is shown by the bar graph. A cell with long neurites was defined as a neuron with neurites extending three somal diameters or longer (>50 cells were counted, n = 3). Tubulin staining shows that cells with neurites that also have flag signals (yellow cell body) have portions of decreased tubulin signals in their neurites, compared with the relatively constant tubulin signals in cells without flag signals (middle). Right two graphs: intensities of tubulin signals in neurites as a function of their distance from the cell body. Each graph corresponds to the cell with purple arrows shown in the insets of the middle image. Bars, 20 \( \mu m \).

Figure 5. Responses of microtubule bundles to katanin inhibition. (A) Effects of the expression of LAPSER1-flag on the central spindle in Rat 1 cells. Cytokinetically stained cells were immunostained for acetylated-Tubulin (red), γ-Tubulin (green) and DNA (blue). Stably LAPSER1-flag expressing Rat 1 cells showed defects [defined as described previously (2)] in central spindle formation. Numbers show the percent of cells with defects (>50 cells were counted, n = 3). Bars, 5 \( \mu m \). (B) Effects of p80 katanin knockdown on the central spindle. Rat 1 cells were transfected with anti-p80 katanin RNAi molecules. A significant reduction of p80 katanin was evident by western blotting. (C) Central spindle and midbody matrix defects in p80 katanin knockdown Rat 1 cells. Rat 1 cells were transfected with anti-p80 katanin RNAi, fixed and stained for acetylated-tubulin (red) and DNA (blue). Weakened central spindle acetylated-tubulin signals and the absence of DIC dense midbody matrix (arrows) are evident in knockdown cells. Quantification of cells with central spindle defects is shown on the right side (n = 3). Bars, 5 \( \mu m \). (D) LacZ, GFP-LAPSER1 or LAPSER1-flag were expressed in rat cortical primary cultured neurons with adenovirus vector. Cells were cultured 2 days after infection, fixed and stained for LacZ (green), flag (red), neurofilament (red) or β-Tubulin (green) as indicated. While LacZ expressing cells (yellow cell body) and cells without LacZ signals (red cell body) (left upper panel) have extended neurites, GFP-LAPSER1-expressing neurons (yellow cell body) or LAPSER1-flag expressing neurons (data not shown) failed to extend their neurites (left lower panel). Quantification of neurite outgrowth is shown by the bar graph. A cell with long neurites was defined as a neuron with neurites extending three somal diameters or longer (>50 cells were counted, n = 3). Tubulin staining shows that cells with neurites that also have flag signals (yellow cell body) have portions of decreased tubulin signals in their neurites, compared with the relatively constant tubulin signals in cells without flag signals (middle). Right two graphs: intensities of tubulin signals in neurites as a function of their distance from the cell body. Each graph corresponds to the cell with purple arrows shown in the insets of the middle image. Bars, 20 \( \mu m \).
neurons were infected with adeno-GFP-LAPSER1, adeno-LAPSER1-flag or control adeno-LacZ viruses. Three days after plating, the infection efficiency was about 30–40% as determined by GFP signals or staining for flag or LacZ. LAPSER1 signals were detected exclusively in cell bodies. Greater than 70% of LAPSER1-expressing cells showed few or no extension of neurites, whereas neurons without exogenous LAPSER1 signals or cells expressing LacZ showed well-developed neurites (Fig. 5D). By staining for β-tubulin, a small population of neurons with extended axons (the longest neurites), even in the presence of exogenous LAPSER1 signals, had rapid tapering or distally unstable amounts of microtubules in their axons compared with neurons without exogenous LAPSER1. These results are consistent with our prediction that LAPSER1 inhibits katanin. We contend therefore that LAPSER1 inhibits microtubule severing by interactions with p80 katanin in the neuronal cell body, which prevents microtubule transport into the axon and eventually results in defective axonal outgrowth.

The two phenotypes seen in the two different biological systems tested indicate that LAPSER1 inhibits the severing activity of katanin and prevents cells from forming microtubule bundles, and that these inhibitory effects are not cell type-dependent.

**LAPSER1 inhibits microtubule severing at the centrosome during prophase**

We next performed pharmacological analyses of prophase cells (the same procedure as described in Fig. 4A was performed). Cells during prophase were defined by separated centrosomes (>5 μm apart) and by condensed chromosomes with no nuclear envelope breakdown. Nocodazole treatment completely depolymerized the microtubules (data not shown). We observed a slight decrease in nucleation in LAPSER1-expressing Rat 1 cells, but the percentage of cells with visible microtubule nucleation was not significantly different from the controls (Fig. 6A; after recovery). However, ≈70% of the control cells released most of their nucleated microtubules into the cytoplasm, whereas only ≈20% of the LAPSER1-expressing cells showed release after vinblastine treatment. The majority of LAPSER1-expressing cells showed relatively large amounts of microtubules attached to the centrosomal region (Fig. 6A; after vinblastine treatment). These results indicate that LAPSER1 expression inhibits katanin-mediated centrosomal microtubule severing during prophase. Consistent with this, similar results were obtained following p80 katanin knockdown in Rat 1 cells (data not shown).

The inhibition of katanin activity reduces the accumulation of γ-tubulin in mitotic centrosomes (6). The microtubule minus ends generated by katanin-mediated microtubule severing are thought to bind to cytosolic γ-tubulin ring complexes, resulting in a redistribution of γ-tubulin at mitosis. Thus, we expected that LAPSER1 expression would influence γ-tubulin accumulation and the organization of microtubules during prophase.

We stained LAPSER1-expressing cells for γ-tubulin, α-tubulin and acetylated-tubulin. Consistent with our prediction, we observed significant decreases in γ-tubulin accumulation, which we quantified by scaling the maximum diameter of the γ-tubulin spots in prophase centrosomes in both stable LAPSER1-flag expressing Rat 1 cells and Saos-2 cells which adenovirally express LAPSER1-flag. γ-tubulin is known to promote microtubule nucleation by forming a γ-tubulin ring complex at the centrosome. In accordance with the decreased γ-tubulin accumulation, alterations to the microtubule organization during mitosis were prevented in LAPSER1-expressing cells (Fig. 6B-1 and B-2) and acetylated-tubulin signals were also weakened in these cells (Fig. 6B-3).

A checkpoint activity has been proposed that monitors the maturation of centrosomes before entering into mitosis (anaphase-checkpoint) (20). In addition, tubulin deacetylase overexpression induces anaphase arrest (21). Therefore, we evaluated the mitotic index in the presence of nocodazole, which causes mitotic stress. As shown in Fig. 6C, the time-dependent increase of the mitotic index was impaired in adenovirally LAPSER1-flag expressing cells. Moreover, this inhibition was not accompanied by an increase of BrdU uptake, indicating that it was not due to mitotic slippage. Similar results were obtained in stably LAPSER1-flag expressing Rat 1 cells (data not shown).

As the inverse situation, we assayed both γ-tubulin accumulation and the mitotic index in the presence of nocodazole in LAPSER1 knockdown Saos-2 cells. Here we detected an increased accumulation of γ-tubulin and an increased mitotic index, although to a relatively small extent (data not shown). These results suggest that LAPSER1 contributes to the prophase checkpoint via the control of γ-tubulin accumulation and subsequent microtubule organization. In accordance with this proposition, the increased paclitaxel sensitivity in LAPSER1-knockdown cells was recently reported (22). Paclitaxel is a well-known inducer of mitotic stress (23). We thus addressed whether LAPSER1 expression altered the sensitivity to paclitaxel. As shown in Figure 6D, stable LAPSER1-flag expression conferred resistance to paclitaxel in Rat 1 cells. Similar results were obtained with adenovirally LAPSER1-flag expressing Saos-2 cells (data not shown). Taken together, we speculate that LAPSER1 induces prophase arrest in the presence of mitotic stress and thereby prevents cells from entering into prometa/metaphase, and subsequent apoptosis.

**LAPSER1 inhibits microtubule release from centrosomes and controls cell motility**

We next addressed if alterations in LAPSER1 expression affect microtubule severing during interphase (Fig. 7A). We observed a slightly decreased extent of nucleation in this phase and a marginally decreased percentage of the cell population showing nucleation in stably LAPSER1-flag expressing cells. However, ≈45% of the control cells released most of their microtubules into the cytoplasm, whereas ≈10% of the LAPSER1-expressing cells released microtubules, and the majority of these cells had large amounts of microtubules attached to centrosomes (Fig. 7A). These results also suggest that LAPSER1 inhibits severing during interphase via katanin.

One of the predicted outcomes of a decreased microtubule severing is their stabilization, because one fate of severed microtubules is depolymerization (4,6). We tested microtubule...
stability by staining with acetylated-tubulin, a marker of stable microtubules. As shown in Fig. 7B, we detected enhanced acetylated-tubulin signals around the centrosomes in LAPSER1-expressing Rat 1 cells under serum starvation. We also detected this enhancement in the presence of 10% serum in these cells, in adenovirally LAPSER1-flag expressing Saos-2 cells (data not shown) and in HeLa cells expressing GFP-p60 P loop KA (Supplementary Material, Fig. S4).

We next addressed if knockdown of LAPSER1 enhanced the release of microtubules. We employed a microtubule regrowth assay as siRNA-mediated knockdown of LAPSER1 is successful only for human cells (Fig. 7C). The pharmacological regime we applied to Rat 1 cells was found to be inappropriate for human cell lines (Saos-2, HeLa) due to the aggregation of microtubules after vinblastine treatment. Nocodazole treatment completely depolymerized microtubules.
which 2 min after recovery showed significant nucleation around the centrosomes. After 11 min recovery, the control cells showed well-developed asters, which are microtubules emanating from centrosomes. In contrast, the majority of LAPSER1-knockdown cells showed almost no aster formation and only small amount of microtubules appeared around the centrosomes, with a large portion being disconnected from centrosomes. This low quantity of microtubules around centrosomes can be interpreted as sustained microtubule nucleation activity. Microtubule scattering throughout the cytoplasm may reflect enhanced microtubule release from the centrosome. Therefore, endogenous LAPSER1 functions to control the microtubule severing that results in the release of microtubules during interphase.

HeLa cells are of epithelial origin and epithelial cells have been considered to have characteristic cytoplasmic microtubule nucleation activity (24). We thus compared epithelial
Caco-2 cells, which have cytoplasmic microtubule nucleation activity (25), with HeLa cells. After 2 min recovery in the microtubule regrowth assay, a massive nucleation of microtubules throughout the cytoplasm was evident in Caco-2 cells (Fig. S5). In contrast, as shown in Fig. 7C, HeLa cells exclusively showed centrosome-focused microtubule nucleation. Few nucleations at other sites were observed. Also, whereas HeLa cells showed asters at 11 min recovery, a large portion of microtubules were not connected to centrosomes in the majority of Caco-2 cells, and appeared to be organized independently of centrosomes. Therefore, we speculate that HeLa cells are more similar to fibroblasts than to epithelial cells with regards to microtubule organization.

Attenuated cell migration via the prevention of microtubule release from the centrosome has been reported (11). In addition, cells with decreased microtubule stability or cells with decreased microtubule acetylation via alterations of the microtubule-associated proteins Rassf1A or HDAC6 also show enhanced cell migration ability (26,27). These results prompted us to test the effects of LAPSER1 on cell migration. First, we performed transwell assays using exogenous LAPSER1-expressing cells in three different systems: stable expression, a tetracycline-inducible expression and adenoviral expression. In all cases, repressed cell migration was evident (Fig. 8A).

If these effects of LAPSER1 on migration were indeed mediated through the inhibition of katanin, we would expect that blocking katanin should also result in cell migration inhibition. In accordance with this prediction, p80 katanin knockdown inhibited migration of Rat 1 cells (Fig. 8A). To test whether the down-regulation of LAPSER1 caused the opposite effect, we employed HeLa cells. As shown in Figure 8A, LAPSER1-knockdown HeLa cells showed enhanced migration. To determine if this enhancement was due to increased microtubule release from the centrosome, we tested the effects of ninein, a well-characterized microtubule release inhibitor (11). Ninein elicits its inhibitory activity via tethering released microtubules to centrosomes (28). We established stably GFP-ninein expressing HeLa cells (Fig. 8B), as GFP-ninein also effectively inhibits microtubule release during interphase (11). We examined knockdown of LAPSER1 in GFP-ninein expressing cells.

As shown in Figure 8C, in control GFP expressing HeLa cells, we observed enhanced migration as a result of LAPSER1 knockdown which was remarkably repressed in GFP-ninein expressing cells. We also observed similar results in a wound healing assay (Fig. 8D). Therefore, the enhanced migration seen in LAPSER1-knockdown cells is dependent upon microtubule release from the centrosomes. These results indicate that LAPSER1 contributes to the regulation of cell migration via the inhibition of katanin. Interestingly, as reported previously in release-involved migration (11), we found no differences in actin stress fiber formation or adhesion plaque formation between LAPSER1-knockdown cells and control cells (data not shown), suggesting no involvement of the actin cytoskeletal change in release-associated migration.

Mechanisms of inhibition of p60 katanin by LAPSER1

Finally, we addressed the issue of how LAPSER1 regulates p60 activity. First, we tested the effects of p80 katanin knockdown on LAPSER1-induced three major phenotypes, central spindle defects, attenuated centrosomal γ-tubulin accumulation at prophase and the inhibition of migration (Fig. 9A–C). Those three phenotypes were also observed in LAPSER1-flag expressing cells transfected with the control oligomers (compared with the control cells transfected with control oligomers) while the percentages or numbers of LAPSER1-flag expressing cells transfected with p80 katanin siRNAs with these phenotypes did not show significant differences compared with the control cells transfected with p80 katanin siRNAs. These data suggest the dependence of the functions of LAPSER1 on p80 katanin.

It has been reported that in HeLa cells, transient expression of wild-type p60 resulted in massive generation of monopolar spindles (4). Co-transfection of wild-type p60 and the con80 domain of p80 but not the full-length p80 resulted in the spindle pole targeting of both constructs in monopolar spindles independently of their tags (4) although single transfection of either p60 or con80 alone was reported to show diffuse localization rather than polar localization in spindles. The spindle pole targeting of both constructs in co-transfection has been interpreted to be due to the interaction of both constructs.

The localization of katanin at mitotic spindle poles requires intact microtubules (12). Since both con80 and p60 have affinities for microtubules, the increased affinity by the sum of affinities derived from each subunit in the p60/con80 heterodimer has been thought to cause pole targeting (4). Also this increased affinity has been considered to contribute to the increased microtubule severing by the heterodimer (4). Thus, as a second strategy, we performed to monitor this polar targeting of the katanin heterodimer (Fig. 9D) in the monopolar spindles. The procon80 domain of p80 consists of residues 301–655 while con80 consists of residues 412–655 of human p80 katanin. Therefore, procon80 is longer than con80 by 111 amino acids. Since procon80 includes all the amino acid sequences responsible for binding to LAPSER1 (2), we started to examine if we could reconstruct this system using procon80 instead of con80. Co-transfections of GFP-p60+pcDNA4 or GFP-procon80+pcDNA4 did not show polar targeting of GFP signals but diffuse localization in the monopolar or bipolar spindles (co-transfection of GFP-procon80+pcDNA4 did not generate significant monopolar spindles) (Fig. 9D-1 and D-6). Co-transfection of GFP-p60+xpress-procon80 (combination1; comb.1) or GFP-p60+xpress-p60 (comb.2) resulted in focused polar localization of GFP signals in the majority of monopolar spindles (Fig. 9D-2 and D-7). Therefore, we confirmed that procon80 could be used in this system instead of con80. Compared with the adenovirus-mediated control LacZ expression, which did not change the pole localization of GFP signals (Fig. 9D-3 and D-8) in both cases of co-transfection (comb.1 or comb.2), LAPSER1-flag expression changed the pole localization to the granular or speckled pattern (Fig. 9D-4 and D-9). In addition, most of the granular or speckled GFP signals were co-localized with LAPSER1-flag signals detected by anti-flag immunostaining (Fig. 9D-5 and D-10). These data suggest that LAPSER1 binds to p60/procon80 heterodimer and reduces their affinity for microtubules.

The third approach is to monitor the physical binding of procon80 to p60. We performed GST pulldown assay using...
bacterially expressed GST-procon80. As an initial step, we confirmed specific binding of GST-procon80 to LAPSER1-flag adenovirally expressed in COS7 cells (Fig. 9E-1). We also reproduced the specific binding of GST-procon80 to GFP-p60 expressed in COS7 cells (Fig. 9E-2). We did not detect any significant changes of the binding of GST-procon80 to GFP-p60 in the presence of overexpressed LAPSER1-flag compared with LacZ control (Fig. 9E-2). These data suggest that LAPSER1 has no effects on the interaction of p60 with procon80.

Based on the series of results, we speculate that LAPSER1 binds to procon80 domain of the p60/p80 heterodimer and reduces the affinity of the entire heterodimer for microtubules without interfering the heterodimer formation, resulting in the inhibition of microtubule severing by the katanin heterodimer (Fig. 10).

DISCUSSION
Our detailed analysis of the centrosomal localization of LAPSER1 has revealed that this molecule shows a preferential localization to the mother centrioles in a microtubule-independent manner. This is in good agreement with our

Figure 8. LAPSER1 inhibits cell migration by suppressing microtubule release from centrosomes. (A) Migration assay for LAPSER1-expressing cells, LAPSER1-knockdown cells and p80 katanin-knockdown cells. The same numbers of stably LAPSER1-expressing Rat 1 cells (Rat 1/LAPSER1-flag) or control cells (Rat 1/Mock) were used. In the tetracycline-inducible system, cells were used 24 h after induction (Tet–/−) in parallel with uninduced controls (Tet+/+). For knockdown cells, cells were first transfected with RNAi molecules against the indicated targets and then subjected to a migration assay 48 h after transfection. Adenovirally LAPSER1-flag expressing Saos-2 cells (Saos-2/LAPSER1-flag) were studied 24 h after infection. The results shown indicate the number of total cells that passed through the membrane, counted from more than five microscopic fields. (n = 3) RNAi(+/−); the sense and antisense oligomers were annealed and transfected. Control(+/+); only sense oligomers were annealed and transfected for the control. (B) Stable expression of GFP-ninein in HeLa cells. Left upper panel shows control GFP-expressing cells. Right upper panel shows stably GFP-ninein expressing HeLa cells where a characteristic centrosomal localization of GFP-ninein was confirmed. Lower panels show corresponding DIC images. Bars, 10 μm. (C) GFP-ninein expression inhibits LAPSER1 knockdown-induced enhancement of migration. Stably GFP-ninein expressing cells were transfected with anti-LAPSER1 RNAi. Twenty-four hours after transfection, cells were assessed using a migration assay (n = 3). (D) Wound healing assay in which GFP-ninein expressing cells were transfected with anti-LAPSER1 RNAi as in (C). After 72 h, confluent cells were scratched with a pipette tip and the cells were photographed 8 h later. Bar, 50 μm.
Figure 9. Mechanism of inhibition of p60 katanin by LAPSER1. (A) Stable LAPSER1-flag expressing Rat 1 cells (A-2 and A-4) or control cells (A-1 and A-3) were transfected with anti-p80 katanin RNAi molecules (A-3 and A-4) or control oligomers (A-1 and A-2). Cytokinetic cells were immunostained for acetylated-tubulin (red) and DNA (blue) (cytokinesis) or cells in prophase were stained for α-tubulin (red), γ-tubulin (green) and DNA (blue) (prophase). Bars, 5 μm in cytokinesis and 10 μm in prophase. Significant defects in central spindle formation and reduced centrosomal γ-tubulin accumulation were observed in control oligomer transfected and LAPSER1-flag expressing cells (compare experiment 2 with 1 (control)), whereas no such differences were detected between mock and LAPSER1-flag expressing cells when transfected with anti-p80 katanin RNAi molecules (compare experiment 4 with 3 (control)). (B) Quantifications of the results in (A) that were performed similarly as described in Figure 5A and C or Figure 6B. (+/+); control oligomer transfection. (+/−); p80 katanin siRNA transfection (n = 3). (C) The same experimental procedures were performed as in (A) and cells were subjected to migration assay. The significant inhibition of migration observed in control oligomer transfected and LAPSER1-flag expressing cells was no longer observed in p80 katanin RNAi transfected cells. (+/+); control oligomer transfection. (+/−); p80 katanin siRNA transfection. (n = 3) (D) HeLa cells were co-transfected with constructs indicated below, infected by adeno-LacZ (D-3 and D-8) or adeno-LAPSER1-flag viruses (D-4, D-5, D-9 and D-10) 1 day after transfection, fixed and immunostained for α-tubulin (red; D-1–D-4 and D-6–D-9) or flag (red; D-5 and D-10) 1 day after infection. GFP (green). DNA (blue). Merged images of green and red channels.
proposition that LAPSER1 works in concert with katanin at the centrosomes. The co-localization of LAPSER1 with p80 katanin in neurons further indicates that the close association between these two molecules is conserved in different cell types. We additionally observed an inhibitory effect of LAPSER1 on katanin in three different biological contexts. Our current results from both in vitro assays and pharmacological analyses at the cellular level thus also provide solid evidence that LAPSER1 is an inhibitor of katanin.

The expression of LAPSER1 during cytokinesis results in binucleation (2). A previous report using serial electron microscopy reconstruction has shown that there may be a release activity of microtubules from mitotic poles and that these released anti-parallel, non-kinetochore microtubules provide the cytosolic bridge between two separating daughter cells with a platform made of microtubule bundles on which many regulatory proteins are recruited (19). This platform is known as the central spindle, the origin of which remains to be clarified. The microtubule release activity may also involve severing, and we propose that a part of this activity is due to katanin.

This severing activity is inhibited when LAPSER1 is over-expressed, which could result in disruption to the central spindle followed by binucleation. Other recent work in which C. elegans mei-2 embryos, deficient in a p80 katanin homologue, showed defects of microtubule redistribution from the poles to the midzone during female meiosis also supports our contention (7). It was proposed in this earlier study that katanin at the spindle pole severs the pole-proximal microtubules into short fragments that are then transported to the midzone. We speculate that these mechanisms are conserved across species.

In neurons, LAPSER1-expressing cells showed defects in neurite outgrowth and also showed abnormally reduced microtubule bundles in their axons, which is another phenomenon caused by the deformation of microtubule bundles. These results are consistent with other reports showing that axogenesis deficiency due to katanin inhibition is caused by reduced severing followed by insufficient microtubule transport into axons (8,17). Thus, given that LAPSER1 inhibits microtubule bundle formation, both central spindle formation and axogenesis appear to have common mechanisms. Both processes are dependent upon microtubules that are severed at some sites and transported to sites where they are needed.

In a pathological setting, LAPSER1-induced binucleation is growth suppressive. Tetraploidy is thought to give rise to multi-polar spindles, and causes chromosomal instability (29). However, when we compared the fate of LAPSER1-induced binucleation to that of dihydrocytochalasin B (DCB)-induced binucleation in p53 null cells, we found that LAPSER1-induced binucleates did not proceed through the cell cycle to generate 8 N (G2/M phase tetraploid) peak on flow cytometry, whereas DCB-induced binucleates did, as previously reported (Supplementary Material, Fig. S6) (30). Hence, LAPSER1-induced binucleates do not appear to contribute to spindle multi-polarity during the next mitotic phase.

Also disruptions to mitotic processes after the onset of anaphase are thought to induce chromosome instability directly (31). However, we found no chromosome bridge associated with LAPSER1-induced cytokinetic defects. We speculate that this is because LAPSER1 functions exclusively at the central spindles and does not influence the interaction of microtubules with chromosomes in mammalian cells. Therefore, LAPSER1 is not likely to be involved in chromosome segregation. In accordance with this, differences in the release status between kinetochore and non-kinetochore spindle microtubules have been reported that suggest discrete release mechanisms exist (19).

Although thick microtubule bundles do not form during prophase, the phenotypes seen in LAPSER1-expressing cells are in good agreement with our core hypothesis. During prophase, severed microtubules contribute to γ-tubulin accumulation (6), which in turn contributes to microtubule nucleation. In this regard, severing may contribute to radical changes in microtubule organization prior to entry into metaphase. Promotion of γ-tubulin accumulation was the revealed function shown for katanin in proliferating mammalian cells in an early study (6), which is consistent with our observation of the inhibitory effects of LAPSER1 on γ-tubulin accumulation. We speculate that the expression of LAPSER1 results in attenuated microtubule reorganization through the inhibition of γ-tubulin accumulation at the centrosomes.

Premature microtubule organization may be detected by a prophase checkpoint (20,32). Retardation of entry into prometa/metaphase observed in LAPSER1-expressing cells are shown in experiments 5 and 10 (merge). The combinations of constructs are GFP-p60+pcDNA4 (the backbone vector of xpress tagged constructs) (D-1), GFP-p60+xpress-procon80 (D-2–D-5), GFP-procon80+pcDNA4 (D-6) and GFP-procon80+xpress-p60 (D-7–D-10). Massive formations of monopolar spindles (more than 70% of all the cells in prometa/metaphase) were observed when either GFP-p60 or xpress-p60 were expressed and we focused our analysis on monopolar spindles except for the experiment 6 in which we did not observe significant monopolar spindle generation. Spindle pole focused GFP signals that were observed in experiments 2 and 7 were disrupted and granular or speckled pattern was observed in LAPSER1-flag expressing cells (D-4 and D-9), whereas such effects were not observed in LacZ expressing cells (D-3 and D-8). The observed granular or speckled pattern of GFP-p60 or GFP-procon80 signals was for the most part co-localized with LAPSER1-flag signals detected by anti-flag staining in monopolar spindles (D-5 and D-10; merge). In experiments 5 and 10, we identified monopolar spindles by their characteristic rosette-shaped condensed chromosomes (DNA). Note that LAPSER1-flag expression did not change the percentages of monopolar spindles that have been considered to be caused by the diffusely localized exogenously expressed p60 (4), small amount of which we detected in experiments 2–5. (E)(W-E) Bacterially expressed GST-tagged procon80 (GST-procon80) and lysates derived from adenovirally LAPSER1-flag expressing COS7 cells were mixed. The mixture was then pulled down by glutathione-Sepharose beads and subjected to western blotting with anti-flag antibodies. Beads input: Coomassie-stained SDS–PAGE of bacterially expressed GST-procon80 or GST. Lysate: whole cell lysates of GFP-p60 (a, b), GFP-p60+LacZ (c) or GFP-p60+LAPSER1-flag (d) expressing COS7 cells which were used in corresponding pulldown experiments (a; GST and b, c, d; GST-procon80).
During interphase, LAPSER1 expression causes the formation of stable, acetylated-tubulin positive microtubules. We speculate that the enhanced acetylation in LAPSER1-expressing cells is due to inhibited microtubule severing at the centrosomes. This phenotype is the reverse of what is normally observed in migrating cells (34). Microtubule acetylation affects migration directly (35). Also, one previous report has shown that prevention of microtubule release in itself inhibits migration (11). In this study, we provide evidence that LAPSER1 negatively controls cell migration as it is promoted by LAPSER1 knockdown.

LAPSER1 knockdown-induced migration was found to be inhibited by the microtubule release inhibitor GFP-ninein. We thus contend that LAPSER1 may be involved in the control of cell migration via the regulation of microtubule release from the centrosomes.

The observed effects of LAPSER1-C terminus on p60/procon80—but not on p60 alone—mediated microtubule severing in vitro and the dependences of the activities of LAPSER1 on endogenous p80 katanin (Fig. 9A–C) suggest that p80 is a critical factor for LAPSER1 to act as an inhibitor of katanin-mediated microtubule severing. LAPSER1 inhibited the polar localization of p60/procon80 expressed in HeLa cells suggesting that the increased affinity for microtubules by heterodimerization was abrogated by the binding of LAPSER1 to the heterodimer (Fig. 9D and E). Taken together, we propose a mechanism by which LAPSER1 inhibits katanin as shown in Figure 10.

One of the questions that would arise from our results for the function of LAPSER1 is if LAPSER1 works in all the biological contexts in that katanin plays essential roles. It is true that some tumor suppressors function in the developmental processes, but there are also tumor suppressors that have been considered to be not required for the developmental processes and in only aged organisms the phenotypes of disruption of such tumor suppressor genes can be observed. Katanin plays various roles in various biological contexts. For example, we showed the co-localization of p80 katanin and LAPSER1 in neurons at least in some subcellular locations that would include centrosomes but would also include centrosome-unrelated locations (Fig. 2B and C). There are reports that show co-localization of both p60 and p80 in other subcellular places than centrosomes using primary cultured neurons derived from rat embryo (5,16). A non-centrosomal function (axonal branch formation) of katanin was also suggested based on the experimental results in which primary cultured neurons derived from rat embryo were used (36). Therefore, we think that there may be neuronal contexts in which LAPSER1 functions as an inhibitor of katanin in the non-centrosomal compartments as well as at the centrosomes. However, there is a report that brain p60 exhibited punctate centrosome pattern in both adult and embryo, but p80 changed their localization and no longer showed centrosomal pattern in the adult brain (37). Also p60 katanin expression levels have been reported to plunge dramatically in the adult rat central nervous system (5,38). These data suggest that LAPSER1 should be much less expected to function as an inhibitor of katanin in the brain of adult mammals. In contrast, there are reports indicating that in rat non-neuronal tissues, higher levels of the p60

**Figure 10.** The hypothesis for the inhibition of katanin-mediated microtubule severing by LAPSER1 (in the absence of LAPSER1); p60 katanin has (1) the N-terminal short domain (orange) that is required for association with the C-terminal domain of human p80, (2) a microtubules binding domain (blue) and (3) an ATPase domain (AAA). P60 by itself has severing activity. When it forms a heterodimer with p80 katanin, it has increased microtubule-severing activity. The p80 katanin has the N-terminal WD40 domain (WD40) that acts as a negative regulator of microtubule disassembly activity and is also required for centrosome or spindle pole targeting, and the procon80 (procon80) domain that includes p60 binding domain, microtubule binding domain (blue) and LAPSER1 binding domain. P80 katanin by itself has no microtubule severing activity. When p60/p80 heterodimer is formed, the increased affinity for microtubules (two blue arrows) has been considered to contribute to the increased microtubule severing (red arrow). Furthermore, the heterodimers have been considered to assemble into transient dodecamers each of which is composed of six p60 subunits and six p80 subunits (4). (In the presence of LAPSER1); LAPSER1 (LAPSER1) binds to the procon80 portion of heterodimer and maybe blocks the microtubule-binding activity which exists within the procon80 domain (purple arrow), resulting in decreased affinity of the entire complex for microtubules. We speculate that this reduction of affinity for microtubules by LAPSER1 binding may be responsible for at least a part of the observed inhibition of microtubule severing by LAPSER1.

suggests that LAPSER1 increases the sensitivity of the cells to a prophase checkpoint. Therefore, LAPSER1 may regulate the sensitivity threshold of this checkpoint to mitotic stresses arising from the extracellular environment by altering the intracellular microtubule status. Since disturbances in prophase checkpoint may also contribute to genome instability (33), the activity of LAPSER1 in this phase might be a guardian against this.
katanin are preserved and authors speculate the reason to be the presence of proliferating epithelial cells (5,38). We speculate that these data suggest that LAPSER1 works as an inhibitor of katanin relatively selectively in the non-neuronal tissues including prostate in the adult mammals.

Recently two proteins including spastin and fidgetin have been shown to possess microtubule severing activity (39). However, mammalian spastin expression is much more predominant in the nervous tissues compared with the non-nervous tissues (36,38). Also major subcellular localization of mammalian fidgetin has been reported to be nucleus (40,41). Therefore, in mammalian adult non-nervous tissues, katanin might be the major microtubule severing protein and the inhibition of katanin might provide cells with key regulations of biological activities that involve microtubule severing.

In summary, we have elucidated three different mechanisms for the potential tumor suppressive activity of LAPSER1. Although they differ in the pathways involved, a common molecular basis for the inhibitory properties of LAPSER1 is microtubule release from the centrosomes.

MATERIALS AND METHODS

Expression constructs and transfection experiments

All of the constructs used in this study including expression vectors, adenoviruses and stable cell lines expressing LAPSER1 have been reported previously (2). Recombinant baculoviruses were constructed using the Bac to Bac System (Invitrogen). Human p60 katanin and GST-procon80 expression constructs in SF9 cells (constructed on pFastBac-HTB vector) were kindly provided by Dr. Francis J. McNally et al. (4). Protein expression and purification of katanin derivatives were performed according to previously reported methods (4). Purification of bacterially expressed GST-LAPSER1-C terminus was performed according to previously reported methods (2). The GFP-ninein expression construct in the pEGFP-C1 background (Clontech) was a kind gift of Dr. Michel Bornens (11). HeLa cells stably expressing GFP-ninein were obtained by transfection of this construct followed by selection under G418. Knockdown of rat p80 katanin by siRNA transfection in Rat 1 cells were performed as described previously (2). GFP-p60 P loop KA construct followed by selection under G418. Knockdown of human p80 katanin were obtained by transfection of this expression construct in the pEGFP-C1 background (Clontech).

Xpress tagged constructs were expressed from the vector pcDNA4 HisMaxC (Invitrogen). GFP-tagged katanin constructs were expressed from the vector pGEX4T-1 (Amersham).

Cell culture

We used several different cell types in our experiments. Their names and characteristics are summarized in the Supplementary Material, Table.

Human cell lines (Saos-2, HeLa and Caco-2) and rodent fibroblast cell lines (NIH3T3, 3Y1 and Rat 1 derivatives) were maintained in D-MEM (NISSUI) supplemented with 10% fetal bovine serum (2). Rat cortical neurons in primary culture derived from 17 days embryo were purchased from Sumitomo Bakelite (Tokyo, Japan) and cultured as described previously (42). In all experiments, neuronal cells were cultured in neuron medium (Sumitomo Bakelite).

Antibodies and reagents

Mouse monoclonal antibodies against neurofilament (Sigma), α-tubulin (DM1A) (LVC), acetylated-tubulin (Sigma), γ-tubulin (Sigma) and phosphorylated histone H3 (S10) (Upstate) were used. The rabbit polyclonal antibody against LAPSER1 has been described previously (2). Rabbit polyclonal antibodies against β-tubulin (LVC) and γ-tubulin (Sigma) were used. Chicken polyclonal antibodies against p80 katanin were obtained from GeneWay Biotech. Nocodazole, vinblastine, paclitaxel and DCB were obtained from Sigma.

Immunocytochemistry

Indirect immunostaining of cell lines was performed as described previously (2). Briefly, cells were plated onto polystyrene-coated glass-bottom dishes (Matsunami, Japan), followed by 70% methanol fixation for 20 min at −20°C, rinsing for 10 min with PBS and incubation with primary antibodies for 1.5 h at 37°C. After further washing with PBS, cells were stained with secondary antibody and in 2 μM TO-PRO-3 (Molecular Probes) for 1 h. The cells were then washed again in PBS. Fluorescent signals were detected using the confocal laser scanning microscope LSM 510 Meta (Carl Zeiss), using a C-Apocromat 40XW objective lens with a 1.2 aperture. The original magnification was 400 ×. The acquisition software used was the Zeiss LSM Rel 3.2 package. Images were processed using Adobe Photoshop 7.0. For staining of primary cultured neurons, the same procedure as described above was performed except that cells were fixed using 4% paraformaldehyde and 0.2% glutaraldehyde in PBS for 1 h at room temperature followed by perforation with 0.5% Triton X100 in PBS for 20 min at room temperature. Centrosome (centriole) staining using anti-α-tubulin was performed by an established method (13). BrdU uptake experiments were performed using BrdU labeling kit (Takara). The samples from the in vitro microtubule disassembly assay were fixed by methanol and subjected to immunostaining.

Immunoblotting and flow cytometry

Western bloting, GST-pulldown experiments and flow cytometry were performed as described previously (2).

Pharmacological regime for revealing microtubule severing and transport

The pharmacological regime that was developed for analyzing the transport of microtubules from the centrosome has been extensively reported (10,16,18). For quantification, more than 100 cells were counted in each experiment (n = 3).

In vitro microtubule disassembly assay

DAPI (4′, 6-diamidino-2-phenylindole)-based disassembly assays were performed using a Microtubule Destabilization assay kit (BK012, Cytoskelton) according to the manufacturer’s
instructions with minor modification. Briefly, we diluted polymerized microtubules 1:4 v/v into a solution that was suitable for the assay of human katanin (20 mM K-HEPES, pH7.4, 10% glycerol (v/v), 2 mM MgCl2, 0.1 mM EGTA, 1 mM ATP) (4). The final concentration of polymerized microtubules was 7 μM. We also added DAPI to the diluted solution at a 16 μM final concentration. The concentrations of purified human p60 katanin, GST-procon80 and GST-LAPSER1-C terminus were 0.1, 0.15 and 0.15 μM, respectively. Fluorescence intensities were measured by excitation at 360 nm and emission at 460 nm using a SPECTRA MAX GEMINI XS (Molecular Devices).

Cell migration assays
Cell migration assays were performed in Transwell migration chambers (diameter 12 mm, pore size, 12 μm; Costar Corporation, Corning). Cells were transfected, infected or tetracycline-induced at 24 h prior to plating into migration chambers. Approximately 2 x 10^4 cells were added to the upper compartment of the transwell chamber and allowed to migrate for 22 h. Cell migration was determined by counting the number of cells that migrated to the underside of the polycarbonate filters. For the wound healing assay, cells were transfected with siRNA 3 days before use and allowed to grow to confluence that migrated to the lower side of the polycarbonate filters.

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Conflict of Interest statement. None declared.

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