Map7D2 and Map7D1 facilitate MT stabilization through distinct mechanisms to control cell motility and neurite outgrowth

Koji Kikuchi^{1*}, Yasuhisa Sakamoto¹, Akiyoshi Uezu², Hideyuki Yamamoto³, Kei-ichiro Ishiguro⁴, Kenji Shimamura⁵, Taro Saito⁶, Shin-ichi Hisanaga⁶, and Hiroyuki Nakanishi^{1*}

¹Department of Molecular Pharmacology, Graduate School of Medical Sciences, Kumamoto University, 1-1-1 Honjo, Kumamoto 860-8556, Japan

²Department of Cell Biology, Duke University Medical School, Durham, North Carolina 27710, U.S.A

³Department of Biochemistry, Graduate School of Medicine, University of the Ryukyus, Okinawa 903-0215, Japan.

⁴Department of Germline Development, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan

⁵Department of Brain Morphogenesis, Institute of Molecular Embryology and Genetics, Kumamoto University, 2-2-1 Honjo, Chuo-ku, Kumamoto 860-0811, Japan

⁶Department of Biological Sciences, Tokyo Metropolitan University, Hachioji, Tokyo 192-0397, Japan

***Correspondence:** kojik@kumamoto-u.ac.jp (K.K), hkananis@gpo.kumamoto-u.ac.jp (H. N.)

Keywords: Map7D2/Map7D1/Microtubule stabilization/Cell motility/Neurite outgrowth

1 **Abstract**

2 Microtubule (MT) dynamics are modulated through the coordinated action of various
3 MT-associated proteins (MAPs). However, the regulatory mechanisms underlying MT
4 dynamics remain unclear. Herein, we show that MAP7 family protein Map7D2 facilitates MT
5 stabilization to control cell motility and neurite outgrowth. Map7D2, was highly expressed in
6 the brain and testis, directly bound to MTs through its N-terminal half similarly to Map7, and
7 promoted MT stabilization *in vitro*. Map7D2 localized prominently to the centrosome and
8 partially on MTs in N1-E115 mouse neuroblastoma cells, which expresses two of the four
9 MAP7 family members, Map7D2 and Map7D1. Map7D2 loss decreased the intensity of MTs
10 without affecting stable MT markers acetylated and detyrosinated tubulin, suggesting that
11 Map7D2 stabilizes MTs via direct binding. In addition, Map7D2 loss increased the rate of
12 random cell migration and neurite outgrowth, presumably by disturbing the balance between
13 MT stabilization and destabilization. The other MAP7 family protein expressed in N1-E115,
14 Map7D1, exhibited similar subcellular localization and gene knock-down phenotypes. However,
15 in contrast to Map7D2, Map7D1 was required for the maintenance of acetylated tubulin levels.
16 Taken together, our data suggest that Map7D2 and Map7D1 facilitate MT stabilization through
17 distinct mechanisms for the control of cell motility and neurite outgrowth.

1 Introduction

2 Microtubule (MT) dynamics play crucial roles in a variety of cellular processes, including
3 mitosis, vesicle and organelle transport, as well as cell motility and morphology (Cleary, 2021;
4 Etienne-Manneville, 2013; Roll-Mecak, 2020). MT dynamics are altered in response to various
5 intrinsic or extrinsic signals and are then modulated through the coordinated actions of various
6 MT-associated proteins (MAPs), which control the processes of dynamic instability (Cleary,
7 2021; Roll-Mecak, 2020). Therefore, it is important to identify and characterize MAPs in order
8 to understand the regulatory mechanisms of MT dynamics. We previously performed a
9 comprehensive proteomic analysis of MT co-sedimented proteins from the brain and identified
10 a series of functionally uncharacterized MT-binding proteins (Sakamoto, 2008). The list
11 included MAP7 family members Map7, Map7D1, and Map7D2, but not Map7D3. Among the
12 MAP7 family, Map7 has been extensively characterized. Several lines of evidence suggest that
13 Map7 has the ability to stabilize and reorganize MTs. Ectopic expression of Map7 induces MT
14 bundling and resistance to nocodazole treatment-induced MT depolymerization (Masson, 1993).
15 Map7 expression is upregulated during MT reorganization in response to the differentiation of
16 keratinocytes (Fabre-Jonca, 1999) and the establishment of apicobasal polarity in human colon
17 adenocarcinoma cell lines such as Caco-2 and HT-29-D4 cells (Carles, 1999; Masson, 1993). In
18 addition, recent studies have shown that Map7 and the *Drosophila* Map7 homolog, Ensconsin
19 (Ens), are involved in Kinesin-1-dependent transport by promoting the recruitment of a
20 conventional Kinesin-1, Kif5b, and its *Drosophila* homolog, Khc, to MTs during various
21 biological processes (Barlan, 2013; Hooikaas, 2019; Kikuchi, 2018; Metzger, 2012; Sung, 2008;
22 Tymanskyj, 2018). The competition between Map7 and other MAPs for MT binding regulates
23 the loading of motor proteins, thereby controlling the distribution and balance of motor activity
24 in neurons (Monroy, 2018; Monroy, 2020). While a considerable body of evidence has
25 highlighted the important roles of Map7 in the regulation of MT dynamics, the function of
26 MAP7 family member Map7D2 in the regulation of MT dynamics and its relationship with
27 other MAP7 family members remain unclear.

28 In this study, we determined the tissue distribution and biochemical properties of
29 Map7D2 for the first time. Map7D2 is expressed predominantly in the glomerular layer of the
30 olfactory bulb and the Sertoli cells of testes. Further, it directly associates with MTs through its
31 N-terminal half, similarly to Map7, significantly enhancing MT stabilization. We also examined
32 the cellular functions of Map7D2 using the N1-E115 mouse neuroblastoma cell line, which

expresses both Map7D2 and Map7D1, but not Map7 nor Map7D3. Map7D2 predominantly localizes to the centrosome and partially on MTs, and suppresses cell motility and neurite outgrowth by facilitating MT stabilization via direct binding. Finally, we determined the functional differences between Map7D2 and Map7D1 with regard to MT stabilization in N1E115 cells. Although Map7D1 exhibits similar subcellular localization and gene knock-down phenotypes to Map7D2, Map7D1 is required to maintain the amount of acetylated tubulin in contrast to Map7D2. These results suggest that Map7D2 and Map7D1 facilitate MT stabilization through distinct mechanisms for the control of cell motility and neurite outgrowth.

Results

Map7D2 is highly expressed in the glomerular layer of the olfactory bulb and the Sertoli cells of testes.

We previously performed a comprehensive proteomic analysis of MT co-sedimented proteins from the rat brain and identified a number of novel factors (Sakamoto, 2008). In the present study, we focused on Map7D2, which is one of the MAP7 family members (Fig. S1A). To analyze the tissue distribution of Map7D2, we first performed northern blotting analysis using total RNA extracted from various rat tissues. Northern blotting analysis showed that the approximately 4.2-kb mRNA was hybridized only in the brain and testis, being more abundant in the former (Fig. 1A). Of note, no detectable signal was observed in other rat tissues examined, including the heart, spleen, lung, liver, skeletal muscle, and kidney. Next, we investigated the tissue distribution of Map7D2 at the protein level by immunoblotting. For the immunoblotting analysis, we raised an anti-Map7D2 polyclonal antibody using aa 1-235 of rat Map7D2 (rMap7D2) as an epitope (Fig. S2A). Using lysates from HeLa cells transfected with an empty vector, hMap7-V5His₆, or rMap7D2-V5His₆, we confirmed that the antibody detected Map7D2, but not Map7 (Fig. S2A). In addition, we evaluated antibody specificity by siRNA-mediated knock-down of endogenous Map7D2. For this experiment, we used a mouse neuroblastoma cell line, N1-E115, in which the expression of *Map7d2* and *Map7d1*, but not *Map7* and *Map7d3*, was detected by quantitative real-time PCR (RT-qPCR) (Fig. S2B). We designed three independent siRNAs against *Map7d2* or *Map7d1*. The immunoreactive band disappeared following treatment with each *Map7d2* siRNA, but not the control or *Map7d1* siRNA (Fig. S2C), indicating that the antibody specifically recognized Map7D2. We then performed immunoblotting analysis using lysates from various rat tissues. Consistent with the northern

blotting analysis, Map7D2 was detected at the protein level only in the brain and testis, while no immunoreactive bands were detected in other rat tissues (Fig. 1B).

We further analyzed the expression patterns of Map7D2 in the brain and testis by immunofluorescence. Based on RNA-seq CAGE, RNA-Seq, and SILAC database analysis (Fig. S3; Expression Atlas, <https://www.ebi.ac.uk/gxa/home/>), Map7D2 expression was confirmed in the four brain tissue regions of postnatal day 0 mice, the cerebellum, cerebral cortex, hippocampus, and olfactory bulb. Among these regions, Map7D2 was most highly expressed in the Map2-negative area of the olfactory bulb, the glomerular layer (Fig. 1C). Only weak signals were detected in the cerebellum, cerebral cortex, and hippocampus (Fig. 1C). Next, we analyzed Map7D2 expression in the seminiferous tubules of adult mice. Map7D2 signals were merged with Tubb3 signals, a marker for Sertoli cells (Fig. 1C), indicating that Map7D2 is expressed predominantly in Sertoli cells. Taken together, these data suggest that *in vivo*, Map7D2 may function in the glomerular layer of the olfactory bulb and the Sertoli cells of the testis.

Map7D2 has an ability to stabilize MTs.

MAP7 family members share two conserved regions (Fig. S1A). The amino acid sequences of the N-terminal (aa 53-138) and C-terminal (aa 389-562) regions of human Map7D2 (hMap7D2) were 64.0% and 42.9% identical to those of human Map7 (hMap7), respectively, while other regions showed no significant homology to hMap7 (Fig. 2A). Using the rat brain cDNA library, we obtained rMap7D2 cDNA by PCR. The cloned cDNA encoded a protein consisting of 763 aa with a molecular weight of 84,823 (DDBJ/EMBL/GenBank accession number AB266744) (Fig. 2A). The full-length aa sequence of rMap7D2 was 68.1% identical to that of hMap7D2. For subsequent experiments, we used the rMap7D2 that we cloned.

We sought to determine whether rMap7D2 directly binds to MTs. To this end, we performed an MT co-sedimentation assay using recombinant rMap7D2. When His₆-rMap7D2 was incubated with MTs, followed by ultracentrifugation, it was recovered with MTs in the pellet (Fig. 2B). The dissociation constant (K_d) was calculated to be approximately 6×10^{-7} M (Fig. 2B). This value is comparable to those of well-known MAPs Tau and CLIP-170 (Gustke, 1994; Lansbergen, 2004). The stoichiometry of His₆-rMap7D2 binding to tubulin was calculated to be one His₆-rMap7D2 molecule per about ten tubulin α/β heterodimers. This value was also

comparable to that of Map7 (Bulinski, 1994). It has been reported that Map7 binds to MTs through a conserved region on the N-terminal side, while Map7D3 binds via a conserved region on the C-terminal side (Yadav et al., 2014). To further examine the location of the MT-binding domain of rMap7D2, the N-terminal (aa 1-421) and C-terminal (aa 422-763) halves were subjected to an MT co-sedimentation assay (Fig. 2A). The N-terminal half was co-sedimented with MTs, whereas the C-terminal half was not (Fig. 2C). These results indicate that the MT-binding domain of rMap7D2 is located at the N-terminal half, similarly to that of Map7, but not Map7D3.

Next, we tested whether rMap7D2 affects MT dynamics. The MT turbidity assay was used to analyze the effect of rMap7D2 on the kinetics of MT assembly. The addition of rMap7D2 significantly enhanced the amount of polymerized MTs in a time-dependent manner, whereas tubulin self-polymerized even in the absence of rMap7D2 (Fig. 2D). Identical results were observed by fluorescence microscopy analysis using rhodamine-labeled tubulin (Fig. 2E). Furthermore, we investigated the ability of Map7D2 to bundle MTs in HeLa cells. Consistent with the *in vitro* data, overexpression of Myc-rMap7D2 induced MT bundling in HeLa cells (Fig. 2F). Taken together, these results indicate that Map7D2 facilitates MT stabilization.

Map7D2 localized prominently to the centrosome and partially to MTs.

Following the biochemical characterization of Map7D2, we sought to determine its functions within the cell. To this end, we used N1-E115 cells that express Map7D2 and Map7D1 (Fig. S2B and C). First, we analyzed the subcellular localization of Map7D2 in N1-E115 cells. N1-E115 cells can undergo neuronal differentiation in response to DMSO under conditions of serum starvation (Kimhi, 1976), and most of the cells extend neurites up to 12 h after treatment with 1% DMSO (Fig. S4A) (Smit, 2003). In both proliferating and differentiated cells, Map7D2 localized prominently to the centrosome and partially to MTs (Fig. 3A-C). These localizations were confirmed in N1-E115 cells stably expressing EGFP-rMap7D2 (Fig. 3D and E). Furthermore, during cytokinesis, Map7D2 accumulated at the midbody, where MT bundling occurs (Fig. 3B). Similarly, localization of Map7D2 was also observed at neurites, where MT bundling is also known to occur (Fig. 3C). Together with the biochemical properties, these subcellular localization data suggest that Map7D2 is involved in MT stabilization within the cell.

1 Since N1-E115 cells express another Map7 family member, Map7D1, we also
 2 determined its subcellular localization. Map7D1 exhibited similar localization to that of
 3 Map7D2 in both proliferative and differentiated states (Fig. S4B-D). Interestingly, Map7D1
 4 knock-down upregulated Map7D2 expression, as confirmed with three different siRNAs (Fig.
 5 S2C), indicating that this effect was not due to the off-target effects of a particular siRNA.
 6 Endogenous Map7D2 expression was suppressed in N1-E115 cells stably expressing
 7 EGFP-rMap7D2 and was restored by specific knock-down of EGFP-rMap7D2 using *gfp* siRNA
 8 (Fig. 3D). These results suggest that the expression of Map7D2 was influenced by changes in
 9 that of Map7D1. In contrast, Map7D2 knock-down did not affect Map7D1 expression (Fig.
 10 S2C), and identical results were observed in the *Map7d2* knock-out (*Map7d2*^{-/-}) N1-E115 cells
 11 we generated (Fig. S5A and B). As Map7D2 has the potential to functionally compensate for
 12 Map7D1 loss, we decided to analyze the phenotypes of single and double knock-downs for
 13 Map7D2 and Map7D1 in the following experiments.

14

15 **Map7D2 is required for MT stabilization in the control of cell motility and neurite** 16 **outgrowth.**

17 It is well known that acetylation and detyrosination of α -tubulins are associated with stable MTs
 18 (Baas, 2016; Janke, 2017). Therefore, we examined the effects of Map7D2 or Map7D1
 19 knock-down on the levels of acetylated and detyrosinated tubulins. Neither Map7D2
 20 knock-down (Fig. 4A), nor *Map7d2* knock-out affected the total levels of these modified
 21 tubulins (Fig. 4B). In contrast, Map7D1 knock-down reduced the total level of acetylated but
 22 not detyrosinated tubulin (Fig. 4A), with double knock-down of Map7D2 and Map7D1 having
 23 the same impact (Fig. 4A). Consistently, immunostaining analysis also showed that Map7D1
 24 knock-down greatly decreased the intensity of acetylated tubulin around the centrosome in
 25 N1-E115 cells (Fig. 4C and D). Map7D1 knock-down decreased the intensity of α -tubulin and
 26 increased that of Map7D2 (Fig. 4C and D), indicating that Map7D1 is required for the
 27 maintenance of stable and acetylated MTs. Under Map7D2 knock-down, the intensity of
 28 α -tubulin and Map7D1 decreased without affecting that of acetylated tubulin (Fig. 4C and D).
 29 Together with our biochemical data for Map7D2, these results suggest that Map7D2 facilitates
 30 MT stabilization via direct binding, in contrast to Map7D1.

1 Dysregulation of MT stabilization is known to affect various biological functions, for
2 instance, it can lead to increased cell motility and neurite outgrowth (Alesi, 2016; Biernat, 2002;
3 Grenningloh, 2004; Hubbert, 2002). Therefore, we analyzed whether random cell migration or
4 neurite outgrowth of N1-E115 cells is affected by single or double knock-down and *Map7d2*
5 knock-out. As expected, each single knock-down and the *Map7d2* knock-out enhanced not only
6 the migration speed and distance during random cell migration (Fig. 5A-C), but also the rate of
7 neurite outgrowth (Fig. 5D). Furthermore, double knock-down of Map7D2 and Map7D1 tended
8 to result in increased cell motility and neurite outgrowth (Fig. 5B-D). Taken together, these
9 results suggest that Map7D2 and Map7D1 facilitate MT stabilization through distinct
10 mechanisms, thus controlling cell motility and neurite outgrowth.

11 **Discussion**

12 In the present study, we provide the first comprehensive analysis of Map7D2 biochemical
13 properties (Fig. 2). The N-terminal and C-terminal regions of Map7D2 exhibited high homology
14 to those of Map7 (Fig. S1). The N-terminal homologous region is basic and highly charged.
15 Most MT-binding domains characterized thus far are confined to positively charged regions
16 (Aizawa, 1990; Lewis, 1989; Noble, 1989; Pierre, 1992). Consistently, the MT-binding region
17 of Map7 was shown to be located at the N-terminal positively charged region (Masson, 1993).
18 Since we demonstrated that the N-terminal half of rMap7D2 directly bound to MTs (Fig. 2C), it
19 is likely that Map7D2 also associates with MTs through the positively charged N-terminal
20 region. Although the physiological role of the C-terminal region of Map7D2 is currently
21 unknown, it is conceivable that this region may play an important role as it is conserved
22 between Map7D2 and Map7. A region within the C-terminus of Map7 is required for complex
23 formation with Kif5b, the heavy chain of Kinesin-1 (Fig. S6), and is involved in
24 Kif5b-dependent transport by loading Kif5b onto MTs (Hooikaas, 2019; Kikuchi, 2018;
25 Metzger, 2012; Tymanskyj, 2018). Interestingly, we found that Map7D2 also formed a complex
26 with Kif5b (Fig. S6). Furthermore, it was recently reported that Map7D2 contributes to
27 Kinesin-1-mediated transport in the axons of hippocampal neurons (Pan, 2019). Taken together,
28 the biochemical properties are largely conserved between Map7D2 and Map7.

29 In contrast, the cellular functions of Map7D2 may differ from those of Map7. Our
30 group and Hooikaas et al. have previously reported that Map7 and Map7D1 have functional
31 overlaps in HeLa cells (Hooikaas, 2019; Kikuchi, 2018). For instance, both form a complex

1 with Dishevelled, a mediator of Wnt5a signaling, while Map7D2 does not (Kikuchi, 2018). In
2 addition, Map7D2 exhibits distinct localization patterns in cultured hippocampal neurons,
3 localizing to the proximal axon (Pan, 2019). In the present study, we propose the molecular
4 mechanism how Map7D2 and Map7D1 regulate MT stabilization in N1-E115 cells (Fig. 5E).
5 Map7D2 and Map7D1 both strongly localize to the centrosome and partially on MTs in
6 proliferating as well as in differentiated N1-E115 cells (Fig. 3 and Fig. S4B-D). Further, the
7 knock-down of either resulted in a comparable reduction of MT intensity (Fig. 4C and D),
8 enhancing the rate of cell motility and neurite outgrowth (Fig. 5A-D). Mechanistically, Map7D1
9 is required for the maintenance of MT acetylation, which is enriched in stable MTs, whereas
10 Map7D2 is not (Fig. 4). Taking these findings into consideration with our biochemical data, we
11 propose that, in contrast to Map7D1, Map7D2 facilitates stabilization by directly binding MTs
12 to then control cell motility and neurite outgrowth.

13 We also determined the tissue distribution of Map7D2, which has not been described
14 to date (Fig. 1). The tissue distribution of Map7 was previously analyzed using gene-trap mice
15 (Komada, 2000). At the mRNA level, *Map7* is expressed in a variety of epithelial tissues, dorsal
16 root ganglia, trigeminal ganglia, and primitive seminiferous tubules during embryonic
17 development. We also reported that both Map7 and Map7D1 are expressed in the epithelia of
18 the mouse fallopian tube at the protein level (Kikuchi, 2018). Consistent with Map7 expression
19 in primitive seminiferous tubules, *Map7* homozygous gene-trap mice exhibited defects in
20 spermatogenesis (Komada, 2000). Map7D2 was expressed predominantly in the glomerular
21 layer of the olfactory bulb and Sertoli cells of the testis (Fig. 1C). The glomerular layer is
22 known to be the region where axons accumulate and does not express Map2, a marker of
23 neuronal cell bodies and dendrites (Fig. 1C). As Map7D2 localizes to the proximal axon in
24 cultured hippocampal neurons (Pan, 2019), Map7D2 may have similar localization and function
25 in olfactory bulb neurons. The function of Map7D2 in Sertoli cells was not clarified in the
26 present study. Therefore, whether Map7D2 is involved in mammalian neurogenesis and
27 spermatogenesis represents a question for future research.

28

1 **Materials and methods**

2 **Molecular cloning, expression, and purification of rMap7D2**

3 Based on the information of DDBJ/EMBL/GenBank accession number XM_228973,
 4 oligonucleotide primers (5'-ATGTCGACATGGAGCGCAGCGGTGGGAACGGCG-3' and
 5 5-ATGTCGACTCAACAGAAGGTGTTCAGCGTAGTTTC-3') were designed, and rat
 6 *Map7d2* (rMap7d2) cDNA was obtained by PCR using rat cDNA as a template. Expression
 7 vectors for rMap7d2 were constructed in pCMV5-Myc (Nakanishi, 1997), pQE9 (Qiagen),
 8 pGEX-5X-3 (Cytiva), pcDNA3.1/V5-His (Thermo Fisher Scientific), pCLXSN-GFP (Reiley,
 9 2005), and pEGFP-N3 (Clontech). His₆-tagged or GST-fused proteins were expressed in
 10 *Escherichia coli* and purified using TALON metal affinity beads (CLONTECH) or
 11 glutathione-Sepharose beads (Cytiva), respectively. GST-rMap7D2 (full length) was further
 12 purified by gel filtration using a HiLoad 16/60 Superdex 200 column (Cytiva).

13

14 **Antibodies**

15 A rabbit polyclonal anti-Map7D2 antibody was raised against GST-rMap7D2 (aa1-235). All the
 16 primary antibodies used are listed in Supplemental Table S1. Secondary antibodies coupled to
 17 horseradish peroxidase (HRP) were purchased from Sigma-Aldrich. Alexa Fluor-conjugated
 18 secondary antibodies used for immunofluorescence experiments were purchased from Thermo
 19 Fisher Scientific.

20

21 **MT binding assay**

22 The MT co-sedimentation assay was performed as previously described, with a slight
 23 modification (Yamamoto, 2002). MTs were prepared by incubating tubulin in polymerization
 24 buffer (80 mM PIPES/NaOH, pH 6.8, 1 mM MgCl₂, 1 mM EGTA, and 1 mM GTP) containing
 25 10% glycerol for 20 min at 37°C. After incubation, taxol was added at a final concentration of
 26 15 μM. Various amounts of rMap7D2 were incubated with 0.4 mg/mL of MTs in
 27 polymerization buffer containing 15 μM taxol for 20 min at 37°C. After incubation with MTs,
 28 the mixture (200 μL) was placed over a 700-μL cushion of 50% sucrose in polymerization

1 buffer containing 15 μ M taxol. After the sample was centrifuged at $100,000 \times g$ for 30 min at
 2 37°C , the supernatant was removed from the cushion, and the original volume was restored with
 3 SDS sample buffer. Comparable amounts of the supernatant and pellet fractions were subjected
 4 to SDS-PAGE, followed by CBB protein staining. The amount of protein was estimated using a
 5 densitometer. ELISA for MT binding was performed in a 96-well microtiter plate as previously
 6 described (Pedrotti, 1994). Briefly, wells were coated by incubating with 0.2 mg/mL of MTs in
 7 polymerization buffer containing 15 μ M taxol for 2 h at 37°C and then blocked via incubation
 8 with 5% glycine. Increasing amounts of rMap7D2 were added to each well and incubated for 20
 9 min at 37°C . The plate was washed and further incubated with an anti-Map7D2 antibody. After
 10 washing, the plates were incubated with a secondary antibody conjugated to horseradish
 11 peroxidase. SuperSignal ELISA Pico (Pierce) was used as a chemiluminescent peroxidase
 12 substrate.

13

14 **MT polymerization assays**

15 MT assembly was assayed by measuring turbidity at 350 nm using a spectrophotometer, as
 16 previously described (Gaskin, 1974). Briefly, GST-rMap7D2 (0.14 mg/mL) was incubated with
 17 2 mg/mL tubulin in polymerization buffer at 37°C . The sample was continuously monitored at
 18 350 nm using a Hitachi U-2000 spectrophotometer. MT assembly was further assayed by
 19 fluorescence microscopy using rhodamine-labeled tubulin (Hyman, 1991). Briefly,
 20 GST-rMap7D2 (0.07 mg/ml) was incubated at 37°C for 20 min with 0.8 mg/mL tubulin (1 : 9 =
 21 rhodamine-labeled tubulin : unlabeled tubulin) in polymerization buffer. Incubation was stopped
 22 through the addition of 1% glutaraldehyde. The sample was spotted onto a glass slide and
 23 viewed under a fluorescence microscope.

24

25 **Northern blotting**

26 An RNA blot membrane (Clontech) was hybridized with the ^{32}P -labeled full coding sequence of
 27 rMap7D2, according to the manufacturer's protocol.

28

1 Cell culture and transfection

2 HeLa and N1-E115 cells were cultured at 37°C in DMEM supplemented with 10% fetal bovine
3 serum and penicillin-streptomycin. The methods employed for plasmid or siRNA transfection
4 were previously described (Kikuchi, 2010). Plasmid transfection of N1-E115 cells was
5 performed using Lipofectamine LTX according to the manufacturer's instructions.
6 Differentiation of N1-E115 neuroblastoma cells was induced by decreasing the serum level to
7 0.5% fetal bovine serum and adding 1% dimethyl sulfoxide (DMSO) (hereafter, the
8 above-described medium was referred to as differentiation medium). Stealth double-stranded
9 RNA was purchased from Thermo Fisher Scientific (USA). All siRNAs used in this study are
10 listed in Supplemental Table S2. Three individual siRNAs for mouse *Map7d2* or *Map7d1* were
11 designed based on the respective sequences. Double-stranded RNA targeting luciferase was
12 used as a control. The cells were cultured for 72 h and subjected to various experiments. In Fig.
13 4A, C, D, and Fig. 5B-D, to exclude siRNA off-target effects, a mixture of three individual
14 siRNAs for Map7D1 or Map7D2 was used. For the generation of N1-E115 cells stably
15 expressing EGFP-rMap7D2, clones were selected by adding G418 at 24 h post-transfection.
16 EGFP-rMap7D2 expression was confirmed by immunoblotting using antibodies against GFP
17 and Map7D2.

18

19 Generation of *Map7d2* knock-out N1-E115 cell lines by CRISPR-Cas9

20 Two sgRNA sequences were designed using the CHOPCHOP CRISPR/Cas9 gRNA finder tool
21 (<http://chopchop.cbu.uib.no/>). The short double-stranded DNA for each sgRNA
22 (5-CACCGTGAAGAGAGCACATGTGCC-3 and 5-AAACGGCACATGTGCTCTCTTCAC-3,
23 or 5-CACCGCAGGATCACCAGGGCCTGG-3 and 5-AAACCCAGGCCCTGGTGATCCTGC-3') were inserted into the *BbsI* site of pX330 (Cong,
24 2013). To construct the *Map7d2* knock-out vector, the 5' and 3' arms of each gene were
25 amplified by PCR using N1-E115 genomic DNA and cloned into the pCR4 Blunt-TOPO vector
26 (Thermo Fisher Scientific). The puromycin resistance marker was inserted between the 5' and 3'
27 arms (Fig. S6A). N1-E115 cells were transfected with 1 µg of each of the two pX330-sgRNA
28 plasmids and the knock-out vector using Lipofectamine LTX (Thermo Fisher Scientific).
29 Knock-out clones were selected by adding puromycin (Sigma-Aldrich) at 24 h post-transfection.

1 Successful knock-out was confirmed by immunoblotting using an anti-Map7D2 antibody and
2 genomic PCR.

3

4 **Animals**

5 Mice (C57BL6/N; Japan SLC, Japan) were used in this study. Animal care and experiments
6 were conducted in accordance with the guidelines for the care and use of laboratory animals of
7 the Center for Animal Resources and Development, Kumamoto University. All experiments
8 were approved by the experimental animal ethics committee of Kumamoto University
9 (A2019-127 and A2021-018). Mice were kept in a light- and temperature-controlled room with
10 a 12-h light/dark cycle at 22 ± 1 °C.

11

12 **Quantitative real-time PCR**

13 Each RNA sample was subjected to reverse transcription using murine leukemia virus reverse
14 transcriptase (Thermo Fisher Scientific), and the generated cDNA was used as a template for
15 qRT-PCR. Each reaction mixture was prepared using the KAPA SYBR Fast qPCR kit (Kapa
16 Biosystems), and the PCR reaction was performed on ViiA7 (Thermo Fisher Scientific). The
17 primers used for RT-qPCR are listed in Supplementary Table S3.

18

19 **Immunoblotting and immunoprecipitation**

20 For immunoblotting, cells were washed once with PBS and lysed with Laemmli's sample buffer.
21 After boiling, the lysates were separated by SDS-PAGE, transferred to PVDF membranes
22 (Millipore), and immunoblotted with antibodies. For immunoprecipitation analysis, the HeLa
23 cells were washed once with PBS at 24 h post-transfection and lysed with $1 \times$ NP40 buffer [20
24 mM Tris-HCl (pH 8.0), 10% glycerol, 137 mM NaCl, 1% NP40] supplemented with protease
25 inhibitors and phosphatase inhibitors for 20 min on ice. The supernatant was collected after
26 centrifugation and incubated with the appropriate antibodies. After incubation, 15 μ L of protein
27 A or G Sepharose beads was added, and the mixtures were rotated for 1 h at 4°C. The beads

were washed once with 1×NP40 buffer, twice with LiCl buffer [0.1 M Tris-HCl (pH 7.5), 0.5 M LiCl], once with 10 mM Tris-HCl (pH 7.5), and were finally resuspended in Laemmli's sample buffer.

Immunofluorescence staining

For immunofluorescence staining, cells were grown on coverslips and fixed in 100% methanol at -20 °C for 5 min. After blocking with 1% BSA in PBS for 1 h at room temperature, the samples were incubated with primary antibodies overnight at 4°C, followed by incubation with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h. For immunofluorescence tissue staining, tissues were fixed in 4% paraformaldehyde in PBS at 4°C overnight, and then immersed sequentially in 10, 20, and 30 % sucrose in PBS at 4°C. After sucrose equilibration, tissues were immersed in OCT (Sakura Finetechnical) at room temperature for 5 min, followed by embedding in OCT and freezing in liquid nitrogen. Sections (10 µm) were stored at -80°C. The sections were washed once with PBS for 10 min and twice with 0.1 % Triton X-100 in PBS for 10 min. After blocking with Blocking One (Nacalai) for 1 h at room temperature, the samples were incubated with primary antibodies overnight at 4°C, followed by incubation with Alexa Fluor-conjugated secondary antibodies (Thermo Fisher Scientific) for 1 h. Nuclei were stained with DAPI for 30 min at room temperature. The samples were viewed under a fluorescence microscope (Olympus, BX51) or a confocal microscope (Olympus, FV1000 or Leica, TCS SP8). Images were processed and analyzed using Fiji software (National Institutes of Health).

Random cell migration assay and neurite outgrowth assay

For the random cell migration assay, cells were seeded onto a laminin-coated (10 µg/mL) glass-bottom dish and recorded under an inverted microscope system equipped with an incubator (Olympus, LCV110). For the neurite outgrowth assay (Fig. S5C), the underside of 3 µm pore transwell membranes (Corning) was coated with 500 µL of 10 µg/mL laminin in PBS into a well of a 24-well plate. After coating, the membranes were removed from the laminin and placed into the well of a 24-well dish containing 500 µL differentiation media. One hundred

microliters of cell suspension (containing $1-2 \times 10^5$ cells) was added to the insert chamber on top of the membrane. The cells were allowed to extend neurites through the membrane pores to the lower chamber (underside of the membrane) for 6 h at 37°C. The cells were then fixed and stained with an anti- α -tubulin antibody. Images were processed and analyzed using Fiji software (National Institutes of Health).

Statistics

The experiments were performed at least three times (biological replicates), and the results are expressed as the average $\pm$ S.D. or the median, first and third quartiles, and 5-95 % confidence intervals for the box-and-whisker plot. Differences between data values were tested for statistical significance using the Student's *t*-test. Statistical significance was set at $P < 0.05$.

Other Procedures

Tubulin was prepared from fresh porcine brains by three cycles of polymerization and depolymerization, followed by DEAE-Sephadex column chromatography (Shelanski, 1973; Williams R.C.Jr. and Lee, 1982).

1 **Acknowledgements**

2 We thank past and present members of our laboratory for helpful discussions. This work was
3 partly carried out at the Institute of Molecular Embryology and Genetics, the Gene Technology
4 Centre, the Center for Animal resources and Development, and Research Facilities of the
5 School of Medicine, Kumamoto University.

6

7 **Author Contributions**

8 K.K. designed the research and carried out most of the experiments, except for Fig. 1A, B, and 2.
9 Y.S., A.U., H.Y., and H.N. carried out Fig. 1A, B and 2, and raised the rabbit polyclonal
10 anti-Map7D2 antibody. K.I. provided technical assistance for the immunofluorescence analysis
11 using the mouse testis. K.S. provided technical assistance for the immunofluorescence analysis
12 using the mouse brain. Y.S., T.S., S.H., and H.N. provided reagents, materials, and analysis
13 tools. K.K. wrote the paper, and Y.S., and H.N. edited the paper. All of the authors discussed
14 the results and commented on the manuscript.

15

16 **Funding**

17 This work was supported by JSPS KAKENHI Grant Numbers 22700881, 24700980, 15K07054,
18 and 19K06664, and grants from The Japan Spina Bifida & Hydrocephalus Research Foundation,
19 Astellas Foundation for Research on Metabolic Disorders, the Mochida Memorial Foundation,
20 the Takeda Science Foundation, and the Uehara Memorial Foundation (to K.K.).

21

22 **Conflict of Interest**

23 The authors declare that the research was conducted in the absence of any commercial or
24 financial relationships that could be construed as a potential conflict of interest.

25

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30

31 **Figures**

32 **Figure 1. Tissue distribution of Map7D2.**

1 (A) Northern blot analysis. An RNA blot membrane (CLONTECH) was hybridized with the
2 ³²P-labeled full coding sequences of rMap7D2 according to the manufacturer's protocol. (B)
3 Immunoblotting analysis. Various tissue lysates (20 µg of protein) were subjected to
4 SDS-PAGE, followed by immunoblotting with the anti-Map7D2 antibody. (C) Expression
5 patterns of Map7D2 in the brain and testis by immunofluorescence. Upper panels, frozen
6 sagittal sections of postnatal day 0 mouse brains were stained with anti-Map7D2 (magenta) and
7 antibodies against mature neuron marker Map2 (green). DNA was labeled with DAPI (gray).
8 For a comparison of signal intensities, images were captured under the same parameters.
9 Contrast-enhanced images of Map7D2 staining were shown in the rightmost column. Lower
10 panels, frozen coronal sections of adult mouse testis were stained with anti-Map7D2 (magenta)
11 and antibodies against Sertoli cell marker Tubb3 (green). DNA was labeled with DAPI (gray).
12 Scale bars in upper panels or lower panels: 100 or 50 µm, respectively.

13

14 **Figure 2. Map7D2 has the ability to stabilize MTs.**

15 (A) Schematic structures of hMap7, hMap7D2, and rMap7D2. (B) Co-sedimentation of
16 rMap7D2 with MTs. Left panel, His₆-rMap7D2 (34 µg/mL) was mixed with MTs, followed by
17 ultracentrifugation. Comparable amounts of the supernatant and pellet fractions were subjected
18 to SDS-PAGE, followed by CBB protein staining. S, supernatant; P, pellet. Middle panel,
19 various amounts of His₆-rMap7D2 were mixed with MTs, followed by ultracentrifugation.
20 Amounts of free and bound His₆-rMap7D2 were calculated by determining protein amounts
21 from the supernatant and pellet fractions, respectively, with a densitometer. Right panel,
22 Scatchard analysis. (C) Location of the MT-binding domain. GST-rMap7D2-N (80 µg/mL) or
23 GST-rMap7D2-C (200 µg/mL) was mixed with MTs, followed by ultracentrifugation.
24 Comparable amounts of the supernatant and pellet fractions were subjected to SDS-PAGE,
25 followed by CBB protein staining. S, supernatant; and P, pellet. (D) Turbidity measurement.
26 GST-rMap7D2 was mixed with tubulin. The sample was incubated at 37°C and continuously
27 monitored at 350 nm using a spectrophotometer. (○) without GST-rMap7D2; and (●) with
28 GST-rMap7D2. *, *P* < 0.003 (the F-test). (E) Immunofluorescent observation. GST-rMap7D2
29 was incubated for 20 min at 37°C with rhodamine-labeled tubulin. After fixation, the sample
30 was spotted on a slide glass and viewed under a fluorescence microscope. (F) HeLa cells
31 transiently overexpressing Myc-rMap7D2. Myc-rMap7D2 was transfected into HeLa cells, and

the cells were then double-stained with anti-Myc and anti- α -tubulin antibodies. Arrowheads show MT bundles. Scale bars, 50 μ m in E and 10 μ m in F.

Figure 3. Subcellular localization of Map7D2 in proliferative and differentiated N1-E115 cells.

(A-C) Localization of Map7D2 in interphase (A), mitosis (B), and differentiation state (C) of N1-E115 cells. Cells were double-stained with anti-Map7D2 and anti- α -tubulin antibodies. In A, the insets show enlarged images of regions indicated by a white box. In B, the inset shows metaphase cells. In C, images of differentiated cells were captured by z-sectioning, and each inset show enlarged images of regions indicated by a white box at each focal plane. Arrowheads show the centrosomal localization of Map7D2. (D) Generation of N1-E115 cells stably expressing EGFP-rMap7D2. To check the expression level of EGFP-rMap7D2, lysates derived from the indicated cells were probed with anti-GFP (top panel) and anti-Map7D2 (middle panel) antibodies. The blot was reprobed for γ -tubulin as a loading control (bottom panel). Of note, stable expression of EGFP-rMap7D2 reduced endogenous Map7D2 expression, and specific knock-down of EGFP-rMap7D2 using *gfp* siRNA restored endogenous Map7D2 expression. (E) Confirmation for subcellular localization of Map7D2 using N1-E115 cells stably expressing EGFP-rMap7D2. Images were captured by z-sectioning. Top panels show images taken with the lower or upper focal plane, and bottom panels show the image reconstructed in the z-axis direction. Arrow head shows centrosomal localization of Map7D2. Scale bars: 10 μ m.

Figure 4. Map7D2 is required for MT stabilization within the cell.

(A) Immunoblot analysis for acetylated (Ace-) and detyrosinated (Detyr-) tubulin in cells treated with each siRNA. Lysates derived from the indicated cells were separated by SDS-PAGE and subjected to immunoblotting with anti-Map7D1, anti-Map7D2, anti-Ace-tubulin, or anti-Detyr-tubulin antibodies. The blot was reprobed for Clathrin heavy chain (HC) or α -tubulin as a loading control. (B) Immunoblot analysis for Ace- and Detyr-tubulins in *wild-type* and *Map7d2*^{-/-} cells. Three independent *Map7d2*^{-/-} clones were used in this study. Lysates derived from the indicated cells were separated by SDS-PAGE and were immunoblotted with

1 anti-Map7D1, anti-Map7D2, anti-Ace-tubulin, or anti-Detyr-tubulin antibodies. The blot was
 2 reprobed for α -actin or α -tubulin as a loading control. (C) Immunofluorescence staining for
 3 α -tubulin, Ace-tubulin, and Map7D1 or Map7D2 in cells treated with each siRNA. For a
 4 comparison of signal intensities, images were captured under the same parameters. The insets
 5 show enlarged images of regions indicated by a white box. Of note, Ace-tubulin was present
 6 predominately around the centrosome in N1-E115 cells. (D) Quantification for
 7 immunofluorescence staining shown in C. Left panels, the intensities of α -tubulin, Ace-tubulin,
 8 and Map7D1 around the centrosome in the indicated cells were measured via ROI analysis
 9 (control, n = 197 cells; siMap7d2, n = 192 cells from three independent experiments). Right
 10 panels, the intensities of α -tubulin, Ace-tubulin, and Map7D2 around the centrosome in the
 11 indicated cells were measured by ROI analysis (control, n = 193 cells; siMap7d1, n = 227 cells
 12 from three independent experiments). *, $P < 1 \times 10^{-13}$; **, $P < 1 \times 10^{-8}$ (the Student's *t*-test). Scale
 13 bars: 10 μ m in C and 5 μ m in D.

14

15 **Figure 5. Map7D2 suppresses random cell migration and neurite outgrowth.**

16 (A) Bright-field images of migrating N1-E115 cells. Arrowheads show lamellipodia formed in
 17 the direction of migration. (B) Tracking analysis of random cell migration in the indicated cells.
 18 Each color represents the trajectory of 12 randomly selected cells. (C) Velocity and net distance
 19 measured in the indicated cells (control: n = 114 cells; siMap7d1: n = 100 cells; siMap7d2: n =
 20 71 cells; siMap7d1/d2: n = 107 cells; Map7d2^{-/-}: n = 60 cells from three independent
 21 experiments). *, $P < 1 \times 10^{-4}$; **, $P < 0.002$ (the Student's *t*-test). (D) Neurite outgrowth assay in the
 22 indicated cells. Neurites and cell bodies were visualized by α -tubulin staining (upper). The
 23 neurite outgrowth from each cell was distinguished by acquiring images with Z-sectioning. Data
 24 are from three or four independent experiments and represent the average $\pm$ S.D. (lower). *,
 25 $P < 0.002$; **, $P < 0.0002$ (the Student's *t*-test). (E) Proposed model for the distinct mechanisms of
 26 Map7D2 and Map7D1 for MT stabilization. See Discussion for further detail. Scale bars in A
 27 and D: 20 μ m.

28

A **B** **C**

A **B** **C** **D** **E** **F**

A **B** **C** **D**

A **B** **C** **D** **E**

Different actions of Map7D2 and Map7D1 to stabilize MTs