α-TubK40me3 is required for neuronal polarization and migration by promoting microtubule formation

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Tri-methylation on lysine 40 of α-tubulin (α-TubK40me3) is a recently identified post-translational modification involved in mitosis and cytokinesis. However, knowledge about α-TubK40me3 in microtubule function and post-mitotic cells remains largely incomplete. Here, we report that α-TubK40me3 is required for neuronal polarization and migration by promoting microtubule formation. α-TubK40me3 is enriched in mouse cerebral cortex during embryonic day (E)14 to E16. Knockdown of α-tubulin methyltransferase SETD2 at E14 leads to the defects in neuronal migration, which could be restored by overexpressing either a cytoplasm-localized SETD2 truncation or α-TubK40me3-mimicking mutant. Furthermore, α-TubK40me3 is preferably distributed on polymerized microtubules and potently promotes tubulin nucleation. Downregulation of α-TubK40me3 results in reduced microtubule abundance in neurites and disrupts neuronal polarization, which could be rescued by Taxol. Additionally, α-TubK40me3 is increased after losing α-tubulin K40 acetylation (α-TubK40ac) and largely rescues α-TubK40ac function. This study reveals a critical role of α-TubK40me3 in microtubule formation and neuronal development.
Microtubules are highly dynamic cytoskeleton composed of α/β-tubulin heterodimers and involved in a variety of cellular processes, especially in neuronal development. Microtubule functions are spatiotemporally regulated by multiple post-translational modifications (PTMs) of tubulins. Importantly, a few PTMs, such as acetylation, detyrosination, and glutamylation, are found to be enriched in neurons and the physiological roles of these PTMs in neuronal development and functions are gaining more and more attention. A recent study reports that in proliferative cells, α-tubulin could be tri-methylated at lysine 40 (α-TubK40me3), the same residue that has been also shown undergoing acetylation, by SET domain containing 2 (SETD2), a methyltransferase responsible for tri-methylation of histone 3 lysine 36 (H3K36me3). α-TubK40me3 occurs on the spindle and midbody and the loss of α-TubK40me3 causes mitotic and cytokinesis defects, indicating that α-TubK40me3 contributes to genomic stability during cell division. However, the functions of α-TubK40me3 in microtubule formation and post-mitotic cells are largely unclear.

Neurons are post-mitotic cells rich in microtubules. During the development of central nervous system (CNS), formation of the six-layered structure of cerebral cortex is a tightly regulated process which is essential for the establishment of proper neuronal circuits and brain functions. Projection neurons within the cerebral cortex are derived from neuronal progenitor cells (NPCs) in the ventricular zone (VZ) and subventricular zone (SVZ), and then radially migrate along the basal processes of radial glial progenitors (RGPs) to their final destinations in the cortical plate (CP) in mice. To initiate the radial migration, new-born projection neurons undergo morphological transition from multipolar stage to bipolar stage in the intermediate zone (IZ) of the cerebral cortex, and then radially migrate along the basal processes of radial glial progenitors (RGP) to their final destinations in the cortical plate (CP).

We profiled the level of α-TubK40me3 in different developmental stages of mouse cerebral cortex using a specific homemade antibody against α-TubK40me3 and we show that α-TubK40me3 is enriched in NPCs and neurons from embryonic day 14 (E14) to E16 in cerebral cortex. In utero knockdown of SETD2 at E14 leads to impaired morphological transition and thereby the migration of cortical neurons, which could be restored by overexpressing NES-SETD2(1469-1724), a cytoplasm-localized SETD2 truncation retaining enzyme activity or α-tubulinKK40F, a tri-methylation mimicking mutant of α-tubulin. Importantly, α-TubK40me3 is preferably distributed on polymeric microtubules in cells, and in vitro microtubule reconstitution assays shows that α-TubK40me3 could potentiate tubulin nucleation and slightly facilitate microtubule growth. Consistently, the microtubule abundance that is reflected by the number of EB3 comets and quantified under electron microscopy, is reduced in the neurites of cultured neurons upon downregulation of α-TubK40me3. Furthermore, promoting tubulin nucleation using a low concentration of Taxol could rescue the defects of neuronal polarization caused by impaired α-TubK40me3. Interestingly, the duration of high-level α-TubK40me3 in the developing cerebral cortex is extended in MEC-17 knockout mice, and the impaired morphological transition and migration of α-TubK40me3-deficient neurons could be largely restored by overexpressing NES-SETD2 and α-tubulinKK40F, implying a compensatory effect by α-TubK40me3. All together, these results suggest that α-TubK40me3 plays an essential role in the polarization and migration of cortical neurons by promoting tubulin nucleation and controlling microtubule formation.

**Results**

A high level of α-TubK40me3 occurs during E14 to E16 in the cerebral cortex. To investigate the existence of α-TubK40me3, we generated a polyclonal antibody against peptide around tri-methylated K40 of α-tubulin (Extended Data Fig. 1a). Dot blotting and immunoprecipitation showed a good recognition on tri-methylated K40 peptide but not un-methylated or mono-methylated K40 peptide, and minimal cross-reactivity with dimethylated K40 peptide and α-tubulinKK40F mutant (Extended Data Fig. 1b, c), confirming a high specificity of homemade α-TubK40me3 antibody. Similar to a previous report, both our homemade antibody and commercial H3K36me3 antibody could recognize α-TubK40me3 in immunoprecipitation (Extended Data Fig. 1d), however, our homemade antibody could not directly detect α-TubK40me3 in immunoblotting. Immunostaining with homemade α-TubK40me3 antibody showed no cross-reactive signals at DAPI-labeled chromatin but intense signals at α-tubulin-labeled spindle and midbody (Extended Data Fig. 1e), where a large amount of tubulin nucleation and microtubule formation take place at the metaphase and telophase. Thus, our homemade antibody is capable for specific detection of α-TubK40me3 in both immunoprecipitation and immunostaining.

To investigate the potential function of α-TubK40me3 in the development of CNS, we profiled the level of α-TubK40me3 at different developmental stages of mouse cerebral cortex using a speciﬁc homemade antibody against α-TubK40me3 and we show that α-TubK40me3 is enriched in NPCs and neurons from embryonic day 14 (E14) to E16 in cerebral cortex. In utero knockdown of SETD2 at E14 leads to impaired morphological transition and thereby the migration of cortical neurons, which could be restored by overexpressing NES-SETD2(1469-1724), a cytoplasm-localized SETD2 truncation retaining enzyme activity or α-tubulinKK40F, a tri-methylation mimicking mutant of α-tubulin. Importantly, α-TubK40me3 is preferably distributed on polymeric microtubules in cells, and in vitro microtubule reconstitution assays shows that α-TubK40me3 could potentiate tubulin nucleation and slightly facilitate microtubule growth. Consistently, the microtubule abundance that is reflected by the number of EB3 comets and quantified under electron microscopy, is reduced in the neurites of cultured neurons upon downregulation of α-TubK40me3. Furthermore, promoting tubulin nucleation using a low concentration of Taxol could rescue the defects of neuronal polarization caused by impaired α-TubK40me3. Interestingly, the duration of high-level α-TubK40me3 in the developing cerebral cortex is extended in MEC-17 knockout mice, and the impaired morphological transition and migration of α-TubK40me3-deficient neurons could be largely restored by overexpressing NES-SETD2 and α-tubulinKK40F, implying a compensatory effect by α-TubK40me3. All together, these results suggest that α-TubK40me3 plays an essential role in the polarization and migration of cortical neurons by promoting tubulin nucleation and controlling microtubule formation.

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To investigate the potential function of α-TubK40me3 in the development of CNS, we profiled the level of α-TubK40me3 at different developmental stages of mouse cerebral cortex using a specific homemade antibody against α-TubK40me3 and we show that α-TubK40me3 is enriched in NPCs and neurons from embryonic day 14 (E14) to E16 in cerebral cortex. In utero knockdown of SETD2 at E14 leads to impaired morphological transition and thereby the migration of cortical neurons, which could be restored by overexpressing NES-SETD2(1469-1724), a cytoplasm-localized SETD2 truncation retaining enzyme activity or α-tubulinKK40F, a tri-methylation mimicking mutant of α-tubulin. Importantly, α-TubK40me3 is preferably distributed on polymeric microtubules in cells, and in vitro microtubule reconstitution assays shows that α-TubK40me3 could potentiate tubulin nucleation and slightly facilitate microtubule growth. Consistently, the microtubule abundance that is reflected by the number of EB3 comets and quantified under electron microscopy, is reduced in the neurites of cultured neurons upon downregulation of α-TubK40me3. Furthermore, promoting tubulin nucleation using a low concentration of Taxol could rescue the defects of neuronal polarization caused by impaired α-TubK40me3. Interestingly, the duration of high-level α-TubK40me3 in the developing cerebral cortex is extended in MEC-17 knockout mice, and the impaired morphological transition and migration of α-TubK40me3-deficient neurons could be largely restored by overexpressing NES-SETD2 and α-tubulinKK40F, implying a compensatory effect by α-TubK40me3. All together, these results suggest that α-TubK40me3 plays an essential role in the polarization and migration of cortical neurons by promoting tubulin nucleation and controlling microtubule formation.
where NPCs were localized. As expected, the intensity of α-TubK40me3 in P0 cortex was relatively low (Fig. 1c). Taken together, these data reveal that α-TubK40me3 exists in NPCs and neurons, and is spatiotemporally regulated during neuronal and cortical development.

Knockdown of SETD2 at E14 results in defects of neuronal morphology transition and thereby radial migration. Since SETD2 served as the methyltransferase of α-tubulin, we profiled the expression pattern of SETD2 in the cerebral cortex. Quantitative real-time PCR (qPCR) detected that the mRNA level of SETD2 was significantly increased in E14 cortex. This suggests that SETD2 plays a critical role in regulating the expression of α-TubK40me3 during neuronal development.
SETD2 was continuously increased from E12 and reached a peak at E16, which was corresponding to the high level of α-TubK40me3 at E14 and E16. The SETD2 mRNA remained at a high level until P3 and afterwards was gradually decreased (Fig. 2a). Immunostaining showed that SETD2 was expressed both in the VZ/SVZ, IZ and CP of somatosensory cortex at E14 and E16 (Fig. 2b), consistent with the distribution pattern of α-TubK40me3 at the same stages (Fig. 1c). In detail, SETD2 puncta were not only localized in the nucleus but also in the cytoplasm of cells in VZ/SVZ and CP (Fig. 2c), supporting its role in catalyzing α-TubK40me3 and fulfilling the non-chromatin function in these cells.

E14-E16 is a critical period for morphological transition and radial migration of cortical neurons19. Then, we addressed whether the high level of α-TubK40me3 in this period was involved in the regulation of neuronal morphology and migration. The levels of SETD2 and α-TubK40me3 were dramatically decreased in Neuro-2a cells expressing short-hairpin-interfering (sh) RNAs for mouse SETD2 (mSETD2) (Extended Data Fig. 2a, b), suggesting that acute knockdown of SETD2 by shRNA is a practical strategy to study α-TubK40me3-related functions.

Using in utero electroporation (IUE), mixed plasmids containing shRNA and GFP reporter were co-transfected to radial glia progenitors in the cerebral cortex of E14 mice. Immunostaining showed that the levels of SETD2 and α-TubK40me3 were significantly decreased in GFP-positive (GFP⁺) SETD2-deficient neurons of somatosensory cortex at E16 (Extended Data Fig. 2c–f). The level of H3K36me3 was also reduced in GFP⁺ SETD2-deficient neurons though the extent of reduction was smaller than α-TubK40me3 (Extended Data Fig. 2e, f). The distribution of GFP⁺ cells in somatosensory cortex, which was divided into CP, IZ, and VZ/SVZ, was analyzed at E16 (Fig. 2d). Approximate 27% of GFP⁺ control cells expressing control shRNA (shNC) migrated into CP, but less than 10% of GFP⁺ SETD2-deficient cells expressing mSETD2 shRNA1 or shRNA2 were able to migrate into CP and most of them were arrested in IZ (Fig. 2d, e). Most SETD2-deficient cells in IZ exhibited highly branched leading processes and multipolar morphology, whereas most control cells in IZ were bipolar with one unbranched leading process (Fig. 2f, g). The percentage of GFP⁺ cells in VZ/SVZ showed no difference between control and SETD2 knockdown (Fig. 2e), neither the percentage of cells labeled by K67, a marker for the proliferative cells, PH3, a marker for the mitotic cells, or cleaved caspase 3, a marker for the apoptotic cells (Extended Data Fig. 3a–d), indicating that proliferation and apoptosis of NPCs were not affected by acute SETD2 knockdown at E14. Furthermore, we did immunostaining using anti-Nestin antibody to investigate the integrity of radial glial progenitors. The result showed that basal processes of radial glial progenitors projected properly to serve as the guide for migrating neurons in SETD2 knockdown brains (Extended Data Fig. 3e), ruling out the possibility that the impaired neuronal migration is due to the disruption of guiding tracks.

To further exclude the influence of SETD2 knockdown on NPCs, we performed IUE with doublecortin promoter-driven Cre (DCX-Cre) plasmid in Setd2fl/flox/fl mice (Extended Data Fig. 4a, b) to achieve neuron-specific deletion of SETD2. Immunostaining showed that the level of α-TubK40me3 was significantly decreased in Tuj1⁺-positive (Tuj1⁺) neurons but not in Tuj1⁻ negative (Tuj1⁻) NPCs (Extended Data Fig. 4c, d). Meanwhile, the same defects of neuronal migration and morphology transition were observed after DCX-Cre transfection (Extended Data Fig. 4e–h), supporting that the effects of SETD2 in these processes are neuron-intrinsic. Taken together, these data suggest that SETD2 controls neuronal morphology transition and thereby neuronal migration during development of cerebral cortex.

Cytoplasmic-localized SETD2 truncation retaining enzyme activity and tri-methylation-mimicking α-tubulinK40F rescues neuronal defects induced by SETD2 knockdown at E14. Given that acute knockdown of SETD2 by shRNA at E14 reduced the level of α-TubK40me3 and H3K36me3, we designed experiments to exclusively increase the level of α-TubK40me3 to explore whether the impaired neuronal morphology and migration could be rescued. SETD2 is a multi-domain protein, in which the SET and its associated domains are responsible for binding α-tubulin and catalyzing α-TubK40me3 (Extended Data Fig. 5a–c), and the C-terminal domains containing the SR1 domain is required for histone methylation in vivo20. Thus, we constructed a truncated SETD2 (1469-1724) to selectively promote the enrichment of α-TubK40me3 but not H3K36me3. The level of α-TubK40me3 was increased in HEK293 cells transfected with SETD2(1469-1724) (Fig. 3a). However, overexpressed SETD2(1469-1724) was preferably distributed in the nucleus than the cytoplasm of HEK293 cells (Fig. 3b). To further exclude its possible effect on H3K36me3 in the nucleus, tandem nuclear export signals (NES) were added to the N-terminus of SETD2(1469-1724) to sequester the truncation in the cytoplasm (Fig. 3b), which significantly promoted the level of α-TubK40me3 without affecting H3K36me3 (Fig. 3a).

Additionally, previous study reported that a SET domain mutant (R1625C) of SETD2 failed to catalyze both α-TubK40me3 and H3K36me3, and the SR1 domain mutant (R2510H) was able to catalyze H3K36me3 but not α-TubK40me3 in cells8. The binding capability and catalytic activity of these mutants was tested in the in vitro methylation assay. Unexpectedly, R2510H mutation did not disrupt the interaction between SETD2 and α-tubulin (Extended Data Fig. 5b), and both R1625C and R2510H efficiently catalyzed α-TubK40me3 in vitro (Extended Data Fig. 5d). Thus, we chose the cytoplasmic enzyme-activity-retaining SETD2 truncation to perform the rescue experiments.

Using IUE, SETD2 shRNA and NES-SETD2 (1469-1724) were co-delivered with GFP reporter into radial glia progenitors at E14. Of note, the targeting sequence of mSETD2 shRNA2 was not comprised in SETD2(1469-1724). Immunostaining showed that Flag-NES-SETD2(1469-1724) was localized in the cytoplasm of GFP⁺ cells in brain slice at E16 and restored the level of α-TubK40me3 but not H3K36me3 in GFP⁺ SETD2-deficient neurons (Extended Data Figs. 2e, f and 5e). At the same time, NES-SETD2(1469-1724) could rescue the defects of neuronal morphology transition and migration in somatosensory cortex (Fig. 3c–f), suggesting that the effects of SETD2 on cortical development during E14-E16 was dependent on its cytoplasmic methyltransferase-activity. To further confirm the contribution of α-TubK40me3 in the development of cerebral cortex, we expressed α-tubulinK40F, a tri-methylation-mimicking mutant by increasing the hydrophobicity and local bulkiness of distal R group of lysine21–23, and α-tubulinK40A as a negative control in SETD2-deficient neurons (Fig. 3g). Both mutants were able to be assembled into microtubule network properly in HEK293 cells (Extended Data Fig. 5f). Immunostaining showed that the defects in neuronal morphology transition and migration caused by SETD2 knockdown were efficiently rescued by α-tubulinK40F, but not α-tubulinK40A at E16 (Fig. 3g–j). Taken together, these data indicate that SETD2-mediated α-TubK40me3 is a critical PTM for neuronal migration in cerebral cortex during E14–E16.

α-TubK40me3 promotes tubulin nucleation and microtubule polymerization. The underlying mechanism of α-TubK40me3 affecting neuronal development was explored. In cells, tubulins either exist in soluble form or polymerize into microtubules. We separated soluble and polymerized tubulins in HEK293 cells and detected the level of α-TubK40me3 in these two pools.
Fig. 2 SETD2 is highly expressed during cortical development and required for neuronal migration. a qPCR showed that the relative level of SETD2 mRNA was high in embryonic and early post-natal mouse cerebral cortex. All data were normalized to the P60 cerebral cortex and represented as the mean ± s.e.m. (n = 3 biological replicates). b Immunostaining showed a distribution of SETD2 in CP, IZ and VZ/SVZ of mouse somatosensory cortex at E14 and E16. Brain slices were stained for SETD2 (red), Tuj1 (green) and DAPI (blue) (n = 3 biological replicates). Scale bar: 25 μm. c SETD2 was localized in the NPCs of VZ/SVZ and the neurons of CP at E14 cerebral cortex. Dashed line indicated the lineament of cell and arrowheads indicated the SETD2 puncta in the cytoplasm. Brain slices were stained for SETD2 (red), Tuj1 (green) and DAPI (blue) (n = 3 biological replicates). Scale bar: 2.5 μm. d Representative images of coronal brain sections in somatosensory cortex at E16 showed the distribution of GFP+ cells (green) electroporated with GFP reporter as well as control shRNA (shNC), mSETD2 shRNA1 or mSETD2 shRNA2 at E14. Sections were stained for DAPI (blue). Scale bar: 25 μm. e Quantitative analysis of d showed that the percentage of GFP+ cells in IZ was significantly increased after SETD2 knockdown. All data were shown as the mean ± s.e.m. (n = 17, 10, 11, respectively). Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. ***P < 0.001 versus shNC. f Representative images of shNC and shRNA2-expressing neurons (green) at E16 in the IZ. Brain slices were stained for DAPI (blue). Scale bar: 10 μm. g Quantitative analysis of f showed that the percentage of GFP+ neurons at the multipolar stage in IZ was significantly increased after SETD2 knockdown. All data were shown as the mean ± s.e.m. (n = 12, 16, respectively). Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. ***P < 0.001 versus shNC. Source data are provided as a Source Data file.
Immunoprecipitation and immunoblotting showed that α-TubK40me3 was preferably distributed on polymerized microtubules (Fig. 4a), consistent with the result that α-TubK40me3 is mainly found in spindle and midbody (Extended Data Fig. 1e). Importantly, compared with α-TubK40ac, α-TubK40me3 showed an even stronger preference for polymerized microtubules (Fig. 4a). To further clarify whether α-TubK40me3 actively promoted tubulin assembly, we first prepared highly methylated tubulin by in vitro methylation reaction using purified adult mouse brain tubulin as substrates. Original tubulin only contained a low level of α-TubK40me3 and after reaction, the methylation level of tubulin was significantly increased (Extended Data Fig. 6a–c). Separation of soluble tubulin and polymerized microtubule after in vitro microtubule assembly revealed that methylated tubulin was able to generate more microtubule (Fig. 4b). Furthermore, SETD2 knockout significantly decreased the level of polymerized microtubules and resulted in the reduction of α-TubK40ac-labeled stable microtubules in HEK293 cells (Fig. 4c, d and Extended Data Fig. 6d). Thus, α-TubK40me3 favors microtubule formation.

Previous study proposed that microtubule formation consists of two main steps: tubulin nucleation and microtubule elongation. Tubulin nucleation is the early step to produce the seeds, and microtubule elongation is the late step for tubulin dimers to add on the existing seeds. We conducted in vitro tubulin nucleation to explore if α-TubK40me3 affected tubulin nucleation. Time-lapse total internal reflection fluorescence (TIRF) imaging showed that the number of short microtubules was gradually increased in a time-dependent manner. Notably, the number of short microtubules was largely increased in the
Fig. 3 Cytoplasmic enzyme-activity-retaining SETD2 truncation and tri-methylation-mimicking mutant of α-tubulin rescue neuronal defects induced by SETD2 knockdown. a. Immunoprecipitation by α-TubK40me3 antibody and following immunoblotting with α-tubulin showed that the level of α-TubK40me3 was increased in HEK293 cells transfected with Flag-SETD2(1469-1724) and Flag-NES-SETD2(1469-1724), while the level of H3K36me3 was not significantly changed (n = 3 biological replicates). b. Immunostaining of Flag (green) and α-tubulin (red) showed the subcellular localization of Flag-SETD2(1469-1724) and Flag-NES-SETD2(1469-1724) in HEK293 cells (n = 3 biological replicates). Cells were stained for DAPI (blue). Scale bar: 5 μm. c. Representative images of coronal brain sections in somatosensory cortex at E16 showed the distribution of GFP+ cells (green) electroverted with GFP reporter as well as shNC, mSETD2 shRNA2 or mSETD2 shRNA2 together with Flag-NES-SETD2(1469-1724) at E14. Sections were stained for DAPI (blue). Scale bar: 25 μm. d. Quantitative analysis of e showed that the migration defects induced by SETD2 knockdown was rescued by expressing Flag-NES-SETD2 (1469-1724) (n = 6, 12, 13, respectively). e. Representative images of GFP+ neurons (green) in IZ. Brain slices were stained for DAPI (blue). Scale bar: 10 μm. f. Quantitative analysis of e showed that the percentage of neurons at the multipolar stage in IZ was rescued by expressing Flag-NES-SETD2 (1469-1724) (n = 6, 12, 13, respectively). g. Representative images of coronal brain sections in somatosensory cortex at E16 showed the distribution of GFP+ cells (green) electroverted with GFP reporter as well as shNC, mSETD2 shRNA2 or mSETD2 shRNA2 together with Flag-α-TubK40me3/K40A at E14. Sections were stained for DAPI (blue). Scale bar: 25 μm. h. Quantitative analysis of g showed that the migration defects induced by SETD2 knockdown was rescued by expressing α-tubulinK40F but not α-tubulinK40A (n = 28, 22, 10, 12, respectively). i. Representative images of GFP+ neurons (green) in IZ. Brain slices were stained for DAPI (blue). Scale bar: 10 μm. j. Quantitative analysis of i showed that the percentage of neurons at the multipolar stage in IZ was rescued by expressing α-tubulinK40F but not α-tubulinK40A (n = 28, 22, 10, 12, respectively). All data were shown as the mean ± s.e.m. and analyzed using two-way ANOVA with Bonferroni’s post-hoc test. *P < 0.05, ***P < 0.001 versus shRNA; ***P < 0.05, ###P < 0.001 versus shRNA2. Source data are provided as a Source Data file.

α-TubK40me3 potently promotes tubulin nucleation and slightly reduces microtubule dynamics, thereby facilitates microtubule formation.

α-TubK40me3 promotes microtubule formation in neurons. To explore whether α-TubK40me3 affected microtubule formation in neurons, we used cultured system and first detected the expression of SETD2 and α-TubK40me3 in neurons. The level of α-TubK40me3 in cultured cortical neurons was continuously increased during development (Extended Data Fig. 7a). Immunostaining showed that SETD2 puncta were localized in the nucleus, soma and neurites of neurons, and acute knockdown by shRNA significantly reduced SETD2 in neurons (Extended Data Fig. 7b). Since cultured hippocampal neurons displayed distinct polarized morphology, they were isolated from E14 mice and transfected with mSETD2 shRNA and GFP reporter by electroporation to examine whether downregulation of α-TubK40me3 impaired the neuronal morphogenesis. The majority of control neurons was polarized and developed one distinct and SMI-312+ axon at day in vitro (DIV) 3 (Fig. 5a). In contrast, the polarization percentage was significantly decreased in SETD2 knockdown neurons, most of which were multipolar with neurites but had no SMI-312+ axon (Fig. 5a, b). Additionally, the number of neurite tips, include all neurites and their protrusions, was largely increased in SETD2 knockdown neurons (Fig. 5b). These defects of neuronal polarization and arborization could be efficiently rescued by NES-SETD2(1469-1724) and α-tubulinK40F (Fig. 5a, b). Thus, α-TubK40me3 also plays critical roles in the development of cultured neurons, similar to that in cerebral cortex.

Then, we examined the regulation of neuronal microtubule formation by α-TubK40me3. Firstly, we compared the distribution of α-TubK40me3 in soluble tubulin and polymerized microtubule in cultured neurons. The result also showed that α-TubK40me3 was preferably distributed on polymerized microtubules in neurons (Fig. 5c). The subcellular localization of α-TubK40me3 was detected in neurons using proximity ligation assay (PLA). The PLA signals with only α-TubK40me3 antibody and α-tubulin/β-tubulin antibodies were regarded as the negative and positive control, respectively (Extended Data Fig. 7c). The PLA signals representing α-TubK40me3 with α-tubulin antibodies were present in the soma, dendrites and axon shaft of cultured hippocampal neurons, but not in the growth cone (Fig. 5d), where microtubules were relatively dynamic, in line with the biochemical data that α-TubK40me3 preferentially distributed on polymerized and stable microtubules (Figs. 4a and 5c). As expected, the PLA signals in cultured neurons were decreased after SETD2 knockdown both in cell body and neurites (Extended Data Fig. 7d, e), supporting the efficiency of SETD2 knockdown on α-TubK40me3 and the specificity of PLA signals representing α-TubK40me3. We further expressed EB3-ttdTomato to label microtubule plus-ends and perform time-lapse imaging in control and SETD2-deficient hippocampal neurons at DIV 3. Quantitative data showed that the number of EB3+ neurons was significantly decreased both in the soma and neurites after SETD2 knockdown, and this reduction was rescued by the expression of NES-SETD2(1469-1724) and α-tubulinK40F (Fig. 5e, f). However, the velocity, growth lifetime, catastrophe frequency and percentage of anterograde movement in EB3+ neuron was not changed in the neurites of SETD2-deficient neurons (Extended Data Fig. 7f), suggesting that α-TubK40me3 does not affect microtubule plus-end dynamics and orientation. Further detection with electron microscopy showed that the number of microtubules at different segments of neurites was significantly decreased in cultured hippocampal neurons at DIV 2 after SETD2 knockdown by lentivirus (Fig. 5g, h and Extended Data Fig. 7g). Since Taxol promotes tubulin nucleation and stabilizes microtubules25,26, we wonder whether it could regulate the neuronal polarization and arborization induced by SETD2 deficiency. As expected, the defects of neuronal polarization and arborization caused by SETD2 knockdown were efficiently rescued by 0.5 nM Taxol (Fig. 5i, j). Taken together, these data support that α-TubK40me3 promotes microtubule formation and maintains microtubule number in neurons.

α-TubK40me3 is able to rescue the defects of radial migration and morphological transition in α-TubK40ac-deficient cortical neurons. Since α-TubK40me3 and α-TubK40ac occurs at the same lysine residue of α-tubulin, a crosstalk possibly exists between them. Despite the fact that SETD2 was highly expressed...
in cerebral cortex throughout embryonic stages (Fig. 2a), the high level of α-TubK40me3 only occurred during E14-E16 and rapidly decreased at E18 (Fig.1a). qPCR showed that the mRNA level of MEC-17 and α-tubulin deacetylase HDAC6 was gradually increased from E14 to E16 and reached the peak at E18 and P0, respectively (Extended Data Fig. 8a), indicating a multifactorial regulation of α-TubK40ac at late embryonic stages in cerebral cortex. These results raise a possibility that the increased α-TubK40ac after E16 occupies lots of K40 sites and results in the reduction of α-TubK40me3. To test this hypothesis, we examined the level of α-TubK40me3 in the cerebral cortex of MEC-17 knockout mice at E16 and P0. In wildtype (WT) mice, the level of α-TubK40me3 was reduced at P0, which was accompanied with increased α-TubK40ac, while α-TubK40me3 was no longer reduced at P0 and even maintained at a high level until adult in MEC-17 knockout mice (Fig.6a and Extended Data Fig. 8b–d). Moreover, upregulating α-TubK40ac by MEC-17 overexpression in HEK293 cells significantly reduced the level of α-TubK40me3 (Fig. 6b). Thus, increased α-TubK40ac causes decreased α-TubK40me3 during cortical development, conversely loss of α-TubK40ac results in enhanced α-TubK40me3.

According to our previous study, reduced α-TubK40ac by MEC-17 knockdown leads to impaired migration of cortical neurons15. However, the neuronal defects of radial migration observed in acute knockdown experiments seemed mild-to-null in MEC-17 knockout mice (Extended Data Fig. 8e, f).
Considering the role of α-TubK40me3 in microtubule formation and neuronal development, an increased level of α-TubK40me3 may compensate for the defects of α-TubK40ac loss in MEC-17 knockout mice. We adopted MEC-17 shRNA to downregulate the level of α-TubK40ac (Extended Data Fig. 8g, h). After IUE, the defects in radial migration and morphological transition of cortical neurons by MEC-17 knockdown at E14 were largely rescued by increased level of α-TubK40me3, which was generated by the expression of either NES-SETD2(1469-1724) or α-tubulinK40F (Fig. 6c–f), indicating a compensatory effect of α-TubK40me3 for α-TubK40ac. Conversely, the neuronal defects by SETD2 knockdown at E14 were not rescued by expressing α-tubulinK40Q (Fig. 6g–j), a widely used variant to mimic α-TubK40ac, similar to the effect of delivering α-tubulinK40A (Fig. 3g–j). In addition, α-tubulinK40Q was able to assemble into microtubule network properly in HEK293 cells (Extended Data Fig. 5f). Taken together, these data suggest that α-TubK40me3 is able to rescue the defects of radial migration and morphological transition of cortical neurons caused by α-TubK40ac deficiency.
Discussion

The spatiotemporal regulation of tubulin PTMs is crucial for modulating microtubule properties and neural development. Here, we found that SETD2-mediated α-TubK40me3 promotes microtubule formation and is required for proper neuronal development. In developing cortical neurons, α-TubK40me3 promotes microtubule formation and facilitates the morphological transition and radial migration during E14-16. Downregulation of α-TubK40me3 results in a reduction of microtubule number and causes defects of neuronal polarization (Fig. 7). These findings greatly expand our knowledge about the functions and mechanisms of α-tubulin methylation in post-mitotic cells.

α-TubK40me3 promotes microtubule formation. The “tubulin code” hypothesis, as an analogy to the concept of “histone code,” has been proposed to describe how various PTMs of tubulins regulate microtubule-associated cellular processes. SETD2-mediated α-TubK40me3 is a recently identified tubulin code. Unlike most tubulin C-terminal PTMs, tri-methylation and acetylation on lysine 40 occur in the lumen of microtubules. Since lysine 40 is localized in one of the two loops that form the lateral contact of α/β-tubulin heterodimers, recent studies propose a model that α-TubK40ac could weaken the lateral interaction between protofilaments and protect long-lived microtubules from mechanical breakage. Notably, the existence of α-TubK40me3 on the same residue provides an additional possibility to regulate microtubule properties. Unlike acetylation that neutralizes the positive charge of lysine, methylation does not alter the charge on lysine and may operate through non-electrostatic mechanisms such as hydrophobic interaction. The lateral interface between neighboring protofilaments is described as the “lock and key” model, which includes the M-loop (S7-H9 loop) on one side, sandwiched by the H1-S2 loop and H2-S3 loop on the other side. α-TubK40me3 on the H1-S2 loop may change its conformation and bulkiness or increase the hydrophobic interaction to further lock the M-loop, resulting in strengthened lateral interaction of α/β-tubulin heterodimers and enhanced tubulin nucleation.

In cells, we found that α-TubK40me3 was enriched in specialized regions consisting of copious microtubules such as spindle, midbody and shaft of neuronal axons, where extensive microtubule formation takes place. Most importantly, the time-lapse imaging of EB3 comets and electron microscopy showed that loss of α-TubK40me3 resulted in a decreased number of microtubules without affecting the growth velocity and direction of growing microtubules in neurites, suggesting an essential role of α-TubK40me3 in microtubule formation but not elongation in neurons. These findings indicate the requirement of α-TubK40me3 for microtubule formation in post-mitotic cells.

α-TubK40me3 is required for neuronal polarization and migration. In the present study, the distinct expression pattern of SETD2, which is defined as a dual-functional methyltransferase for histone and α-tubulin, was observed in the VZ/SVZ, IZ, and CP regions of developing cerebral cortex. Previous study reveals that homoygous disruption of Setd2 leads to embryonic lethality at E10.5–E11.5, which is accompanied by defects of neural development including unclosed neural tube and forebrain hypoplasia. Mutations in SETD2 have been linked to brain disorders such as intellectual disabilities and autism spectrum disorders. SETD2-mediated H3K36me3 is a well-characterized epigenetic marker associated with active gene transcription and plays important roles in neural development. Acute knockdown of SETD2 by shRNAs led to severe defects in neuronal development, along with significantly disrupted α-TubK40me3 and less affected H3K36me3. The smaller impact on H3K36me3 level after acute SETD2 knockdown might be due to the relatively higher abundance of SETD2 in nucleus or relatively low turnover rate of H3K36me3-labeled histones. As expected, the role of SETD2-mediated α-TubK40me3 in neuronal development is also supported by the evidence that the defects in neuronal polarization and migration by SETD2 knockdown could be rescued by a cytoplasmic-localized truncation of
SETD2 retaining enzyme activity as well as the α-TubK40me3-mimicking mutant. Both immunoblotting and immunostaining showed that the level of α-TubK40me3 was dramatically increased during E14-E16 in the developing cerebral cortex, which is a critical period for neuronal migration. Local polymerization and stabilization of microtubules is required for the leading process formation and morphological transition from multipolar stage to bipolar stage to initiate the radial migration. Previous study also reveals that the nucleation of non-centrosomal microtubules in the axon is required for neuronal polarization. In the present study, the reduced microtubule number in the neurites after SETD2 knockdown imply an essential role of α-TubK40me3 in neuronal microtubule formation. Further evidence that application of 0.5 nM Taxol to promote tubulin nucleation and stabilize microtubules completely rescues the defect of neuronal polarization caused by SETD2 knockdown finally supports an importance of α-TubK40me3 in facilitating microtubule formation during neuronal development.

A crosstalk exists between α-TubK40me3 and α-TubK40ac. The temporal regulation of α-TubK40me3 in cerebral cortex was not correlated to the expression profile of SETD2, indicating that
Fig. 6 α-TubK40me3 is increased after MEC-17 knockout and able to rescue the defects of α-TubK40ac-deficient neurons. a Immunoprecipitation by α-TubK40me3 antibody and following immunoblotting with α-tubulin showed that the level of α-TubK40me3 was not decreased at P0 in the cerebral cortex of MEC-17 knockout mice (n = 3 biological replicates). b Immunoprecipitation and quantitative analysis showed that the level of α-TubK40me3 was significantly reduced after overexpression of MEC-17 in HEK293 cells. All data were shown as the mean ± s.e.m. (n = 4 biological replicates). Data were analyzed using paired two-tailed Student’s t test. ***P < 0.001 versus GFP. c Representative images of coronal brain sections in somatosensory cortex at E16 showed the distribution of GFP reporter as well as shNC, mSETD2 shRNA2 or mSETD2 shRNA2 together with Flag-α-TubK40Q at E14. Sections were stained for DAPI (blue). Scale bar: 30 μm. d Quantitative analysis of c showed that the migration defects induced by MEC-17 knockdown was highly rescued by expressing either NES-SETD2(1469-1724) or α-tubulinK40F at E14. All data were shown as the mean ± s.e.m. (n = 18, 22, 18, 14, respectively). Data were analyzed using two-way ANOVA with Bonferroni post-hoc test. ***P < 0.001 versus shNC; ###P < 0.001 versus MEC-17 shRNA. e Representative images of GFP+ neurons (green) in white matter (WM). Brain slices were stained for DAPI (blue). Scale bar: 20 μm. f Quantitative analysis of e showed that the percentage of GFP+ neurons at the multipolar and branched uni/bipolar stage was largely rescued by expressing either NES-SETD2(1469-1724) or α-tubulinK40F. All data were shown as the mean ± s.e.m. (n = 12, 14, 8, respectively). Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. ***P < 0.001 versus shNC; ###P < 0.001 versus MEC-17 shRNA. g Representative images of coronal brain sections in somatosensory cortex at E16 showed the distribution of GFP+ cells (green) electroporated with GFP reporter as well as shNC, MEC-17 shRNA or MEC-17 shRNA together with NES-SETD2(1469-1724) or α-tubulinK40Q at E14. Sections were stained for DAPI (blue). Scale bar: 25 μm. h Quantitative analysis of g showed that the migration defects induced by SETD2 knockdown was not rescued by expressing α-tubulinK40Q. All data were shown as the mean ± s.e.m. (n = 16, 12, 18, respectively). Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. ***P < 0.001 versus shNC. i Representative images of GFP+ neurons (green) in IZ. Brain slices were stained for DAPI (blue). Scale bar: 10 μm. j Quantitative analysis of i showed that the percentage of GFP+ neurons at the multipolar stage was not rescued by expressing α-tubulinK40Q. All data were shown as the mean ± s.e.m. (n = 14, 12, 18, respectively). Data were analyzed using two-way ANOVA with Bonferroni’s post-hoc test. ***P < 0.001 versus shNC. Source data are provided as a Source Data file.

Fig. 7 A proposed model for the role of α-TubK40me3 in regulating microtubule formation as well as neuronal polarization and migration. In cerebral cortex, developmentally upregulated α-TubK40me3 facilitates tubulin nucleation and promotes the neuronal exit from multipolar stage, thereby initiating the radial migration. In the absence of α-TubK40me3, tubulin nucleation is not easy to form and the number of microtubules is reduced in neurons, leading to defects in neuronal polarization and migration.

additional regulatory mechanisms might exist. Despite the highest mRNA level of α-tubulin deacetylase HDAC6 at P0, the level of α-TubK40ac was significantly increased from E16 to P0, implying that the post-transcriptional mechanisms, probably PTMs and subcellular localization of relevant enzymes, are also involved in regulating α-TubK40ac level. Our study suggests that increased level of α-TubK40ac after E16 in cerebral cortex results in the downregulation of α-TubK40me3 possibly due to the competition for the K40 site. The preponderance of α-TubK40ac in this competition may be further evidenced by relatively higher expression level or catalytic efficiency of MEC-17 than SETD2 in cytoplasm. Furthermore, the spatiotemporal distribution and related mechanisms of α-TubK40me3 and α-TubK40ac in various microtubule-associated processes remain to be explored.

Due to the catalytic preference of MEC-17 for microtubules over free tubulins, α-TubK40ac mostly occurs on post-assembly microtubules and is widely thought as a marker for stable microtubules[24,28] and dampens microtubule plus-end dynamics[36,39]. Given that α-TubK40ac weakens the lateral interaction between protofilaments[24,28] and dampens microtubule plus-end dynamics[36,39], these results imply that α-TubK40ac functions through protecting long-lived microtubules from mechanical breakage and regulating plus-end dynamics. In contrast, polymerization of microtubules is not
required for α-TubK40me3 catalysis by SETD2, because SETD2 methylates both soluble tubulins and polymerized microtubules. In the present study, we found that methylated tubulins are more prone to nucleate and promote microtubule formation, leading to the fact that α-TubK40me3 are mostly detected on polymerized microtubules. These findings support that α-TubK40me3 and α-TubK40ac work collaboratively to ensure microtubule functions.

Our previous study suggests that α-TubK40ac restricts axon outgrowth and overgrowth by dampening microtubule plus-end dynamics in neurons. The axon outgrowth was observed in the cerebral cortex and cultured neurons of MEC-17 knockout mice, despite under both conditions the level of α-TubK40me3 was maintained at a high level. Interestingly, loss of increased α-TubK40me3 does not compensate for the overbranching defect due to loss of α-TubK40ac. More importantly, the axon outgrowth was efficiently rescued by overexpression of α-TubK40ac-mimicking α-tubulinK40Q. Mechanistically, loss of α-TubK40ac led to hyperdynamics in microtubule plus-ends, including increased growth rate and catastrophe frequency, and suppression of microtubule plus-end hyperdynamics could rescue axon outgrowth in MEC-17-deficient neurons.

However, α-TubK40me3 mainly promotes microtubule formation without altering microtubule plus-end dynamics in neurons. Thus, the enhanced axon outgrowth and branching in MEC-17 knockout neurons is derived from loss of α-TubK40ac itself. Therefore, α-TubK40me3 is able to rescue the defects of morphological transition and migration but not axon outgrowth in MEC-17-deficient neurons.

Methods

Plasmids. The expression constructs for human SETD2 (NM_014159) 1469–1724 and 1392–2564 amino acids were subcloned from SETD2-GFP plasmid (Addgene), and the PCR-amplified fragment linked with Flag-encoding sequence was inserted into pcAG-IRS-eGFP vector for overexpression or pGEX-AT-1 vector for protein purification. Tandem NES of mouse STAT1 (SLAAEFRHLQLK) was added to the N-terminus of SETD2 truncation. The expression construct for rat TUBA1A (NM_022298.1), of which amino acid sequence is totally conserved between rat and mouse, was modified from previously described plasmids by inserting Flag tag into the N-terminus of α-tubulin. The R1625C and R2510H mutations of α-TubK40me3 were generated by PCR-mediated mutagenesis kit (Toyobo) and verified by sequencing. The end binding protein 3 (EB3) (NM_001007656.1) was cloned from the cDNA of rat dorsal root ganglion into the pcDH-CMV vector with tIdTomato tag on the C-terminus. All shRNA constructs were inserted into the pGPU6-GFP-Neo vector. The shRNA sequences for SETD2 or MEC-17 were provided in Supplementary Table 1.

Animals and genotyping. All animal experiments were approved by Institutional Animal Care and Use Committee, CAS Center for Excellence in Molecular Cell, Chinese Academy of Sciences. The C57BL6 mice of either sex at different developmental stages were provided by the Shanghai Laboratory Animal Center of Chinese Academy of Sciences. For genotyping of SETD2fl/fl and Mec-17 knockout mice, the genomic DNA was extracted from mouse tail by Proteinase K and amplified by 2 × Taq Plus Master Mix (Vazyme) with primer pairs listed in Table S4. The PCR products were analyzed by agarose gel electrophoresis. Genotyping primers were provided in Supplementary Table 2.

Generation of the antibody against α-TubK40me3. The antibody against α-TubK40me3 was custom-designed and generated (GL Biochem, Shanghai, China). Briefly, the tri-methylated peptide Ac-GQPMPSDKM(KMe3)TIGGGDC-amide conjugated to keyhole limpet hemocyanin was used as the immunogen to generate α-TubK40me3 antibody in rabbits. Purification of α-TubK40me3-specific antibody was performed using serial columns with un-methylated and tri-methylated peptides.

Cell culture and transfection. HEK293, Neuro-2a and ND7/23 cells (Shanghai cellbank, Chinese Academy of Sciences) were cultured in Dulbecco’s Modified Eagle Medium (DMEM; Gibco) supplemented with 10% fetal bovine serum (Biochrom) and antibiotics (Gibco). The cells were transiently transfected with plasmids using Lipofectamine 2000 (Invitrogen) and then used for different assays after 48 h.

Hippocampal and cortical tissues were dissected from mouse brain at E14 in Hanks balanced salt solution (HBSS) and digested with 10 U/μl papain (Worktingham Bio Corp) and 0.1 mg/mL DNase I (Sigma) for 12 min at 37 °C. After digestion, the tissues were washed in HBSS and then dissociated by repeated passages through a 1 ml pipette. The indicated plasmids mixed in Nucleofector buffer (0.15 mL) were electroporated into hippocampal and cortical neurons by Amaxa Nucleofector II (Lonza) using program O-005. The neurons were plated on poly-D-lysine (Sigma) coated dishes with Minimum Eagle’s Medium (MEM; Gibco) containing 5% fetal bovine serum. After 3 h, the medium was replaced by Neurobasal medium (Gibco) containing 2% B27 supplement (Gibco) and 2 mL L-Glutamin (Gibco). The neurons were used for immunostaining or time-lapse imaging after 3 days. Fixed neurons which developed one distinct and SMI-312 γ-axon at DIV 3 were defined as the polarized neurons, and all neurites and their protrusions labeled by GFP were counted as neurite tips.

For lentivirus infection, the virus at multiplicity of infection (MOI) = 10 was applied to neurons 3 h after plating and removed after 12 h treatment. The percentage of GFP+ neurons was observed at DIV 2 and then the neurons were fixed for electron microscopy.

qPCR. Total mRNAs of mouse cerebral cortex at different developmental stages were extracted with TRIzol reagent (Invitrogen) and then used for reserve transcription to produce complementary DNAs (cDNAs) by SuperScript II reverse transcriptase (Invitrogen). The primers used to amplify the SETD2, MEC-17 or HDAC6 cDNA were listed at below. For the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, the primers were 5′-GCTTGGCCACCAAGGATCA-3′ and 5′-TACGTTGCTGGGTATGGC-3′. The relative mRNA level of individual gene was calculated by a standard curve and normalized to the mRNA level of GAPDH. All values at different stages were normalized to the P60 cerebral cortex.

Protein purification and enzymatic modification. GST-tagged human SETD2 and its truncation and mutant proteins were expressed in E. coli Rosetta 2 at 37 °C for 4 and then induced with 0.5 mM Isopropyl-β-D-Thiogalactoside (Sigma) at 16 °C for 20 h. Cells were collected by centrifugation at 6000 × g for 10 min at 4 °C. Cell pellets were resuspended with lysis buffer (50 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.5 mM NaCl, 1 mM PMSF) and then sonicated at 4 °C. Cell fragments were harvested by centrifugation at 10 000 × g for 10 min at 4 °C and the supernatant was used for experiments. The control glyceraldehyde-3-phosphate dehydrogenase protein was purificated by GST-tagged truncated or mutant SETD2 proteins for 4 h at 4 °C. Proteins were extracted with TRIzol reagent (Invitrogen) and then used for reserve transcription to produce complementary DNAs (cDNAs) by SuperScript II reverse transcriptase (Invitrogen). The primers used to amplify the SETD2, MEC-17 or HDAC6 cDNA were listed at below. For the control glyceraldehyde-3-phosphate dehydrogenase (GAPDH) cDNA, the primers were 5′-GCTTGGCCACCAAGGATCA-3′ and 5′-TACGTTGCTGGGTATGGC-3′. The relative mRNA level of individual gene was calculated by a standard curve and normalized to the mRNA level of GAPDH. All values at different stages were normalized to the P60 cerebral cortex.

Tubulin purification and enzymatic modification. Tubulin was purified from the brain of C57BL6 mice (8 weeks) by two cycles of polymerization/depolymerization as previously described. The 2 mg tubulin was methylated by adding 0.1 mg per mg tubulin of Acetylcholinesterase (Catalyzed by choline-HCl, pH 8.0, 2 μM BAPTA, 0.5 mM NaCl, 1 μM PMSF) and then sonicated at 4 °C. Cell fragments were harvested by centrifugation at 10 000 × g for 10 min at 4 °C and the supernatant was used for experiments. The control glyceraldehyde-3-phosphate dehydrogenase protein was purificated by GST-tagged truncated or mutant SETD2 proteins for 4 h at 4 °C. Proteins were extracted with liquid nitrogen and stored in −80 °C.

Immunoprecipitation and GST pull-down assay. The brain tissues and HEK293 cells were lysed in ice-cold RIPA buffer (50 mM Tris-Cl, pH 7.4, 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, 10% glycerol, 1 mM PMSF, 1 μg/mL aprotinin, 1 μg/mL leupeptin and 1 μg/mL pepstatin). For brain tissues at different developmental stages, the volume of lysis buffer was adjusted to ensure the same input of total α-tubulin. After centrifugation at 15 000 × g for 10 min, the supernatant was diluted 10 times and incubated with α-TubK40me3 antibody (1:200; homemade) or H3K36me3 antibody (1:200; Abcam) overnight at 4 °C and then precipitated by protein G agarose (Sigma). For denatured immunoprecipitation, brain tissues were first lysed and boiled in 1% SDS to fully linearize proteins and expose epitopes, and the lysates were diluted 10 times with ice-cold RIPA buffer. Then, immunoprecipitation was performed with α-TubK40me3 antibody overnight at 4 °C and then precipitated by protein G agarose. The immunoprecipitates and 1% total lysates were analyzed by immunoblotting.

For GST pull-down assay, purified tubulin from mouse brain was incubated with GST-tagged truncated or mutant SETD2 proteins for 4 h at 4 °C. The protein complex was precipitated by GST-beads and analyzed by immunoblotting.

Immunoblotting and dot blotting. The protein samples were separated by SDS-PAGE, transferred to the nitrocellulose membrane (Whatman), blocked by 5% skim milk for 60 min at room temperature, probed with specific antibodies and
then visualized with enhanced chemiluminescence (Tanon). For dot blotting, indicated peptides were dissolved in PBS and dropped on the nitrocellulose membrane. The primary antibodies include anti-α-Tubulin (1:10000; Sigma T9026), α-TubK40ac (1:5000; Sigma T7451), α-TubK40me3 (1:1000; homemade), H3K36me3 (1:10000; Abcam ab9050), SETD2 (1:500; Sigma HPA042451), Tuji (1:1000; Abcam ab107216), Flag (1:1000; Sigma F7425/F35613), pH3 (1:1000; Millipore St-100), β-actin (1:1000; Chemicon MAB3501), GAPDH (1:10000; Abcam ab8245) and GST (1:1000; Abcam ab6612). The blotting times were always within the linear range of detection and protein bands were quantified using ImageJ software (NIH).

**Immunostaining.** The HEK293 cells or cultured neurons were fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature, blocked by 1% bovine serum albumin (BSA) for 30 min at room temperature, and then incubated with primary antibodies diluted in the buffer containing 1% BSA and 0.3% Triton X-100 against α-tubulin (1:10000; Sigma T5168), α-TubK40me3 (1:10000; homemade), H3K36me3 (1:1000; Abcam ab9050), SETD2 (1:10000; Abcam ab107250), Tuji (1:1000; Abcam ab107216), Flag (1:1000; Sigma F7425/F35613), pH3 (1:1000; Millipore St-100), β-actin (1:1000; Chemicon MAB3501), GAPDH (1:10000; Abcam ab8245) and GST (1:1000; Abcam ab6612). The blotting exposure times were 14 min. NATURE COMMUNICATIONS | https://doi.org/10.1038/s41467-021-24376-2 | www.nature.com/naturecommunications

**Measurement of the soluble and polymerized tubulin fractions.** HEK293 cells and neurons were then incubated in BRB80 for 15 min at 27 °C to remove unpolymerized tubulin. Cold BRB80 was added to resuspend the microtubule pellet, followed by depolymerization at 4 °C for 20 min and a second round of polymerization with 1 mM GMPCPP at 37 °C for 30 min. Finally, microtubule seeds were obtained after centrifugation at 126,000 × g for 5 min at 27 °C, resuspended using 37 °C pre-warmed BRB80, frozen by liquid nitrogen and stored in –80 °C. For the assembly of sample chamber, cleaned and silanized glass coverslip was attached onto microscopic slides by double-sided tape to create a reacting space. Low or high level of methylated tubulin and HiLyte-488 labeled tubulin (at a 1:91 ratio) were mixed with reaction buffer containing 5 mM Dithiothreitol (DTT), 0.1% methylcellulose, 75 mM KCl, 0.25 mg/ml bovine serum albumin (BSA), 25 mM glucose, 100 µM calcein, 300 µM glucose oxidase and 1 mM GTP. For microtubule growth assay, microtubule seeds were flowed through the channel for 10 min at 37 °C before adding reaction buffer.

**Image collection.** Sealed chamber was put into Zeiss cell observer spinning disk system with a pre-warmed 37 °C temperature-controlled workstation. Images were acquired with a 100 × oil objective (NA = 1.40) and an Orca CCD camera (Hamamatsu). Tubulin nucleation and growing microtubules from seeds were imaged using the Zeiss cell observer spinning disk system (Zeiss) with Zen 2.1 software under 60 × oil lens and 60 frames were collected for 2 min at 2 s interval. The number of EB3 comets in the cell body was averaged from 3 independent frames. The neurite segments were selected from the immediate region of the longest neurite in neurons. The kymograph was generated using ImageJ software (NIH), and then the number and direction of EB3 comets in the neurite were analyzed. The mean velocity, growth lifetime, and catastrophe frequency of EB3 comets in particular kymograph was calculated using Image-Pro Plus 5.1 software.

**Proximity ligation assay.** The proximity ligation assay was performed according to the Duolink manufacturer’s instructions (Sigma). In brief, E14 hippocampal neurons grown on glass slides at DIV 3 were fixed with 4% PFA for 20 min at room temperature, blocked by 1% BSA for 30 min at room temperature and incubated with the indicated primary and secondary antibodies diluted in the buffer containing 1% BSA and 1% Triton X-100 for 2 h at 4 °C for 10 min, the supernatant was then centrifuged at 126,000 × g for 5 min at 27 °C to remove unpolymerized tubulin. Cold BRB80 was added to resuspend the microtubule pellet, followed by depolymerization at 4 °C for 20 min and a second round of polymerization with 1 mM GMPCPP at 37 °C for 30 min. Finally, microtubule seeds were obtained after centrifugation at 126,000 × g for 5 min at 27 °C, resuspended using 37 °C pre-warmed BRB80, frozen by liquid nitrogen and stored in –80 °C.

**Time-lapse imaging.** To detect the movement of EB3 tagged with tdTomato (EB3-tdTomato), E14 hippocampal neurons were transfected with indicated plasmids and then plated on glass-bottom dishes. At DIV 3, the dishes were placed on a temperature-controlled workstation (37 °C, 5% CO2) with an inverted microscope. Randomly selected neurons (at least 35 neurons for each condition from 4 individual experiments) were imaged using the Zeiss cell observer spinning disk system (Zeiss) with Zen 2.1 software under 60 × oil lens and 60 frames were collected for 2 min at 2 s interval. The number of EB3 comets in the cell body was averaged from 3 independent frames. The neurite segments were selected from the immediate region of the longest neurite in neurons. The kymograph was generated using ImageJ software (NIH), and then the number and direction of EB3 comets in the neurite were analyzed. The mean velocity, growth lifetime, and catastrophe frequency of EB3 comets in particular kymograph was calculated using Image-Pro Plus 5.1 software.

**Electron microscopy.** For transmission electron microscopy, cultured E14 hippocampal neurons on glass-bottom dishes were then incubated in BRB80 for 30 min at 37 °C, fixed with 4% PFA for 20 min at room temperature, blocked with 1% BSA for 30 min at room temperature and incubated with 50% PFA + 0.1% Triton X-100 for 30 min at 4 °C. The cell culture dish was then washed with PBS, then post-fixed with 1% OsO4 for 30 min at room temperature, dehydrated with graded ethanol series and embedded in Epon 812 resin. The sections were cut on glass slides and stained with uranyl acetate and lead citrate. The sections were then imaged using a Tecnai G2 Spirit transmission electron microscopy (FEI).

**Statistical analysis.** All data were collected from at least 3 independent experiments and presented as the mean ± s.e.m. No statistical methods were used to predetermine the sample sizes. Statistical analysis was performed using Prism 5 software (GraphPad). For Western blot and in vitro microtubule reconstitution assay, data were analyzed using paired Student’s t test. In other experiments, comparison between two groups was performed with unpaired Student’s t test and comparison among multiple groups was evaluated by two-way analysis of variance (ANOVA) with Bonferroni’s post-hoc test. Differences were considered significant at the level of P < 0.05.
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Author contributions

XX did most experiments in cerebral cortex and neurons. S.G.W. did the in vitro methylation and microtubule reconstitution assay, and majority of biochemical experiments. M.Y.L. performed IUE. L.D. helped detection of MT dynamics. J.I.C. provided induced ES cells. W.G.Z. provided the Set2lox/lox mice. X.Z. initiated the project. X.Z. and X.Y.P. helped the project design. L.B., W.F.F., X.X. and S.G.W. designed the project and wrote the paper. L.B. supervised the whole project.

Competing interests

The authors declare no competing interests.

Additional information

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