Direct induction of microtubule branching by microtubule nucleation factor SSNA1

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Microtubules are central elements of the eukaryotic cytoskeleton that often function as part of branched networks. Current models for branching include nucleation of new microtubules from severed microtubule seeds or from γ-tubulin recruited to the side of a pre-existing microtubule. Here, we found that microtubules can be directly remodelled into branched structures by the microtubule-remodelling factor SSNA1 (also known as NA14 or DIP13). The branching activity of SSNA1 relies on its ability to self-assemble into fibrils in a head-to-tail fashion. SSNA1 fibrils guide protofilaments of a microtubule to split apart to form daughter microtubules. We further found that SSNA1 localizes at axon branching sites and has a key role in neuronal development. SSNA1 mutants that abolish microtubule branching in vitro also fail to promote axon development and branching when overexpressed in neurons. We have, therefore, discovered a mechanism for microtubule branching and implicated its role in neuronal development.

Cell-shape control is critical in a number of physiological processes. Microtubules, the major cytoskeletal component determining cell shape, are mostly nucleated at the centrosome in proliferating cells. During specialized cell-shaping events, such as mitosis or cell polarization, cytoskeletal remodelling is thought to be driven by local nucleation of microtubules using a centrosome-independent mechanism.

Neuronal cells are a distinctive example of cells with highly complex morphologies. Neurons are shaped in an extremely polarized fashion with a unique-shaped axon protruding from the cell body and stretching over long distances. Individual cells develop branch points from their axons to connect to neighbouring cells, creating an intricate communication network in the nervous system. As the shape of axons is determined by microtubules, these branch points require remodelling of microtubules to split the cytoskeletal path into separate branches. As the centrosome is not necessary for the morphological development of the axon, it is possible that axonal transformation occurs in a locally regulated manner within the axon. At axon branching points, the local destabilization and fragmentation of microtubules is mediated by the microtubule-severing enzyme spastin, which leads to the emergence of short microtubules. However, the process of splitting the microtubule networks and, specifically, how the local rearrangement of spastin-processed tubulin oligomers or short microtubule fragments occurs has been enigmatic.

Due to its importance in various cell activities, the microtubule cytoskeleton has been well characterized in vitro. In the classical view, microtubules are considered as cylindrical polymers made of ~13 protofilaments. However, in living cells, it has been suggested that microtubules form higher-order branched networks to regulate their distribution within complex cytoskeletal networks.

The branched networks could be envisioned either through the attachment of new microtubule modules onto the side of an existing microtubule, or through direct branching of microtubules. So far, only one example of branching microtubule network has been shown, involving the microtubule nucleators augmin and γ-tubulin, which allow microtubules to grow out from nucleation points that attach to the side of existing microtubules.

We now demonstrate that SSNA1 is a powerful microtubule-nucleating and -branching factor. In vitro reconstitution of SSNA1-mediated microtubule nucleation showed an induction of branched microtubules, where new daughter microtubules directly branch out from existing microtubules. SSNA1 attaches along single protofilaments, guiding them to grow away from a microtubule and template a branched microtubule. Mutation of residues essential for the oligomerization and the microtubule-branching activity of SSNA1, which we designed by structure-guided in vitro experiments, leads to defective axonal branching in primary neurons, showing that the...
simple scaffolding mechanism of SSNA1 can lead to vast morphological changes in neurons.

Results

SSNA1 localizes at axon branching sites in primary neurons. A previous study implicated SSNA1 in the promotion of axon branching, but the underlying mechanism remained elusive. To investigate how SSNA1 plays a role in neuron development, we transduced wild-type murine primary hippocampal neurons with lentiviral particles encoding GFP-2A-mouse SSNA1. Overexpression of SSNA1 led to the promotion of axon outgrowth (Fig. 1a,c), in agreement with a previous report. In addition, we found a striking accumulation of SSNA1 at axon branches (Fig. 1b, panel 4, and Supplementary Fig. 1). In agreement with its localization, SSNA1 overexpression led to increased and more complex branching as characterized by the Strahler number (Fig. 1d). Since SSNA1 localizes at the cytosolic compartments where microtubule dynamics are dynamic, we hypothesized that clusters of SSNA1 at branching sites in neurons might facilitate local microtubule nucleation.

SSNA1 induces direct microtubule branching. To assess the influence of SSNA1 on microtubules, we prepared recombinant SSNA1 (from Chlamydomonas reinhardtii, CrSSNA1, Supplementary Fig. 2A–C) and tested its interaction using cryo-electron microscopy (cryo-EM; Fig. 2a and Supplementary Fig. 2D). CrSSNA1 induced a formation of direct microtubule branches that split from a single microtubule (Fig. 2a, arrowheads), in contrast to an attachment of a second microtubule on the microtubule surface. Branching occurs by splitting the lattice of the microtubule, and protofilaments of mother microtubules directly continue into the outer surface of the branched microtubule. The bending angle was variable (Fig. 2b, 47° ± 15°, and Supplementary Fig. 2D,E), which suggests a rather flexible junction, in contrast to the more rigid, 70° Arp2/3-mediated actin branching. Moreover, microtubules occasionally formed fork-like structures with several branches or junctions (Fig. 2b and Supplementary Fig. 2D). This has so far not been observed in any other system, and further underpins the uniqueness of SSNA1-mediated microtubule branching.

Cryo-ET shows diverging microtubules with a break in the microtubule lattice. To further understand the organization of microtubule branches, we performed cryo-electron tomography (cryo-ET) on branched microtubules (Fig. 3 and Supplementary microtubule branches, we performed cryo-electron tomography of SSNA1-mediated microtubule branching. To explore the dynamic behaviour of SSNA1 causing this unique action in microtubules, we tested the interaction of SSNA1 with unpolymerized tubulin using fluorescence microscopy (Fig. 4a). Considering the average cellular concentration of SSNA1 of 187 nM, we mixed 200 nM CrSSNA1 and 8 μM tubulin in the presence of polyethylene glycol (PEG). Above a concentration of 5% PEG, we observed condensates of CrSSNA1 clustering with tubulin (Fig. 4a–c). Interestingly, several microtubules emerged from these CrSSNA1–tubulin clusters (Fig. 4a,b), reminiscent of aster

Fig. 1 | The effect of SSNA1 overexpression on primary hippocampal neurons. a, Immunostaining of MAP2 (green) and Tau (red) in control (GFP overexpression) and SSNA1-wild-type overexpression. b, Immunostaining of SSNA1 (red) and βIII-tubulin (green) in neurons overexpressing SSNA1 wild type shows the localization of SSNA1 at axon branch sites. c, Scatter dot plots of axon length under overexpression of SSNA1. The longest protrusion from the soma was defined as the axon, and cells with very short protrusions were also included in the counting, so that underdeveloped neurons could be assessed as well. The promotion of axon development occurs only in overexpression of wild-type SSNA1. Experiments were performed in triplicates, shown in magenta, green and yellow. Every cell is represented by a single point: control (n = 505 cells), wild type (n = 499 cells), pooled from 3 independent experiments, and the overlaid box-and-whisker plots cover 50% (boxes) and 90% (whiskers) of the entire population, with median values indicated as lines within the boxes. The results show statistical significance (P < 0.0001) as tested using the Kruskal–Wallis test, followed by Dunn’s multiple comparison post hoc test. d, Pie graphs showing the distribution of the number of branches under overexpression conditions (control (n = 496 cells), wild type (n = 490 cells), pooled from 3 independent experiments) and Strahler number (degree of sub-branch formations on the existing branches; control (n = 266 cells), wild type (n = 289 cells) pooled from 3 independent experiments). Distributions of the branches and the Strahler number in SSNA1-expressing neurons differ significantly from the control (GFP overexpression) according to χ² two-sample test (χ² = 20.7, P < 0.01 and 18.6, P < 0.005, respectively). See Supplementary Table 3 for source data.
SSNA1 forms a fibril-like assembly on the surface of the microtubule with 11-nm periodicity. Although cryo-ET did not visualize the decoration of SSNA1 on microtubules, we observed that the free ends of microtubules are often extended with thin fibrils (Supplementary Fig. 2D, red arrowheads). These fibrils extend from splitting microtubules, seemingly to work as a ‘guide rail’ for the growth of branched microtubules (Fig. 2b; and Supplementary Fig. 2D, ‘guide rail’). Computational averages of the cryo-EM images of microtubules allowed the visualization of CrSSNA1 directly attached to the surface of microtubules, revealing a ladder-like pattern (Fig. 5a, compare to ‘microtubule-only control’) with a periodicity of ~11 nm (Fig. 5b). We observed that CrSSNA1 facilitated preferential assembly of 13-protofilament microtubules similar to doublecortin and EBs. In contrast, 14-protofilament microtubules are predominantly polymerized in the absence of SSNA1 (Fig. 5c).

SSNA1 forms a head-to-tail fibril with 11-nm periodicity and covers the C-terminal tail of microtubules. SSNA1 is a protein with a relative molecular mass of 14,000 Da predicted to adopt a tropomyosin-like single parallel coiled-coil configuration (Fig. 3F). Fibril formation has previously been observed as a result of head-to-tail self-assembly. Accordingly, we observed that CrSSNA1 readily forms short fibrillar appearances with occasional long fibril formations (Fig. 5g, ‘FL’). Furthermore, the shorter fibrils of CrSSNA1 were converted into longer, organized bundles of fibrils after ~24 h incubation (Supplementary Fig. 3F). A closer look at these bundles revealed a striped, knob-like pattern, which leads to the formation of a sheet (Supplementary Fig. 3E, 24h) with a 11-nm periodicity (Supplementary Fig. 3F, inset), and the inter-fibril distance of 3.5 nm. This pattern is comparable to that observed on the microtubule surface (Fig. 5a), indicating that the fibrils are covering microtubules along their long axis, giving a 11-nm spaced ladder-like pattern.
To further characterize the interaction between SSNA1 and microtubules, we obtained a cryo-EM three-dimensional (3D) structure of CrSSNA1 in complex with microtubules (Fig. 5d–f and Supplementary Fig. 4A) with an overall resolution of 6.1 Å (Supplementary Fig. 4B). Due to the symmetry mismatch between microtubules (4- or 8-nm periodicity) and SSNA1 (11-nm periodicity), SSNA1 was averaged out, and the fibril appeared as a ‘cloud’ of protein density running parallel to the microtubule surface, with an apparent local resolution of ~11 Å (Supplementary Fig. 4C).

However, it was possible to visualize thin lines of additional densities running parallel to the microtubule surface (Fig. 5e,f), which we interpreted as SSNA1 filaments. The SSNA1 filaments run between two protofilaments, proximal to the unstructured, highly acidic carboxy-terminal tails (E-hooks) of tubulin (Fig. 5f labelled ‘C’). Removal of E-hooks resulted in weakening of SSNA1 crosslinking with microtubules as determined by EDC (~49% less crosslinked; Supplementary Fig. 4E). E-hooks create a negative electrostatic cloud by their periodical arrangement on the microtubule surface.29

Fig. 4 | Nucleation and branching of microtubules mediated by CrSSNA1 under various conditions. a, Aster-like formation of microtubules (20% HiLyte488 tubulin) occurs within 3 min after mixing tubulin with a lower concentration (200 nM) of CrSSNA1 (upper) under conditions mimicking molecular crowding (7.5% PEG, typically used as a crowding agent), where tubulin alone does not form any polymers. Microtubules propagate out from tubulin concentrate, serving as a nucleation centre. These experiments were performed three independent times with similar results. b, 200 nM CrSSNA1 and 8 μM tubulin self-associate, forming clusters in the presence of PEG with concentration >5%. c, SSNA1 antibody recognizes the microtubule nucleation centre. d, A plot of the percentage of the concentrates growing into asters with microtubules as a function of time (min) in the presence of 50, 100 and 200 nM CrSSNA1. The error bars are mean ± s.d. from n = 3 independent experiments. As little as 50 nM of CrSSNA1 is sufficient to observe aster formation in the presence of 7.5% PEG. e, Counts of microtubules observed per field of view, in the presence of different concentrations of CrSSNA1. The box plots cover 50% (boxes) and 90% (whiskers) of the entire population, with median values indicated as lines within the boxes. Sample size: 0 nM: n = 42 fields of view; 50 nM: n = 29; 100 nM: n = 30; 200 nM: n = 25. Data were pooled from three independent experiments except for the first point (0 nM) for which data were pooled from four independent experiments. f, Green coloured dynamic microtubules on red-microtubule GMPCPP seeds in the presence of a higher concentration of CrSSNA1 (30 μM) without molecular crowding agents to achieve globally concentrated conditions. ‘branch-like’ nucleation is observed. Branches were categorized as ‘splitting’, ‘end-joining’, ‘side branching’ or ‘dynamic branching’. g, Ratios of different branch types (n = 895 branches, mean ± s.d. pooled from 3 independent experiments). ‘?’ shows the bundled microtubules, which are difficult to categorize. ‘X’ shows microtubules without branching. Branch-like nucleation can be seen from the locally concentrated SSNA1 condition described in a–e; however observations of individual microtubules are challenging due to the high local protein concentrations. h, A negative-stain EM image of SSNA1-mediated branched microtubules in the presence of 200 nM CrSSNA1 and 7.5% PEG, representative of 3 independent experiments. See Supplementary Table 3 for source data.
which could attract the SSNA1 fibrils. This could explain why SSNA1 interacts with microtubules despite the symmetry mismatch. It also suggests that the head-to-tail assembly of SSNA1 fibrils could guide protofilament assembly and microtubule polymerization by covering and neutralizing the E-hooks as shown previously.

The head-to-tail fibril formation of SSNA1 is essential for microtubule branching. On the basis of the observation that SSNA1 fibrils appear to guide the protofilaments of microtubules, we hypothesized that the microtubule branching activity is mediated by the formation of long SSNA1 fibrils that curve away and guide the protofilament out of the lattice (guide rail, Fig. 2b and Supplementary Fig. 2D). To test this, we created a series of truncated SSNA1 fragments that abolish fibril formation. On the basis of a PHYRE2 analysis and previous reports, we found that SSNA1 contains a well-conserved α-helical region (residues 6–104) followed by an unstructured C-terminal tail (Supplementary Fig. 3E). A series of amino-terminal truncations showed that the first 19 residues were not necessary for fibril formation, as Cr<sub>SSNA1</sub>(20–111) (20-C) formed cable-like bundled fibrils, which were less ordered compared to full-length protein (Cr<sub>SSNA1</sub> FL), but displayed an ~11-nm pattern (Fig. 5g,h, Supplementary Fig. 5A and Supplementary Table 1). In contrast, Cr<sub>SSNA1</sub>(21–C), a truncation missing one more residue, Glu 20, was unable to form fibrils (Fig. 5g,h, Supplementary Fig. 5A and Supplementary Table 1). This observation correlates with the ability of Cr<sub>SSNA1</sub>(20–C), but not Cr<sub>SSNA1</sub>(21–C), to mediate microtubule branching (Supplementary Fig. 5A and Supplementary Table 1). The key role of the residue Glu 20 for fibril-formation and microtubule-branching activity of SSNA1 was further underpinned by point mutations E20A and E20A/D21A, which drastically reduced microtubule branch formation (Supplementary Fig. 5A and Supplementary Table 1). These mutants may form fibrils, but with much lower frequency and without a distinct higher-order organization.

In the C-terminal region of Cr<sub>SSNA1</sub>, three distinctive lysine residues (Lys 105, Lys 106 and Lys 107) mark the beginning of the unstructured Cterminus. Cr<sub>SSNA1</sub> truncations 1–104 and 1–105 (Fig. 5g,h, Supplementary Fig. 5A and Supplementary Table 1) showed that Cr<sub>SSNA1</sub>(1–104) can no longer form fibrillar oligomers, or induce microtubule branching (Fig. 5g,h), while Cr<sub>SSNA1</sub>(1–105) was purified as fibrils and showed microtubule branching activity (Supplementary Fig. 5A) at a similar efficiency to Cr<sub>SSNA1</sub> FL. These results indicate that the positive charge of the lysine residues is essential for the ability of Cr<sub>SSNA1</sub> to form fibrils. We confirmed this by generating a triple point mutant, K105A/K106A/K107A, which indeed abolished fibril formation and microtubule branching (Supplementary Fig. 5A and Supplementary Table 1) for both the full length and the 1–107 fragment. Altogether, our mutational analyses indicate that the key interaction for longitudinal fibril formation is mediated by Glu 20 of one unit and the C-terminal tail (Lys 105–107) of the adjacent interacting unit (Fig. 5i). To confirm this, we created mutants in which the charges of residues Glu 20/Asp 21 and Lys 105/Lys 106/Lys 107 were swapped. When two of the opposite charges were swapped (E20K/D21K/K105E/K106E or E20K/D21K/K106E/K107E), both cable-like formation and microtubule branching activity of SSNA1 were retained. In contrast, swapping of the two negative residues at the N terminus and the three positive residues at the C terminus (E20K/D21K/K105E/K106E/K107E), resulting in a change of net charge from +1 to −1, abolished microtubule branching (Supplementary Fig. 5B). However, this construct was still able to form SSNA1 fibrils and cable-like structures, indicating that the microtubule branching activity depended not only on fibril formation of SSNA1, but also on the presence of an extra negative charge at the unstructured SSNA1 Cterminus. This was confirmed by the mutant E20A/D21A/K105A/K106A/K107A, termed 5A, showing a complete loss of microtubule branching activity (Fig. 5g).

Microtubule-branching-deficient SSNA1 mutants abolished the promotion of axon branches. Having gained insights into the molecular organization of SSNA1 and its effect on microtubule nucleation and branching, we hypothesized that the promotion of axon growth and branching observed in neurons overexpressing wild-type SSNA1 might be altered when microtubule-branching-deficient SSNA1 versions are expressed. Our results indeed showed that, in contrast to the SSNA1 wild type (Figs. 1 and 6a–c), SSNA1 with mutations abolishing microtubule branching in vitro also failed to promote the growth of axons or axon branches (Fig. 6a–c and Supplementary Fig. 6A–E) in primary neurons. Notably, a dominant-negative effect was also observed when the 5A mutant was overexpressed for the number of total neurite processes (Fig. 6c), showing a decreased number of major and minor branches. This dominant-negative effect was also found when the two negative residues at the N terminus and the three positive residues at the C terminus were swapped (swap-KK/EEE). Notably, swapping only two opposite charges and leaving the third C-terminal lysine intact (swap-KK/EE) could still promote axon growth (Supplementary Fig. 6D,E). Together these findings show that the ability of SSNA1 to induce fibril formation and microtubule branching at the molecular level correlates with its function of mediating axon branching and development, suggesting the intriguing possibility that it locally generates branched microtubules at axon branch sites.

Morphological change of microtubule networks in non-neuronal cells. To test whether the function of SSNA1 is conserved in different cell types, we used fibroblasts, which are structurally less specialized than neurons, and tested whether overexpression of SSNA1 has the capacity to change the microtubule organization (Fig. 6e–j). Super-resolution light microscopy with DNA-PAINT showed that individual microtubules are well resolved in the control cells with a wide-ranging network (Fig. 6e–g). In contrast, microtubules were rather short in SSNA1-overexpressing cells (Fig. 6h–j), suggesting that SSNA1 can promote nucleation, generating more but shorter microtubules. We also occasionally found microtubules forming three-way intersections, as if one microtubule emerged out of another in both control and SSNA1-overexpressing cells (Fig. 6g,j, arrowheads). These events occurred more often in SSNA1-overexpressing cells (2.8 ± 1.2 occurrences per 100 µm of microtubule) than control cells (1.0 ± 0.35 occurrences per 100 µm of microtubule). Although the limited resolution in light microscopy prevented us from discerning whether microtubules branched with a shared lattice or if two microtubules only attached to each other, the observations were consistent with our in vitro studies by electron microscopy.

Discussion

During cell polarization, the dynamics and distribution of the microtubule cytoskeleton is tightly regulated. Although the centrosome has a major role as a microtubule-organizing centre in less differentiated cells, the inactivation of centrosomes in neurons does not affect axon growth, a process strongly dependent on microtubule assembly. Thus far, the molecular mechanisms regulating axonal microtubule nucleation, especially in the form of branching, have remained a mystery. Here, we show that SSNA1 accumulates at axon branches and promotes axon branching in primary neurons, and can nucleate microtubules in vitro. Mutations interfering with SSNA1 in vitro nucleation activity also affect the occurrence of axon branches in neurons. Together, these results suggest that SSNA1 could act as a microtubule nucleator at axon branch sites.

Strikingly, our work revealed that SSNA1 independently mediates microtubule branching by causing protofilaments to splay apart from the lattice. To our knowledge, no other microtubule-binding protein shows this activity. When tested, EB3 and ch-TAG, known regulators of microtubule dynamics, under the same conditions,
Fig. 5 | Molecular characterization of the branching action of SSNA1. a, Left: representative class average of the SSNA1-induced microtubules. Right: SSNA1 decoration emphasized by computationally subtracting microtubule densities35. Bottom left: average of microtubules without decoration for comparison. b, The power spectrum of microtubule class averages shows an additional 11 nm periodicity in the presence of SSNA1. c, Distribution of protofilament (pf) numbers of microtubules reconstituted from brain tubulin in the absence (left) and in the presence of SSNA1 (right) shifting the majority from 14- to 13-protofilament microtubules. d, Greyscale slice from the density map of the plus-end-on view of the SSNA1–microtubule 3D reconstruction. SSNA1 decoration and the secondary structures of tubulin density are well resolved. e, Rendering of the microtubule surface decorated with SSNA1. The resolution of the microtubule surface (~10 Å) is not as high as the core (<8 Å) due to the SSNA1 decoration. f, Tubulin atomic model (PDB ID: 3jal) fitted to the map. The SSNA1 coiled-coil fibril is indicated as a tube representation. Note that the periodical feature of SSNA1 is averaged out because of the symmetrical mismatch between tubulin dimers (8 nm) and SSNA1 fibrils (11 nm). g, Morphological observation of SSNA1 and its branching activity. Left: observation of the purified protein at 0 h incubation (that is, immediately after purification). Right: a magnified view of the co-polymerized microtubules. Microtubule branching was observed with SSNA1-FL, while other protein fragments do not facilitate branching. For the proteins that do not cause the branching, examples of typical crossing of microtubules (white and beige bars at the scheme within the image), instead of branching, are shown. Detailed observations are available in Supplementary Fig. 5A. h, A graphical scheme of the SSNA1 constructs used in g. i, A scheme of the SSNA1 self-assembly and microtubule nucleation mediated by SSNA1. While SSNA1 oligomers alone can also undergo a slow self-assembly process, the SSNA1 oligomers interact with tubulin dimers to promote their co-polymerization. The polymerized SSNA1 may further act as a guide rail (bottom inset) for protofilament splitting, resulting in microtubule branch formation. A class average indicating the guide rail mechanism is shown. Other class averages are available in Supplementary Fig. 2E.
Fig. 6 | The effect of various SSNA1 constructs on primary hippocampal neurons and fibroblast cells. a, Immunostaining of MAP2 (green), Tau (red) and GFP (blue, expression control) in SSNA1-overexpressing cells. For the SSNA1 wild type, the axon is indicated with a dashed line. b, Scatter dot plots of axon length under overexpression of various SSNA1 constructs. The control and wild-type profiles shown in Fig. 1 are placed as a negative and positive control, respectively. The promotion of axon development occurs only in overexpression of wild-type SSNA1, while no apparent effect was observed for the constructs that fail to mediate microtubule branching. Every cell is represented by a single point; control (n = 496 cells), wild type (n = 490), 1–104 (n = 788), 21-C (n = 610), 5A (n = 642) from 3 independent experiments, shown in magenta, green and yellow, and the overlaid box-and-whisker plots cover 50% (boxes) and 90% (whiskers) of the entire population, with median values indicated as lines within the boxes. c, Pie graphs showing the distribution of the number of branches and Strahler number under different overexpression conditions. The GFP-expression-control and SSNA1-wild-type-overexpression profiles in Fig. 1 are placed as controls. d, A schematic model describing how SSNA1-mediated microtubule nucleation could contribute to axon branch formation. Spastin has been shown to localize at axon branches1 and to interact with SSNA115. Taken together with our finding of SSNA1 localization at axon branches, it is possible that the two proteins work sequentially by spastin severing microtubules to provide tubulin oligomers, and SSNA1 nucleating microtubules at the branching site. e, A DNA-PAINT image of a 500-nm slice of the microtubule network in untreated cells (control). f, Zoomed-in view microtubules at the branching site. g, Individually recognized microtubules are highlighted in various colours. Three-way intersections are indicated with red arrowheads. h, i, j, Corresponding view of a 500-nm slice of the microtubule network in SSNA1 overexpressing cells. For analysis, 3 independent SSNA1-overexpressing and control cells were assessed each, containing the total microtubule lengths of 5,700 µm, 7,900 µm and 7,700 µm, respectively. See Supplementary Table 3 for source data.

did not induce branching (Supplementary Fig. 6E,G). In addition, we showed that microtubule branching requires co-polymerization of SSNA1 with microtubules, as well as specific amino-acid interactions. Taken together, these results indicate that the observed branching activity is highly specific to SSNA1.

Our work has uncovered a surprising example of how co-polymerization of a simple coiled-coil protein with tubulin can induce global remodelling of the microtubule network. Association of SSNA1 may reinforce longitudinal connections of tubulin oligomers, facilitate protofilament formation and act as a polymerization seed for microtubule formation. Concomitantly, the preference of SSNA1 for lateral connections may facilitate the lateral associations between microtubule protofilaments. Polymerized SSNA1 may precede microtubule protofilaments, guiding protofilaments out of the microtubule axis, thus providing a template for a new microtubule branch (‘guide rail’ mechanism). In a cellular context, however, the situation is more complex as SSNA1 activity is probably modulated by other factors. Further experiments are necessary to test this mode of action of SSNA1 for microtubule branching in cells.

Our in vitro reconstitutions showed that SSNA1 self-assembles into clusters together with tubulin at a high local concentration. As axons are densely packed with cytoskeletal components, this organization is a plausible prerequisite for the physiological function of SSNA1, allowing SSNA1 to concentrate locally, self-assemble and become a microtubule nucleation centre at designated locations. Alternatively, the requirement of a high local concentration of SSNA1 may be a means to limit the microtubule-remodelling activity of SSNA1 to specific subcellular areas such as axon branches, the midbody in dividing cells and the base of cilia.

As microtubules are much less dynamic in axons compared to less polarized cells22–24, SSNA1-mediated branching may be restricted to locally destabilized sites of the microtubule cytoskeleton. Interestingly, SSNA1 interacts with spastin, a protein important for the initiation of axon branching and thought to increase the pool of soluble tubulin through microtubule fragmentation1.
It is tempting to speculate that short, spastin-severed microtubules (that is, tubulin oligomers) provide the building blocks for SSNA1-mediated microtubule nucleation and branching. Thus, the synergistic action of spastin and SSNA1 could facilitate the formation of axonal branch points (Fig. 6d). Further investigation of SSNA1 activity in situ will provide valuable insights into the initiation and organization of axon branches. In particular, it will be interesting to explore whether SSNA1-mediated microtubule branching is a direct driving force for axon branching, or an intermediate state during early stages of neuronal morphogenesis. Considering the diverse sites at which SSNA1 is localized in various cell types, the microtubule-branching mechanism discovered here could have broad implications for understanding the regulation of various microtubule functions, providing new clues to previously unanswered questions about cytoskeleton and intracellular transport.

Online content
Any methods, additional references, Nature Research reporting summaries, source data, statements of data availability and associated accession codes are available at https://doi.org/10.1038/s41556-018-0199-8.

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References
1. Ishihara, K., Nguyen, P. A., Groen, A. C., Field, C. M. & Mitchison, T. J. Microtubule nucleation remote from centrosomes may explain how asters span large cells. Proc. Natl Acad. Sci. USA 111, 17715–17722 (2014).
2. Meunier, S. & Vernos, I. Acentrosomal microtubule assembly in mitosis: the where, when, and how. Trends Cell Biol. 26, 80–87 (2016).
3. Petry, S. & Vale, R. D. Microtubule nucleation at the centrosome and beyond. Nat. Cell Biol. 17, 1089–1093 (2015).
4. Kaif, K. & De Camilli, P. Branch management: mechanisms of axon branching in the developing vertebrate CNS. Nat. Rev. Neurosci. 15, 7–18 (2014).
5. Lewis, T. L., Courchet, J. & Polleux, F. Cell biology in neuroscience: cellular and molecular mechanisms underlying axon formation, growth, and branching. J. Cell Biol. 202, 837–848 (2013).
6. Stieß, M. et al. Axon extension occurs independently of centrosomal microtubule nucleation. Science 327, 704–707 (2010).
7. Yu, W. et al. The microtubule-severing proteins spastin and katanin participate differently in the formation of axonal branches. Mol. Biol. Cell 19, 1485–1498 (2008).
8. Petry, S., Groen, A. C., Ishihara, K., Mitchison, T. J. & Vale, R. D. Branching microtubule nucleation in Xenopus egg extracts mediated by augmin and TPX2. Cell 152, 768–773 (2013).
9. Decker, F., Orioli, D., Dalton, B. & Brugéus, I. Autocatalytic microtubule nucleation determines the size and mass of Xenopus laevis egg extract spindles. eLife 7, e31149 (2018).
10. Murata, T. et al. Microtubule-dependent microtubule nucleation based on recruitment of gamma-tubulin in higher plants. Nat. Cell Biol. 7, 961–968 (2005).
11. Janson, M. E., Setty, T. G., Paolotti, A. & Tran, P. T. Efficient formation of bipolar microtubule bundles requires microtubule-bound gamma-tubulin complexes. J. Cell Biol. 169, 297–308 (2005).
12. Goshima, G., Mayer, M., Zhang, N., Stuurnu, N. & Vale, R. D. Augmin: a protein complex required for centrosome-independent microtubule generation within the spindle. J. Cell Biol. 181, 421–429 (2008).
13. Kamasaki, T. et al. Augmin-dependent microtubule nucleation at microtubule walls in the spindle. J. Cell Biol. 202, 25–33 (2013).
14. Sánchez-Huertas, C. et al. Non-centrosomal nucleation mediated by augmin organizes microtubules in post-mitotic neurons and controls axonal microtubule polarity. Nat. Commun. 7, 12187 (2016).
15. Pfannenschmidt, F. et al. Chlamydomonas DIP13 and human NA14: a new class of proteins associated with microtubule structures is involved in cell division. J. Cell Biol. 164, 1449–1462 (1999).
16. Lai, C. K. et al. Functional characterization of putative cilia genes by high-content analysis. Mol. Biol. Cell 22, 1104–1119 (2011).
17. Goyal, U., Renvoisé, B., Chang, J. & Blackstone, C. Spastin-interacting protein NA14/SSNA1 functions in cytokinesis and axon development. PLoS ONE 9, e112428 (2014).
18. Blanchon, L. et al. Direct observation of dendritic actin filament networks nucleated by Arp2/3 complex and WASP/Scar proteins. Nature 404, 1007–1011 (2000).

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Author contributions
N.B. and N.M. performed electron and light microscopy, designed mutant constructs and performed biochemistry experiments, analysed the data and prepared the figures. S.B. and M.M.M. performed experiments with neuron primary culture. N.B. and A.H.C. performed light microscopy experiments. N.B. and M.T. cloned and purified proteins. N.M., N.B., H.N., S.B., M.M.M. and C.J. analysed neuron data and G.C.
facilitated the automation of neuron analysis. N.M., N.B. and H.N. performed electron microscopic data collection and analysed the data. H.N. and T.S. performed super-resolution light microscopy experiments and N.M., H.N., G.C., C.B and R.J. analysed the data. Experiments were designed by N.M. and the manuscript was written by N.M. with contributions from the other authors.

**Competing interests**
The authors declare no competing interests.

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Methods

Protein preparation and purification. The DNAs of CrSSNA1 and mouse SSNA1 were obtained by gene synthesis (GeneArt, ThermoFisher) and cloned into self-generated LIC (ligation-independent cloning) vectors. The SSNA1 fragments were prepared as hexahistidine (His) fusion proteins with a TEV protease recognition site. The proteins were expressed in Escherichia coli BL21(DE3) (Merck) by induction with 0.4 mM IPTG (Carl Roth) overnight at 18 °C. Cells were sonicated in lysis buffer (50 mM Na-phosphate buffer pH 7.5, 150 mM NaCl, 10% (v/v) glycerol, 5 mM β-mercaptoethanol) supplemented with protease inhibitors (1 mM pepstatin A, 1 mM AEBSF and 1 mM leupeptin) and clarified. The soluble fraction was purified by Ni-NTA affinity chromatography. The His tag was either removed by TEV cleavage or left on the protein. Biochemical analysis did not show any differences with or without the tag. For CrSSNA1(1–104), an additional step of size-exclusion chromatography (Superdex 200, GE Healthcare) was applied. Circular dichroism spectra were obtained on a JASCO 715 CD spectrometer equipped with a Peltier temperature control, at 4 °C and 37 °C. Tubulin was purified from porcine brains (The Bayerische Landesanstalt für Landwirtschaft) according to a previously published protocol21 or purchased from Cytokeleton. The oligomerizations of SSNA1 variants were monitored for 0 h (immediately after purification), 24 h and 48 h.

The DNAs for mouse chTOG (amino acids 1–505) and human EB3 (amino acids 1–281) were obtained from the Mammalian Gene Collection (MGCC, Source BioScience LifeSciences). mTTOG protein was expressed in E. coli BL21(DE3)pLysS by induction with 0.5 mM IPTG and cells were grown overnight at 16 °C. Cells were sonicated in lysis buffer (50 mM Tris, pH 8.0, 300 mM NaCl, 100 mM imidazole, 1 mM dithiothreitol) supplemented with protease inhibitors (1 mM pepstatin A, 1 mM leupeptin and 1 mM phenylmethylsulfonyl fluoride (PMSF)) and clarified. The protein was purified using Ni-affinity chromatography followed by ion exchange chromatography (Hi Trap S, GE Healthcare) and size-exclusion chromatography (Superdex 200, GE Healthcare). His tag was removed by 3C protease. EB3 was expressed in E. coli BL21(DE3). Cells were sonicated in lysis buffer (20 mM Pipes, 1 mM MgCl2, 1 mM EGTA) supplemented with protease inhibitors (1 mM pepstatin A, 1 mM leupeptin and 1 mM PMSF) and clarified. The protein was purified using Ni-affinity chromatography followed by size-exclusion chromatography (Superdex 200, GE Healthcare). His tag was removed by 3C protease.

Electron microscopy of SSNA1–microtubule complex and image processing. For screening conditions that were also used for light microscopic observations, 8 or 15 µm of tubulin was mixed with 0.1 to 30 µm of SSNA1 in BRB60 buffer (40 mM NaCl, 6.7 mM Pipes-KOH, 1 mM MgCl2, 1 mM EGTA) supplemented with 1 mM GTP or GMPcPP for 5 min, and directly applied on an EM grid for negative staining. The cluster of the microtubules observed in light microscopic environments was only partially preserved under the negative stain condition, due to the fixation process of the sample for negative-stain EM. The centre of the cluster is not visible due to high electron density.

For cryo-EM, we used 15 µl of tubulin and two to five times excess of SSNA1 to maximize the decoration with the protein. Microtubules were stabilized using a non-hydrolysable GTP analogue mimicking GTP-bound conditions, GMPcPP. After 5 min, 5 µl of sample without dilution was applied to glow-discharged grids with holey carbon (Quantifoil, Cu, R1.2/1.3) and vitrification was carried out in liquid ethane using a gas-flow-manual plunger. The cryo-EM specimens were observed on a Tecnai F20 (Thermo Fisher Scientific) at 200 kV with a magnification of 29,000x. Images were taken using a Falcon2 direct detector (Thermo Fisher Scientific), corresponding to 3.46 Å per pixel with a defocus of about −2.5 µm. The total 98 images with the dose of 50 electrons per square ångstrom were collected and subjected to image analysis.

For counting the numbers of the protofilaments of the microtubules, we referred to a well-known specific interference pattern (moire pattern) of the microtubules observed under cryo-EM.

For the measurement of the branching angles, we used 99 branched microtubules. Using FIJI software, two-connector segments were drawn with each segment approximately 50 nm long, placing the junction of the segments at the centre of the branching points. Then the two segments were aligned along the direction of the two branched microtubules. Examples of branches with various angles are shown in Supplementary Fig. 2D.

For the initial analysis visualizing the 11 nm periodicity of SSNA1 on the microtubule surface, a data set acquired on the F20 (described above) was used. The EMAN222 c2helixboxer scheme was used to extract the segments of microtubules. The box size was set to 256 pixels corresponding to 886 A with 90% overlap and 6,160 segments were extracted in total. For classification and averaging of the images, RELION2 software was used.

Data sets were collected using a Titan Artica microscope (Thermo Fisher Scientific) working at 200 kV and equipped with a Falcon3 direct detector (Thermo Fisher Scientific), at a magnification of 92,000x, corresponding to 1.6 Å per pixel and a Titan Krios microscope (Thermo Fisher Scientific) working at 300 kV and equipped with a K2 Summit direct electron detector, and controlled with SerialEM software, at a magnification of 105,000x, corresponding to 1.34 Å per pixel. The final reconstruction included in this report was carried out using the data set taken with the Titan Krios. For the data set that was included in the final reconstruction, 762 images were collected with defocus varying from −1.5 µm to −3.5 µm. The detector was operated in counting mode with a dose rate of 10.1 electrons per pixels per second. A total exposure time of 6 s, corresponding to an accumulative dose of 34.08 electrons per square ångström was fractionated into 24 video frames with 0.25 s exposure time and a dose of 1.42 electrons per square ångström for each frame. The video frames were aligned, and averaged using the UCSF Motioncor2 program.

For the 2D classification of branched microtubules, RELION2 was used. Two hundred and twenty-six branched microtubules not overlapping with other microtubules were selected from the data set recorded with the Titan Krios and boxed out with a box size of 1,000 pixels, corresponding to 1.34 Å. The branch angles were variable, causing structural heterogeneity, limiting the resolution of averages.

For image analysis leading to the 3D reconstruction of the microtubule–SSNA1 complex, quality, defocus and astigmatism of each micrograph were assessed using CTFFinder4. Out of 762 images, 478 images containing microtubules were selected for further processing. A total of 177 selected micrographs were segmented with a box size of 480 Å with 90% of overlap. As microtubules with 13 protofilaments were the majority, we chose to process 13-protofilament microtubules further. The 13-protofilament microtubules contain a seam that breaks the helical continuity of tubulin dimers, which is a building block. To circumvent this problem, a specially designed package described earlier43 was used. The method described with the description described previously was used for the determination of the seam. This reference alignment was performed using 20 Å low-pass-filtered, 2D projections of a microtubule with 13 protofilaments as a reference. The package uses a reference that was computationally synthesized using the atomic structure of tubulin decorated by kinesin. The alignment revealed the polarity and the position of the seam by following the segmented boxes that position along a single microtubule. After the determination of the seam, re-segmentation of the microtubules from the micrograph was performed using the alignment information and with the box size of 600 Å and every 80 Å as an interval. The FREELIGN package was implemented in the package for refinement with options of helical analysis. For this, the known helical parameter of a microtubule with 13 protofilaments was used (helical rise: −9.37308 Å, helical twist: 27.692 degree, helical subunit: 13). Afterwards, the method in refs44-46 was implemented for refinement as this method follows the consistency of patterns within individual microtubules, independent of the kinesin-decorated pattern, as a reference of alignment. We however observed that the SSNA1 decoration on the microtubules affected the accuracy of the particle alignment as well as the seam detection as indicated in the local resolution estimation shown in Supplementary Fig. 4. While the reconstruction of the microtubules could be further improved by a more laborious strategy, SSNA1 on the contrary cannot be better resolved because of the existing symmetry mismatch with the microtubules. The resulting reconstruction is nevertheless informative, as it was used to visualize a local fibril attached to the circumference of microtubules. The global resolution was determined to be 6.1 Å by calculating the Fourier shell correlation of two independent reconstructions. However, we note that the alpha and beta tubulins are not sufficiently separated and the higher resolutions are only effective in the core of tubulin. The reconstruction was filtered based on local resolution estimation by the ‘blocces’ scheme with a scan box size of 50 pixels.

Cryo-ET of SSNA1–microtubule complex and image processing. Ten-nanometre BSA-coated gold (Aurion) was used as a fiducial marker. A 4 µl volume of sample was mixed with 1 µl fiducial marker and then applied to glow-discharged grids (Quantifoil, Cu, R2/2). Plunge freezing immediately followed using Vitrobot (Ted Pella Inc). Tomograms were collected using cryo-ET on a Titan Krios (Thermo Scientific) operated at an acceleration voltage of 300 kV, equipped with a Gatan K2 Summit direct electron detector, with magnification of 64,000x corresponding to 2.23 Å per pixel. Images were collected in a sequential manner, starting at 0° and increasing to +59° with 1° increments. After acquiring +59°, the stage was returned to 0° and the tilt series was collected until −59° with 1° increments as well. Each tilt series was collected with the defocus value set between 3 and 7 µm. Images were acquired as videos in counting mode using a dose rate of 4.7 electrons per pixels per second. The total accumulative dose of the tilt series was 112.46 electrons per square ångström. The video frames were aligned using the UCSF Motioncor2 program.

Tomogram reconstruction was performed using the IMOD package47. Tilt series were aligned using fiducial gold markers and further binned by a factor of 4 (final pixel size of 8.92 Å per pixel). Tomograms were reconstructed by back projection and a simultaneous iterative reconstruction technique with seven iterations in IMOD.
Subtilisin treatment of microtubules and crosslinking. Taxol-stabilized microtubules (20 nM) were mixed with 7.4 mM subtilisin (Sigma Aldrich) and incubated for 0–60 min at 37 °C. The reaction was stopped by adding 2.5 mM PMSE. In 10 min, subtilisin completes the cleavage of β-tubulin E-hooks and the cleavage of α-tubulin E-hooks follows. For the crosslinking assay, 5 μM microtubules were mixed with 25 nM of SSNA1, and 1-ethyl-3-[3-dimethylaminopropyl]carbodiimide hydrochloride (EDC) (Fisher) was added to a final concentration of 5 mM. Samples were incubated at room temperature for 1 h. The densities of SDS–PAGE were measured using Fiji.

Light microscopy of in vitro SSNA1-mediated microtubule nucleation. Flow cells were assembled with cover glass and passivated coverslips as described before18. The use of the GODCAT oxygen scavenging system, common for microtubule growth observation, blocked the effects of CrSSNA1 on microtubule polymerization in our assays. Therefore, instead we used the PCA/PCD/Troleic acid oxygen scavenging system19, which contains 10 mM protocatechuate, 3.4-dioxygenase from the Pseudomonas species, 2.5 M 3,4-dihydroxybenzoic acid ‘PCA’ and 1 mM Troleic (Sigma). Total internal reflection fluorescence microscopy was performed on a DeltaVision Elite imaging system (GE Healthcare). For the formation of ‘aster’, the conditions used were: 8 μM tubulin (20% HiLyte488 Tubulin, Cytoskeleton, 50–200 nM CrSSNA1, 0–10% PEG and 2 mM GTP. Further experiments containing PEG were performed in the presence of 7.5% PEG. For the detection of the localization of CrSSNA1, after 5 min of the incubation of the mixture of the samples, an anti-SSNA1 antibody was added, and then an anti-rabbit antibody labelled with Alexa Fluor 568 (Life Technology) was added for the visualization of the antibody. We observed that PEG causes formations of concordate of SSNA1, which is detectable by 2–8% of PEG, and can modulate microtubule formation with >5% PEG, in good agreement with other proteins previously reported to nucleate microtubules20. With any of the above-mentioned conditions, it is confirmed that spontaneous formations of microtubules do not occur without SSNA1. As little as 50 nM CrSSNA1 was effective to mediate a microtubule formation in the presence of 7.5% PEG.

To mimic the nucleation event, seeds were used as a template. The seeds were formed by incubating 30 μM of tubulin with 15% of atto565-labelled tubulin in the presence of 0.5 μM GMPPCP at 37 °C for 30 min and then centrifuged at 15,800 g for 8 min to remove excess GMPPCP. Pellets were dissolved in BRBB0 buffer. Seeds (1 μM) were mixed with 15 μM tubulin containing 20% HiLyte488 tubulin, 2 mM GTP and 50% (448 out of 895 microtubules) had branch-like protrusions of microtubules. To categorize the types of branch, the snapshots of microtubules in the presence of 30 μM SSNA1 were used, and all of the microtubules (n = 895) were selected out of 89 snapshots from 3–4 independent experiments (n = 47, 21, 21) and categorized into three types—splitting—dynamic microtubules with GTP are growing out from the end of the preformed microtubules; ‘end joining’—two pre-existing microtubules are annealed through dynamic tubulin oligomers; ‘side branching’—dynamic microtubules are growing out of the wall of the pre-existing microtubules; ‘dynamic branching’—newly formed dynamic microtubules branch out; ‘indistinguishable’; and ‘no branch’—microtubules without branching.

Mouse hippocampal primary neuron cultures. Animal care and use for this study were performed in accordance with the recommendations of the European Community (2010/63/UE). Experimental procedures were specifically approved by the ethics committee of the Institut Curie CEEA-I-C no. 118 (authorization no. 04395.03 given by the National Authority) in compliance with the international guidelines. The study is compliant with all relevant ethical regulations regarding animal research.

Mouse hippocampal neurons were cultured as described previously21. Briefly, wild-type dams at 17.5 days of pregnancy were euthanized using cervical dislocation, the embryos were decapitated and their hippocampi were dissected. Hippocampi were digested with 0.25% trypsin–EDTA (Thermo Fisher, 15909046) for 20 min at 37 °C, followed by mechanical dissociation with glass pipettes. Dissociated neurons were then plated in plating medium (MEM supplemented with 10% FBS and 0.6% w/v glucose (Sigma G-8769)) on coverslips coated with poly-d-lysine (no. 354210, Corning). Four hours after plating, media were changed to Neurobasal medium (Gibco, 21103045) containing 10% FBS and 0.6% glucose (Sigma). Cells were plated with poly-d-lysine (no. 354210, Corning) and imaged on a Zeiss Axio Imager.M2 with 20x or 40x objectives. Acquired images were analysed using Fiji22. Cells with very short axons were included in the analysis so that underdeveloped neurons could be assessed as well. Note for the data set overexpressing swap-KK/EE and swap-KK/EEE, primary neurons were prepared at a different time, causing the change in general growth profiles of axons. Control (GFP transfected) was used as a standard for comparison of promotion or reduction of axon development of different mutants. Axons were defined as the longest protrusion from the soma, which were selected using the Simple Neurite Tracer plugin23. The collateral branches longer than 15 μm were defined as major branches. The total number of collateral branches and the total length of all the branches for each axon was determined by tracing of the neuron morphology. In each image, the position of the cell bodies was marked by the nucleus: after applying the mask and subtracting the background, the image was thresholded using Otsu’s method. In the overexpression experiments, the neurons were screened for transduction efficiency, as measured by EGFP expression. The branch network was obtained by segmenting and combining the intensity in the Tau1 and MAP2 fluorescence images: for each channel, the neurites were highlighted by mapping the curvature of the neurites (Compute Curvature, ImageJ) and thresholding the neurites using Otsu’s method. The resulting binary mask was then skeletonized to outline all of the neurites detected. By overlapping the traced axons with this image, only the neurites branching from the axons were kept and measured. The branching complexity of each neuron was summarized with the Strahler number24 (Strahler Analysis plugin), using the location of its corresponding cell body to mark the root branch, which is the start point of the axon.

Immunostaining for DNA-PAINT. DNA-labelled antibodies were prepared as previously reported25. In brief, 300 μl of 1 mg ml−1 secondary donkey anti-rat antibody (Jackson ImmunoResearch, 711-005-152) was reacted with 10x mol excess maleimide–PEG2-succinimidyl ester crosslinker (Sigma Aldrich, 746223), and then 10x mol excess of DNA was added to the antibody–crosslinker. Final usage concentration was 10 μg ml−1.

Mouse embryonic fibroblast cells were transfected with pTRIP_2A_EGFP vector using Lipofectamine 2000 (Thermo Fisher Scientific). Cells were fixed and stained as described previously26, and then were incubated at 4 °C overnight with primary rat α-tubulin (YLI1/2) antibody. Antibody information is provided in Supplementary Table 2. DNA-labelled secondary antibody (10 μg ml−1) was added and incubated for 1 h. Samples were then incubated for 5 min with 90-nm gold particles (Cytodiagnostics, G-90-100) at a 1:10 ratio in PBS, and then residual gold was washed away. Cells were kept at 4 °C until they were used for imaging within 48 h.

DNA-PAINT. Fluorescence imaging was carried out on an inverted Nikon Eclipse Ti microscope (Nikon Instruments) with the Perfect Focus System, applying an objective-type total internal reflection fluorescence (TIRF) configuration with an oil-immersion objective (Apo SR TIRF 100x, NA 1.49, oil). Two lasers were used for excitation: 488 nm (200 mW, Coherent Sapphire) or 488 nm (200 mW, Topica iBeam smart). The laser beam was passed through a cleanup filter (ZET488/10× or ZET561/10×, Chroma Technology) and coupled into the microscope objective using a beamsplitter (ZET488rdc or ZET561rdc, Chroma Technology). Fluorescent light was spectrally filtered with two emission filters (ET525/50 m and ET500p for excitation and ET577/50 m and ET575/50 for Alexa Fluor 647, Chroma Technology) and imaged on a sCMOS camera (Andor Zyla 4.2) without further magnification, resulting in an effective pixel size of 130 nm after 2× binning.

The camera readout sensitivity was set to 16-bit, and the readout bandwidth was set to 200 MHz.

Transfected cells were screened using 488 nm laser excitation at 0.01 kW cm−2. The laser power was switched to 561 nm, the focal plane and TIRF angle were readjusted and imaging was subsequently performed using ~1.5 kW cm−2 for 561 nm laser excitation. The imager strand concentration varied dependent on the
measurement from 2 nM to 5 nM Cy3b-P1 and was adjusted to minimize double-binding events. Imaging was performed in 1 x PCA (Sigma-Aldrich, 37580-2SG-F/1 x PCD (Sigma-Aldrich, F8279-25UN)/1 x PRO (Sigma-Aldrich, 238813-1G) in Buffer C (PBS + 500 mM NaCl) and imaged for 20,000–40,000 frames at 200 ms exposure time. 3D imaging was performed using a cylindrical lens in the detection path as previously reported35.

**Super-resolution data analysis.** Raw data videos were reconstructed with the Picasso software36. Drift correction was performed with a redundant cross-correlation and/or gold particles as fiducials. Using Picasso, the localization information was converted to an image volume with isotropic pixel sampling of 10 nm. The volumes were denoised by applying a Gaussian filter with a standard deviation of 0.3 nm. The topology of the microtubules was derived using stitching open active contour modelling, as implemented in the SOAX software37. Three independent SSNA1-overexpressing fibroblasts and control cells were assessed each, containing the total tube lengths (that is, microtubule lengths) of 7.70 μm, 8.50 μm and 7.70 μm for control cells and 5.70 μm, 7.90 μm and 1.90 μm for SSNA1-overexpressing cells. In each cell, the occurrences of the three-way intersections were counted to be 0.96, 0.78 and 1.2 per 100 μm for control cells and 1.6, 3.2 and 4.2 per 100 μm for SSNA1-overexpressing cells. As the expression level of SSNA1 varies between individual cells, the transfected cells were selected on the basis of the signal of GFP, which was co-expressed with SSNA1. Three independent cells containing the strongest signals out of >500 cells have been selected.

**Statistics and reproducibility.** All microtubule nucleation assays and TIRF-based assays were performed independently at least three times unless otherwise stated. Similar results were observed in all of the replicates performed. Primary neuron preparation was performed from three independent mice.

The χ² two-sample test was performed to determine the significance of differences between two data sets. The Kruskal–Wallis test, followed by Dunn’s multiple comparison post-hoc test, was performed to test the significance across multiple independent samples. Reproducibility was confirmed.

**Reporting Summary.** Further information on experimental design is available in the Nature Research Reporting Summary linked to this article.

**Code availability.** Morphology analysis of the neurons (total number of collateral branches and total length of all the branches for each axon) was performed using Fiji, with the help of scripts written ad hoc for the task. All scripts are available from the corresponding author upon request.

**Data availability**

The cryo-EM structure of the SSNA1–microtubule is available through EMDB (https://www.ebi.ac.uk/pdbe/entry/pdb/EMDB_38780). The additional tomography images are available in Figs. 1, 4, 6 and Supplementary Fig. 6 have been provided as Supplementary Table 3. Other data supporting the findings of this study such as the cryo-tomography data are available from the corresponding author on reasonable request.

**References**

37. Shelanski, M. L., Gaskin, F. & Cantor, C. R. Microtubule assembly in the absence of added nucleotides. Proc. Natl Acad. Sci. USA 70, 765–768 (1973).
38. Chrétién, D., Kenney, J. M., Fuller, S. D. & Wade, R. H. Determination of microtubule polarity by cryo-electron microscopy. Structure 4, 1031–1040 (1996).
39. Tang, G. et al. EMAN2: an extensible image processing suite for electron microscopy. J. Struct. Biol. 157, 38–46 (2007).
40. Scheres, S. H. W. RELION: implementation of a Bayesian approach to cryo-EM structure determination. J. Struct. Biol. 180, 519–530 (2012).
41. Li, X. et al. Electron counting and beam-induced motion correction in near-atomic-resolution single-particle cryo-EM. Nat. Methods 10, 584–590 (2013).
42. Rohou, A. & Grigorieff, N. CTFFIND4: Fast and accurate defocus estimation from electron micrographs. J. Struct. Biol. 192, 216–221 (2015).
43. Shang, Z. et al. High-resolution structures of kinesin on microtubules provide a basis for nucleotide-gated force-generation. eLife 3, e04686 (2014).
44. Zhang, R. & Nogales, E. A new protocol to accurately determine microtubule lattice seam location. J. Struct. Biol. 192, 245–254 (2015).
45. Zhang, R., Alushin, G. M., Brown, A. & Nogales, E. Mechanistic origin of microtubule dynamic instability and its modulation by EB proteins. Cell 162, 849–859 (2015).
46. Alushin, G. M. et al. High-resolution microtubule structures reveal the structural transitions in α-tubulin upon GTP hydrolysis. Cell 157, 1117–1129 (2014).
47. Grigorieff, N. Frealign: an exploratory tool for single-particle cryo-EM. Methods Enzymol. 579, 191–226 (2016).
48. Cardone, G., Heymann, J. B. & Steven, A. C. One number does not fit all: mapping local variations in resolution in cryo-EM reconstructions. J. Struct. Biol. 184, 226–236 (2013).
49. Kremer, J. K., Mastronarde, D. N. & McIntosh, J. R. Computer visualization of three-dimensional image data using IMOD. J. Struct. Biol. 116, 71–76 (1996).
50. Crevenna, A. H. et al. Side-binding proteins modulate actin filament dynamics. eLife 4, e04599 (2015).
51. Wieczorek, M., Rechstedt, S., Chaaban, S. & Brouhard, G. J. Microtubule-associated proteins control the kinetics of microtubule nucleation. Nat. Cell Biol. 17, 907–916 (2015).
52. Kaech, S. & Banker, G. Culturing hippocampal neurons. Nat. Protoc. 1, 2406–2415 (2006).
53. Lahaye, X. et al. The capsids of HIV-1 and HIV-2 determine immune detection of the viral cDNA by the innate sensor cGAS in dendritic cells. Nat. Immunol. 14, 1132–1142 (2013).
54. Magiera, M. M. & Janke, C. Investigating tubulin posttranslational modifications with specific antibodies. Methods Cell Biol. 115, 247–267 (2013).
55. Schindelin, J. et al. Fiji: an open-source platform for biological-image analysis. Nat. Methods 9, 676–682 (2012).
56. Longair, M. H., Baker, D. A. & Armstrong, J. D. Simple Neurite Tracer: open source software for reconstruction, visualization and analysis of neuronal processes. Bioinformatics 27, 2453–2454 (2011).
57. Hollingworth, T. & Berry, M. Network analysis of dendritic fields of pyramidal cells in neocortex and Purkinje cells in the cerebellum of the rat. Phil. Trans. R. Soc. Lond. B 270, 227–264 (1975).
58. Vormberg, A., Effenberger, F., Muellerleile, J. & Cunz, H. Universal features of dendrites through centripetal branch ordering. PLoS Comput. Biol. 13, e1005615 (2017).
59. Schnitzbauer, J., Straus, M. T., Schlichte, A., Schieder, F. & Jungmann, R. Super-resolution microscopy with DNA-PAINT. Nat. Protoc. 12, 1198–1228 (2017).
60. Huang, B., Wang, W., Bates, M. & Zhuang, X. Three-dimensional super-resolution imaging by stochastic optical reconstruction microscopy. Science 319, 810–813 (2008).
61. Xu, T. et al. SOAX: a software for quantification of 3D biopolymer networks. Sci. Rep. 5, 9081 (2015).
Reporting Summary

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Our web collection on statistics for biologists may be useful.

Software and code

Policy information about availability of computer code

Data collection

- For EM data collection - EPU (from FEI)
- For Cryo-Tomography, Data was collected using Serial-EM (http://bio3d.colorado.edu/SerialEM/)
- For Immunofluorescence, Cells were imaged on a Zeiss Axio Imager.M2 microscope with 20x or 40x objectives using the Zeiss Zen software.
- For DNA-PAINT imaging was carried out on an inverted Nikon Eclipse Ti microscope (Nikon Instruments) with the Perfect Focus System, applying an objective-type TIRF configuration with an oil-immersion objective (Apo SR TIRF 100x, NA 1.49, Oil).

Data analysis

- For light microscopy - Zeiss Zen and Fiji V 1.0 (https://fiji.sc/) was used.
- For DNA-PAINT in addition Picasso software suite (https://github.com/jungmannlab/picasso) and the SOAX software (https://www.nature.com/articles/srep09081) was used.
- For electron microscopy - SPIDER (https://spider.wadsworth.org/), RELION (https://www2.mrc-lmb.cam.ac.uk/relion/), Frealign(grigoriefflab.janelia.org/frealign), customized software for microtubule analysis (PMID: 26424086) and for tomographic reconstruction IMOD (bio3d.colorado.edu/imod/) was used.
- Prism 7 and Microsoft Excel 15.35 were used for statistical analysis and graphical data presentation (https://www.graphpad.com/).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors/reviewers upon request. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Research guidelines for submitting code & software for further information.
Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:
- Accession codes, unique identifiers, or web links for publicly available datasets
- A list of figures that have associated raw data
- A description of any restrictions on data availability

Cryo-EM structure of the protein complex is available through EMDB (or PDB) with the accession code: EMD-4188.
Additional tomographic images could be found in figshare.

Field-specific reporting

Please select the best fit for your research. If you are not sure, read the appropriate sections before making your selection.

- [ ] Life sciences
- [ ] Behavioural & social sciences
- [ ] Ecological, evolutionary & environmental sciences

For a reference copy of the document with all sections, see nature.com/authors/policies/ReportingSummary-flat.pdf

Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size

For neuron experiments, primary neuron preps were performed from independent 3 mice and the neurons were grown on independent glass slides. The sample sizes were determined by counting ALL neurons on the entire slides.
For TIRF analysis, triplicate experiments were performed for each conditions, and microtubules and asters (objects of interest) were counted from the entire view of the CCD camera.
For cryo-EM analysis, sample sizes were determined by the number of the images acquired by given available time slots. Typically it would be an over-night session.
For microtubule morphological analysis, sample sizes were determined by the number of images recorded. Typically > 50 images are recorded. In each image, ~10 microtubules were observed.

Data exclusions

There is no data excluded.

Replication

All attempts of replication of the biological experiments were successful. For quantification triplicates datasets (biologically and technical replicates) are collected.

Randomization

Randomization was not performed as all the data were included in the large data analysis.

Blinding

Neuron analysis were done both automatically as well as manually. For the manual analysis, investigators were blinded.

Reporting for specific materials, systems and methods

| Materials & experimental systems | Methods |
|---------------------------------|---------|
| n/a                             | n/a     |
| □ Unique biological materials  | □ ChiP-seq |
| □ Antibodies                   | □ Flow cytometry |
| □ Eukaryotic cell lines        | □ MRI-based neuroimaging |
| □ Palaeontology                |         |
| □ Animals and other organisms  |         |
| □ Human research participants  |         |

Antibodies

Antibodies used

The information about dilution and manufacturer are mention in Supplementary Table 2

Validation

Validations are based on the datasheet from the manufacturer.
Eukaryotic cell lines

Policy information about cell lines

Cell line source(s)  
Mouse embryonic fibroblasts as described in Austen et al (DOI: 10.1038/ncb3268).

Authentication  
No further authentication.

Mycoplasma contamination  
Original cells are not mycoplasma contaminated.

Commonly misidentified lines  
Cells are not listed in the database of commonly misidentified cell lines.

Animals and other organisms

Policy information about studies involving animals. ARRIVE guidelines recommended for reporting animal research

Laboratory animals  
17.5 days embryo from C57Bl6/N mouse strain was used for the preparation of hippocampus neuron.

Wild animals  
This study did not involve any wild animals.

Field-collected samples  
This study did not involve any field-collected samples.