Centrosomin: a Principal Effector of Abrupt that Represses Dendrite Branching by Orienting Microtubule Nucleation

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ABSTRACT

Neuronal dendrite branching is fundamental for building nervous systems. Branch formation is genetically encoded by transcriptional programs to create dendrite arbor morphological diversity for complex neuronal functions. In *Drosophila* sensory neurons the transcription factor Abrupt represses branching via an unknown effector pathway. Targeted screening for branching-control effectors identified Centrosomin, the primary centrosome-associated protein for mitotic spindle maturation. Centrosomin repressed dendrite branch formation, and was utilized by Abrupt to simplify arbor branching. Live imaging showed Centrosomin localized to the Golgi cis face, and that it recruited microtubule nucleation to Golgi outposts for net retrograde microtubule polymerization away from nascent dendrite branches. Removal of Cnn enabled the engagement of wee Augmin activity to promote anterograde microtubule growth into nascent branches leading to increased branching. The findings reveal that polarized targeting of Cnn to Golgi outposts during elaboration of the dendrite arbor creates a local system for guiding microtubule polymerization.

Neurons primarily receive inputs through their dendrite arbors. The shape and complexity of the dendrite arbor, which is elaborated during differentiation, defines the neuron's function. It enables the neuron to properly cover its receptive field and establishes the positions of inputs into the arbor. Disruptions in dendritic branching can precipitate intellectual disability and psychiatric disorders[1,2].

Arbor morphology is regulated for each neuron class to support its structural and functional requirements[3]. Arbor morphology is genetically encoded, linked to class-specification by transcriptional programs. For example, in *Drosophila* the single unbranched dendrite of external sensory neurons is specified over an alternative multipolar dendritic arborization (da) neuron fate by the Prdm transcription factor Hamlet[4]. Similarly, the proneural transcription factor Ngn2 regulates multiple aspects of pyramidal neuron development in the mammalian cortex including the specification of a characteristic apical dendrite[5], and Cux1, Cux2, and SatB2 link dendrite development to cortical layer-specific developmental programs[6-9].

Dendrite development is controlled in a neuron class-specific manner to create differences in arbor morphology and complexity. Class-specific dendrite targeting is regulated via the activity of transmembrane adhesion proteins. For example, in *C. elegans,* class-specific expression patterns of the transcription factors MEC-3, AHR-1, and ZAG-1 regulate the morphology of mechanosensory neurons, and MEC-3 promotes differential expression of the Claudin-like membrane protein HPO-30 to enable lateral branch stabilization[10]. *Drosophila* da neurons exist in four classes, of which class I neurons express Abrupt (Ab) that defines their simple arbor shape; and class IV express Knot and Cut that together promote the complex morphology of this class[11-16]. The EGF-repeat factor Ten-m is co-regulated by both Knot and Ab to control the direction of branch outgrowth in both class I and IV neurons[17].

Contrasting activities of Knot, Cut, and Ab in da neurons emphasize that altering dendrite branching is fundamental to regulate arbor complexity. Knot and Cut promote branch formation[12-14]. Conversely, Ab represses branch formation[15,16]. We understand little about how modulatory control over branching is achieved.

Microtubules polymerize via the addition of Tubulin dimers, primarily at the plus end. In axons microtubules polymerize in an anterograde direction providing a protrusive force for outgrowth. Microtubule polymerization also drives axon branch formation as precursors only transform into branches after microtubule invasion[18]. Mature dendrites have a predominantly minus-ends-out microtubule array[18], nevertheless recent studies identified that anterograde microtubule polymerization events in mature neurons can initiate or extend branches or modulate the size of dendritic spines[19,20]. In addition, the re-initiation of growth and branch formation following injury uses upregulation of microtubule polymerization and polarization in the anterograde direction[21].

Here we examined if class-specific transcription factors regulate branch promotion and repression by controlling microtubule organization during arbor development. In da sensory neurons, microtubule nucleation and polarity can be assayed *in vivo* using transgenic markers. Through the genetic manipulation of class I and class IV da neurons we found that Ab controls class-specific differences in the localization of microtubule minus-end-directed markers in the da neuron arbor. By assaying Ab-mediated changes in the expression of a set of candidate microtubule regulators and through ChIP we identified Cnn (Centrosomin) as

an effector of Ab action. We show that Cnn-centered control mechanisms, analogous to those that cluster microtubule nucleation events to create the mitotic spindle, are used in growing dendrites to regulate branching and to create class-specific arbor complexity.

RESULTS

Neuron class-specific arrangements of the microtubule array in dendrites

If class-specific transcription factors control branching through microtubule organization, class-specific differences may exist in the microtubule arrays of simple (class I) and highly-branched (class IV) arbors (**Fig. 1a**).

To investigate this potential unexplored feature of neuron diversification, we used the Khc::nod::lacZ transgenic reporter in which β-*galactosidase* is linked to a chimeric microtubule minus-end-directed motor. Khc::nod::lacZ concentrates at sites with a high density of microtubule minus ends within the cell to reveal the overall organization of the stable microtubule array[22,23]. This analysis revealed class-specific differences in both the length and the position of Khc::nod::lacZ labelling. In class IV neurons continuous stretches of Khc::nod::lacZ made up the majority of the label (**Fig. 1b-f; Supplementary Fig. 1**); while in class I neurons Khc::nod::lacZ label was predominantly in short foci of <3µm, and arbor coverage by continuous stretches of label (>9µm) was rare (**Fig. 1f-k, Supplementary Fig. 1**). Class I neurons also showed a higher frequency of label in dendritic tips as compared to class IV (**Fig. 1g, Supplementary Fig. 1**).

To test if the class I-specific transcription factor Ab controls the differences in Khc::nod::lacZ localization, we ectopically expressed *ab* in class IV neurons and examined *ab* mutant class I neurons. Ectopic expression of *ab* in class IV neurons reduced coverage of the arbor with continuous stretches of label (>9µm), increased coverage by short foci of <3µm, and targeted label to the dendritic tips (**Fig 1. d-g, Supplementary Fig. 1**). Conversely loss of *ab* lead to the formation of continuous stretches of label (>9µm), (along with an increased frequency of all label including short foci of <3µm), and it also caused a reduction of label at tips (**Fig. 1j-m; Supplementary Fig. 1**)

Lis1 enhances Dynein-mediated minus end-directed transport along microtubules, and previous characterization of tagged Lis1 revealed that this marker is transported towards microtubule minus ends during mitosis and meiosis[24,25]. We found that dendritic localization of tagged Lis1[24,25] also differed between class I and IV neurons, and was regulated by *ab*. In class IV neurons, GFP::Lis1 labelled long stretches of dendrite in the proximal arbor; this labelling was suppressed by ectopic *ab* expression. Conversely Lis1 label was limited to rare foci in the dendrites of class I, the coverage of which was increased in *ab* mutant neurons (**Supplementary Fig. 2**).

Taken together, the observed differential localizations of these microtubule-associated markers suggest alterations in the dendrite arbor microtubule cytoskeleton properties between these da neurons with simple and complex arbor morphologies. Pointedly, the class I specific transcription factor Ab is regulating such differences.

Cnn restricts dendrite branching and acts as an effector of Ab

We reasoned that microtubule-organizer protein activity is part of the Ab transcriptional-effector pathway in class I neurons. We took a two-step approach to identify this activity. First we assayed for microtubule-organizer gene regulation by Ab. Next we used genetic loss- and gain-of-function and genetic interaction analyses to place this effector in the pathway downstream of Ab.

We expressed *ab* ectopically in all da neuron classes, isolated the neurons, and assayed for changes in the gene expression of 42 candidate microtubule-organizers. Of these, the centrosome-associated protein *centrosomin* (*cnn*) showed the highest fold change (4.1 fold, P = 0.0026, **Fig. 2a**). To confirm Cnn as a candidate for further testing, we carried out anti-Ab ChIP-seq on *Drosophila* embryos and found Ab protein bound at the *cnn* locus (**Fig. 2b**). In addition, *cnn* levels were reduced in da neurons isolated from *ab* mutant embryos (**Supplementary Fig. 3**). Taken together, these data suggest Cnn as an effector of Ab, and we examined Cnn function in dendrite branching control.

cnn mutant class I neurons had increased dendrite branching (similar to that previously reported for ab[15,16]) (**Fig. 2c-f**). This was apparent at embryonic stage 17 (st17) (41%, P < 0.0001, **Fig. 2f**) and *cnn* mutants continued to add extra branches during the larval stage (56.3% at 3rd instar P < 0.0001, **Fig. 2g**). In st17 neurons examination of the distribution of each branch order by the Strahler method showed a trend for increased branches at multiple branch orders in the *cnn* mutants as opposed to WT (**Supplementary Fig. 4**). Use of Sholl analysis to examine the distribution of arbor complexity also revealed increased densities of dendrites at medial and distal positions of the dendritic field (**Supplementary Fig. 4**). These findings suggest that loss of *cnn* leads to increases in branch formation at iterative stages of arbor outgrowth.

We next tested if Cnn is part of the effector pathway downstream of Ab in these neurons. Removal of one copy of *cnn* in a hypomorphic *ab* background (ab^{1}/ab^{k02807})[16] caused a strong increase in branch number (*ab-cnn* interaction P = 0.0009, **Fig. 2h**). *cnn* overexpression had no effect in WT class I neurons; however, overexpression of *cnn* in *ab* mutant class I neurons repressed branch number (*ab-cnn* interaction P = 0.0039, **Fig. 2i**).

In addition to class I, we also observed a role of Cnn for suppressing branching in class IV neurons, and we found differences in Cnn utilization between the two classes. *cnn* mutant class IV neurons showed an increase in branch number at st17 (23%, P = 0.0007, **Fig. 2j, Supplementary Fig. 4**). However, in contrast to class I neurons, *cnn* mutant class IV neurons recovered their normal branch tip number by the 3rd instar stage (**Fig. 2l**). Overexpression of *cnn* also demonstrated a difference between class I and IV, as *cnn* overexpression repressed branch number in class IV neurons (34.2%, P < 0.0001, **Fig. 2k**). Finally, we used class IV neurons to confirm Cnn as an Ab effector. Ectopic Ab expression suppresses branch number in class IV neurons[15,16]; yet this phenotype was rescued by simultaneously removing *cnn* (*ab-cnn* interaction: P = 0.0048, **Fig. 2l**).

Our analysis also revealed that Cnn shapes the dendrite arbor in other neuron types. The vertical system 1 (VS1) interneurons of the *Drosophila* adult visual system have a curved primary dendrite shaft from which secondary branches radiate (**Supplementary Fig. 5**)[26], and in *cnn* mutants the frequency of these secondary branches was increased by 51% (P < 0.0001, **Fig. 2m**, **Supplementary Fig. 5**).

Taken together these data reveal Cnn as a new factor active in multiple neuron types to repress dendrite branching. Moreover, they show that where dendrite arbor complexity is inhibited by the transcription factor Ab, Cnn is a principal effector of this program.

Cnn organizes the spatial distribution of dendrite microtubule nucleation events

To address how Cnn represses dendrite branch formation, we first examined its relationship with previously described dendrite branch suppression factors. Dendrite branch-suppression pathways commonly converge to activate the small GTPase RhoA; in addition the microtubule binding factor Map1B (*Drosophila* - Futsch) can also suppresses branching[2,27]. Repression of RhoA with a dominant negative transgene caused reduction of the high levels of F-actin that usually accumulate at the tips of the growing class I dendrites (**Supplementary Fig. 6**) and an 18.0% increase in branch number compared to wildtype (**Fig. 3a**; $P < 0.001$). Similarly, loss of *futsch* caused a 24% increase branching (**Fig. 3b**, $P = 0.0017$). Nevertheless, we were unable to detect genetic interactions between *cnn* and either of these factors suggesting that Cnn-mediated branch suppression is through an alternative mechanism (**Fig. 3a, Supplementary Fig. 7**). To identify components of this new mechanism, we noted that during mitosis Cnn is the most abundant component of the pericentriolar material (PCM) where it acts in conjunction with Plp (Pericentrin like protein) [28-30]. Analysis of *plp* mutants showed a 26.7% increase in dendrite branch number ($P < 0.0001$, **Fig. 3c**), and furthermore that both *cnn* and *ab* showed strong synergistic genetic interactions with *plp* to suppress branch formation ($P = 0.0041$ and 0.0088, respectively, **Fig. 3d,e**). These data reveal that Cnn and Plp act together to suppress branching, and function in the Ab-mediated branch suppression program.

Plp incorporates Cnn, and recruits microtubule nucleation machinery to the PCM [29]. In addition, Plp was recently found to be involved in acentrosomal microtubule nucleation in dendrites[20]. We therefore tested if Cnn organizes microtubule nucleation events within the dendrite. To do this, we utilized EB1::GFP. EB1::GFP forms comets on the plus end of a polymerizing microtubule and the appearance of a comet indicates a site of microtubule nucleation, either *de novo* or as catastrophe rescue. In the primary dendrite of *cnn* mutants at st14 we found a 203% increase in the total number of microtubule nucleation events ($P = 0.0138$, **Fig. 3f-h**). Furthermore, multiple comets appeared in succession from a single point (**Fig. 3f**). Such repeated formation of comets at single positions indicates the presence of a focal *de novo* microtubule nucleation center[20,31]. However in *cnn* mutants the positions of individual microtubule nucleation events were spread along the length of the dendrite (231% increase in individual sites, $P = 1.38E^{-4}$, **Fig. 3i**). These data show that Cnn organizes the spatial arrangement of the nucleation sites for dendrite microtubules, and suggest it concentrates the nucleation events.

Cnn localizes to one side of ManII-positive Golgi outposts

Cnn anchors microtubule nucleation events to the centrosome in order to create the polarized microtubule array of the mitotic spindle[30,32]. We therefore investigated if Cnn is re-utilized in post-mitotic neurons in order to localize microtubule nucleation by an analogous anchoring process. The mammalian Cnn paralog CDK5RAP2 localizes to somatic Golgi during interphase in non-neuronal cells[33]. Moreover, in addition

to their function in dendrite secretory trafficking[34,35], Golgi outposts were recently identified as a site of acentrosomal microtubule nucleation in dendrites[20]. In the neuron cell body, the Golgi apparatus has an ordered and compartmental structure. However in dendrites *medial* and *trans* Golgi compartments are often disconnected creating 'single-compartment' outposts, a finding resolving recent controversies over the differential localization of Golgi markers in dendrites[31].

We first examined Cnn association with the ordered somatic Golgi in the da neuron cell body. GFP::Cnn reproduces Cnn localization[28] and rescues *cnn* loss of function phenotypes [28](**Supplementary Fig. 8**). GFP::Cnn localized immediately adjacent to the *medial* cisternae marker ManII::Ch2, and further from the *trans* Golgi marker RFP::GalT ($P < 0.05$, **Fig. 4a-i**). GFP::Cnn also showed a stronger spatial correlation with ManII than with GalT ($P < 0.0001$, **Fig. 4a-h,j**). Localization of Cnn with the ManII-positive Golgi surface was less stable in *plp* mutants. Remodeling of Cnn distribution on the Golgi surface created transient foci (**Supplementary Fig. 9**), the frequency of which was increased in *plp* mutants ($P < 0.0001$, **Fig. 4k**). Overall, we found a total of 57.9% of ManII-labelled Golgi co-localized with Cnn (n = 114), and live imaging revealed that these Cnn positive and negative populations behaved differently. 90.4% of the Golgi that did not co-localize with Cnn moved during imaging (348s). On the other hand, 100% of the Golgi where ManII::Ch2 and GFP::Cnn co-localized remained stable during this period, which is significant because non-motile Golgi are the population that can act a sites of microtubule nucleation [20].

Next we examined Cnn localization in the dendrites. GFP::Cnn localized as foci along dendrite branches, at branch points, and at tips (**Fig. 5a-d**), and there was a 33.7% increase in the clustering of Cnn foci in *plp* mutants indicating that proper dendritic localization of Cnn within dendrites depends on Plp ($P = 0.010$, **Fig. 5e, Supplementary Fig. 10**). Upon examining outposts in the dendrites, we found that in class I neurons the outposts co-localized with the Khc::nod::LacZ foci characteristic of this class (**Supplementary Fig. 11**). Furthermore, we found that while complex da neuron classes have been shown to contain a mix of single and multicomponent outposts [31], surprisingly predominantly only ManII-positive outposts were detected in the simple arbors of class I neurons (**Supplementary Fig. 12**). GalT-positive outposts were found rarely and only in the proximal dendrite segment. These data suggest that outposts exist primarily in the single (*medial*) compartment form in the simple arbor of the class I neurons (**Supplementary Fig. 12**). 44.5% of these ManII::Ch2-positive outposts co-localized with GFP::Cnn (n= 119) (and 50.3% of GFP::Cnn foci co-localized with the ManII-positive Golgi outposts (n=177)) (**Fig. 5f**). At the outposts the Cnn signal was distributed to only one side, with 92.6% of outposts showing asymmetric Cnn localization (**Fig. 5f**). While multi-compartment Golgi are necessarily polar (Fig 4, [31]), this Cnn localization highlights that outposts also have intrinsic polarity when in the ManII-positive single-compartment form.

Taken together, these data indicate that Cnn is polarized towards the *cis* face of Golgi, and that it associates asymmetrically with outposts in dendrites.

Cnn recruits microtubule nucleation events to Golgi outposts

The co-localization of Cnn with Golgi outposts led us to hypothesize that, in analogy with the role of Cnn in the PCM, the Ab-Cnn pathway might recruit microtubule nucleation events to the outposts to establish a local platform from which a polarized microtubule array extends.

To test this, we examined microtubule nucleation events where two or more EB1::GFP comets were initiated in succession at the same position. Successive comets that nucleated at a ManII-positive Golgi outpost travelled in the same direction. In contrast, comets continuously nucleated at a position where no outpost was present often travelled in opposite directions ($P < 0.0001$, **Fig. 6a-c**). These results confirm that single-compartment Golgi outposts act as a local system for guiding microtubule polymerization during arbor development.

Next we asked if Cnn and Ab are involved in recruiting microtubule nucleation events to the outposts in the terminal dendrite branches (termini). In both *cnn* and *ab* mutants microtubule nucleation events became uncoupled from the outposts. In WT neurons 18.8% of EB1::GFP comets were initiated at outposts. In contrast, in *cnn* mutants this dropped to 6.8%, ($P = 5.46e^{-5}$) and to 4.2% in *ab* mutants ($P = 9.43e^{-3}$) (**Fig. 6d**). Importantly, neither loss of *cnn* nor *ab* caused a reduction in the absolute level of microtubule nucleation in these nascent branches, demonstrating that these factors are required for localizing but not initiating microtubule growth (**Fig. 6e**). In summary, by recruiting microtubule nucleation events to Golgi outposts, the mechanism of Cnn action is to promote the concentration and coupling of microtubule nucleation to a local and polarized source (**Supplementary Fig. 13**).

Cnn counteracts wee Augmin to regulate arbor complexity through control over microtubule polymerization-based extension of dendrite termini

Anterograde polymerization of microtubules in dendrite termini promotes arbor outgrowth and branching[20] (**Fig. 7a**, **Movie 1**) leading us to hypothesize that this dynamic population of polymerizing microtubules in nascent dendrite termini is suppressed by the Ab-Cnn pathway. To test this, we followed the direction of EB1::GFP comet movement (retrograde or anterograde) in the termini. In WT neurons 43.8% of microtubule polymerization events were in the anterograde direction. In *cnn* and *ab* mutants this increased to 56.9% or 58.4%, respectively ($P = 0.0072$, $P = 0.0034$, **Fig. 7b**). To confirm that Ab interacts with Cnn to suppresses these events, we next examined neurons where *cnn* expression levels were reduced in a hypomorphic *ab* background; in these neurons microtubule polymerization in the anterograde direction also increased to 64.8 % ($P = 0.011$, **Fig. 7c**).

The total number of anterograde microtubule polymerization events in the dendrite termini correlates strongly with those microtubule polymerization events in which the formation of a nascent terminal branch or the extension of an existing termini was observed (Pearson $r = 0.90$, $P = 0.0002$, **Fig. 7b-d**). This suggests that Ab and Cnn can repress branch formation by suppress microtubule polymerization events that are associated with branch initiation or extension (**Fig. 7a**). Analysis of EB1::GFP comets in the termini supported this hypothesis. In WT, 8.5% of microtubule polymerization events were associated with the formation of a nascent terminal branch or the extension of existing termini. This increased to 21.0% and 17.8%

in *cnn* and *ab* mutants, respectively (P = 0.0004, P = 0.0068, **Fig. 7b**), and to 19.0% when *cnn* levels were reduced in a hypomorphic *ab* background (P = 0.0339, **Fig. 7c**).

These data (**Fig. 7b-d**) indicate that decoupling microtubule nucleation at Golgi outposts results in a specific bias toward anterograde directional microtubule outgrowth in the dendrite termini. In wildtype dendrite termini approximately 80% of microtubules do not originate from Golgi outposts and in the *cnn* mutant this increases to approximately 95% (**Fig. 6d**). This relationship suggests that the mechanism through which dendrite branching is suppressed by Cnn involves Cnn counteracting the action of an additional factor able to promote anterograde polymerization among those microtubule nucleation events decoupled from the Golgi outposts.

To identify such a factor, we again took a candidate-based approach. Considering the Cnn interaction network shaping the microtubule array of the mitotic spindle, we noted that Augmin-mediated microtubule nucleation during spindle formation occurs away from the centrosome; it utilizes the microtubule network of the spindle as the nucleation site. A balance between these different microtubule nucleation pathways usually provides robustness to protect against disruption of the essential process of spindle formation. Notably, under normal circumstances the PCM pathway for microtubule nucleation dominates[36,37], and as such the activity of Augmin was identified only when loss of *cnn* revealed the presence of a second nucleation pathway[36].

Analysis of *wee Augmin* (*wac*) mutants revealed no effect on the direction of microtubule polymerization in termini, while loss of *cnn* increased the anterograde events. Strikingly however, the increase in anterograde microtubule growth that occurred in *cnn* mutants was suppressed by concomitantly removing *wac* (**Fig. 7d**). These data reveal that during the normal process of dendrite outgrowth Cnn action prevents Wac from promoting microtubule nucleation with an anterograde bias in the dendrite termini.

Why do swings of approximately 15% in the anterograde to retrograde microtubule polymerization balance and in the balance of Golgi-based to non-Golgi-based microtubule nucleation give rise to a much greater (40-50%) expansion in arbor complexity? The reason is compounding of increases at multiple stages of branching in turn lead to the much bigger total increase in dendrite ends in the final arbor. This can be illustrated using a numerical simulation on a simple model of class I neuron dendrite arbor development up to stage 17 (**Supplementary Fig. 14**). In the mutant case, when the initial number of branches arising from the soma is 15% higher, and subsequently all branching probabilities are also increased by 15%, then the average number of end points in the arbor increases from 20 in wildtype to 28 in a mutant case (**Supplementary Fig. 14**).

Taken together the above data support a mechanism in which the formation of a complex arbor is repressed by Cnn via the suppression of anterograde microtubule polymerization in termini. This mechanism predicts that preventing the increased anterograde microtubule polymerization that occurs in *cnn* mutants will suppress the increase in arbor complexity. To test this, we utilized the fact that Wac is required for the amplification of anterograde microtubule polymerization that occurs in *cnn* mutants. As predicted, in *wac* mutants alone no reduction in dendrite branching compared to wildtype was observed. On the other hand, the extra dendrite branching phenotype of *cnn* mutants was fully suppressed when *wac* was simultaneously removed (*cnn*-*wac* interaction P < 0.0001, **Fig. 7e**).

DISCUSSION

Nervous systems assemble from a wide variety of neuron classes, each of which has characteristic morphological complexity and physiological features. Transcription factor networks encode neuron diversity; nevertheless how transcriptional activity translates into changes in arbor morphology has remained to be elucidated. Transcription factors that regulate cell differentiation usually bind to multiple loci simultaneously[17,38], and while binding can be useful to highlight potential candidates, alone it cannot predict central effectors[38]. The finding that dendritic microtubules can have different properties between classes suggested that a class-specific transcription factor may utilize microtubule regulators in order to create differences in arbor complexity. Therefore, the approach we used here was to focus explicitly on identifying putative Abrupt targets that regulate the microtubule cytoskeleton, and to employ subsequent cell biological analysis to explicitly test effector activity. ChIP-Seq revealed binding of Ab to the *cnn* locus, and furthermore the binding of the endogenous protein is corroborated by that of a transgenic Ab-DamID fusion protein in a recently reported independent dataset[17] (**Supplementary Fig. 15**). Subsequent analysis showed that Cnn-mediated microtubule nucleation site selection is used by Ab to repress branching.

During expansion and elaboration of the dendrite arbor Golgi outposts create a local system for guiding microtubule polymerization. These data reveal that Cnn tethers microtubule nucleation events to one face of Golgi outposts and biases the direction of microtubule polymerization away from dendrite tips. This Cnn activity in growing dendrites shows interesting parallels to its function in the PCM where it anchors microtubule nucleation events to the centrosome in order to promote the polarized microtubule array of the mitotic spindle[30]. During the formation of the mitotic spindle, in addition to the main PCM-derived pathway, microtubules are also nucleated directly from the forming microtubule network and on the chromatin itself. Interplay between these different microtubule nucleation pathways provides robustness to the spindle building process[32,37]. Our data reveal a further remarkable analogy to the spindle, as a balance between Cnn suppressing anterograde polymerization and Wac (a second novel factor we find active in the dendrites) supporting anterograde polymerization together regulates arbor complexity. Augmin facilitates integration of new microtubules into preexisting arrays, and it couples nucleation to the specific orientation of these arrays[39]. With respect to the formation of the arbor, we illustrate that initial outgrowth is driven by anterograde directed microtubule polymerization, and this may explain why Wac can amplify the anterograde polymerization in nascent termini to increase branching during dendrite arbor outgrowth.

These data illustrate how arbor complexity can be regulated by controlling an antagonistic relationship between two different microtubule nucleation pathways. In addition, the Cnn pathway described here is likely to control the microtubule array in conjunction with a larger network of dendritic microtubule regulators. Focal concentration of Cnn itself can create a *de novo* microtubule nucleation center[40]. This suggests that Cnn could create foci of microtubule nucleation independent from outposts, and that mechanisms localizing Cnn during the expansion and elaboration of the dendrite arbor are also likely to contribute to arbor patterning. Tubulin must also be transported into the growing dendrite to support local dendritic microtubule nucleation. Tubulin can be transported as both dimers and polymers, and in the latter

case local microtubule severing will create substrates for new microtubule nucleation events[12,41]. In analogy to axons, dendritic microtubule severing may be regulated spatially by local protection of the microtubule network by microtubule binding proteins[42]. Microtubule nucleation and severing events within the dendrite also produce free minus ends that can be stabilized by CAMSAP/Patronin, the loss of which reduces branching[43,44]. This present study also reveals class-specific differences in the localization of dendritic markers associated with microtubule minus-end-directed molecular motor activity, such differences suggest differential distribution of minus ends in the stable microtubule array between neurons classes, and in addition or alternatively that motor movement and transport properties along the dendritic microtubule array differs between classes. Any differences in the transport of motor-based cargos within the arbor are likely to create both functional and morphological differences between neuron classes. We also find differences in the composition of Golgi outposts between neuron types that are likely to affect outpost-associated microtubule nucleation activity[31]. Overall, these studies suggests that the network of microtubule regulators active in dendrite may provide the rich substrate through which intrinsic programs tune arbor complexity, and the integration of the output of this network with dendritic targeting programs will produce final arbor shape.

In humans dendrite abnormalities are associated with a range of developmental neurological disorders, several of which are caused by mutations in microtubule regulators[1,2,45,46]. Mutations in *CDK5RAP2* itself cause primary autosomal recessive microcephaly[47], and the small cortical size in these patients is because CDK5RAP2 (along with Pericentrin) acts at the centrosome to maintain neuronal stem cell self-renewal[48,49]. However, in addition CDK5RAP2 is expressed and may continue to function during early stages of neuron differentiation[45,50]. It will be interesting to determine if primary dendrite patterning is also affected in patients with *CDK5RAP2* mutations.

ACKNOWLEDGMENTS

This work was funded by a Human Frontiers Program Long-Term Fellowship to L-F.Y., and a RIKEN Brain Science Institute (BSI) core grant to A.W.M. We thank Tetsutaro Hayashi and Itoshi Nikaido, RIKEN Advanced Center for Computing and Communication (ACCC) for experimental advice, and Charles Yokoyama and members of the BSI Laboratory for Genetic Control of Neuronal Architecture for discussion.

AUTHOR CONTRIBUTIONS

SE, CY, CD, and AWM designed the project, SE, CY, CD, HT, and AWM carried out genetics, immunohistochemistry, live imaging, neuronal tracing, and interpreted results; RA carried out immunohistochemistry; CD, LFY, SA, RS carried out molecular biology; RS carried out bioinformatics; AWM, and BTN carried out modelling; KW provided reagents, equipment, and technical support; AWM, CD, and LFY wrote the paper. SE and CY contributed equally to this study.

COMPETING FINACIAL INTERESTS

The authors declare no competing financial interests.

LEGENDS

Figure 1. Neuron class-specific arrangements of microtubule minus ends in dendrites

(**a**) Tracings of class I ddaE and class IV ddaC. In this and all subsequent figures, anterior is to the left, dorsal is up. Adapted from[12].

Staining with fluorescence antibodies to detect in the left column: GFP (Green), β-*galactosidase* (magenta/white).

(**b-e**) Class IV 3rd instar, Khc::nod::lacZ label.

(**f**) Coverage index (sum of label length/arbor length) at 3rd instar for Khc::nod::lacZ labels with a length >9μm (n (neurons) =7, 7, 6; class I WT vs class IV WT $P < 0.0001$, class IV WT vs class IV UAS-ab $P < 0.01$)

(**g**) The frequency at which Khc::nod::lacZ labels a dendrite tip at 3rd instar (n (tips) = 195, 436, 94; class I WT vs class IV WT P = 1.13e-4, class IV WT vs class IV UAS-ab P < 4.4e-16)

(**h**-**k**) Class I embryo st17; Khc::nod::lacZ label.

(**l**) Coverage index for class I neurons at st17 (n (neurons) =10, 10; <3μm P = 0.0108, >9 μm P = 0.0007).

(**m**) The frequency at which Khc::nod::lacZ labels a dendrite tip at st17 (n (tips) = 344, 610; P = 0.0009).

Scale bars: (**b-e**) 50μm; (**h-k**) 5μm. Abbreviation: cI: class I; cIV: class IV. Data shown as percentages or or as scatter plots with the mean ±SEM.

Statistical tests used: (**f**) one-way ANOVA followed by Bonferroni's Multiple Comparison Test, (**g**) Fisher's exact test with Bonferroni correction, (**l**) t-test, (**m**) Fisher's exact test.

Figure 2. Cnn restricts dendrite branch formation and facilitates Ab-mediated branch repression

(**a**) Fold change in gene expression in da neurons in which Ab was expressed ectopically.

(**b**) Abrupt ChIP-seq. Top row : Control subtracted ChIP data from anti-Ab immunoprecipitated chromatin collected from two populations of embryos. A magenta asterisk marks Ab binding at the *cnn* promoter. Middle row: Sequence enrichment above background at IDR=0.02 using MACS. Bottom row: Annotated transcripts.

(**c**) Wildtype (WT), (**d**) *cnn*, (**e**) *ab* class I neurons. Scale bar 10μm.

(**f**, **g**) Increase in dendrite tips in class I *cnn* mutants (**f**) st17 (n(neurons)=30, 20) and (**g**) 3rd instar (n (neurons)=33, 28).

(**h**) Removing one copy of *cnn* in a hypomorphic *ab* background leads to a synergistic increase in class I branch tip number st17 (n= (neurons) 16, 11, 16, 16).

(**i**) Overexpression of *cnn* does not alter dendrite tip number in class I neurons, but suppresses the over-branching phenotype of *ab* mutants st17 (n= (neurons) 18, 27, 35, 15).

(**j**) Increase in dendrite tip number in the posterior dorsal quadrant of class IV *cnn* mutants at st17 (n (neurons)=22, 24).

(**k**) Overexpression of *cnn* represses branch tip number of class IV neurons st17 (n (neurons)=8, 9).

(**l**) 3rd instar *cnn* mutants do not show an alteration in class IV branch tip. *cnn* mutation rescues the suppression of tip number caused by ectopic *ab* (n (neurons)=16, 16, 16, 15).

(**m**) Frequency of secondary branches in vertical system 1 (VS1) is increased in *cnn* mutant neurons (n (neurons)=12, 12).

Data drawn as scatter plots with the mean ±SEM. Abbreviation st17: embryo stage 17. 3rd instar: wandering 3rd instar larva.

Statistical tests used: (**a**, **f**, **g**, **j**, **k**, **m**) t-test, (**h**, **i**, **l**) two-way ANOVA.

Figure 3. Cnn organizes dendrite microtubule nucleation

(**a**) Dominant negative RhoA increases branch number, but does not show a genetic interaction with *cnn*. Effect of *RhoA* manipulation on branching $P < 0.0001$; interaction between *cnn* and *RhoA* $P = 0.9008$) (n (neurons)=19, 24, 19, 28).

(**b**) Increase in dendrite branch number in *futsch* mutants (n (neurons)=15, 19).

(**c**) Increase in dendrite branch number in *plp* mutants (n (neurons)=27, 34).

(**d**, **e**) Genetic interactions between *plp* and (**d**) *cnn* (n (neurons)=54, 31, 48, 26) or (**e**) *ab* (n (neurons)=25, 9, 24, 24) lead to synergistic increases in branch number.

(**f**) WT (**g**) *cnn*. Time lapse images of the growth of a class I primary dendrite at st14, the dendrite tip region is shown. The appearance of a comet is marked with blue asterisks. In the *cnn* mutant successive comets initiate in different positions. Each successive comet is numbered. The direction of movement and subsequent positions are marked with an arrow and magenta asterisk, respectively. Images of every 2 seconds are shown. Vertical dashed-lines between panels represent the skipping of frames. Scale bar: 1µm.

(**h**, **i**) The number of microtubule nucleation events and the number of individual sites where microtubule nucleation occurs increases in *cnn* mutants (n (neurons)=6, 7).

Data drawn as scatter plots with the mean ±SEM. Abbreviation: T - tip.

Statistical tests used: (**a**, **d, e**) two-way ANOVA, (**b**, **c**, **h**, **i**) t-test.

Figure 4. Polarized Cnn localization at Golgi

(**a**-**h**) Co-localization of GFP::Cnn with either ManII::Ch2 or RFP::GalT and (**c**,**f**). Fluorescent signals (y-axis in **g**, **h**) were measured along a 1.5µm line; the dashed line in (**c**) shows an example of the position this line. The line was drawn perpendicular to the Golgi face in each test (ManII::Ch2/GFP::Cnn or RFP::GalT/GFP::Cnn). Scale bar: 1µm. Examples of data from single experiments are shown in (**g**, **h**).

(**i**, **j**) Across the line (as illustrated in (**c**)) (**i**) the distance between the peak of expression of each, and (**j**) the correlation between the intensity of GFP::Cnn expression and that if a Golgi marker. (n (Golgi)=27, 29, 29). *plp* mutants do not show any difference in the localization of Cnn to the *cis* face.

(**k**) The fragmentation of GFP::Cnn into foci on the surface of Golgi increases in *plp* mutant neurons (n (Golgi)=158, 186).

Data shown as percentages or as scatter plots with the mean ±SEM.

Statistical tests used (**i**, **j**) one-way ANOVA followed by Holm-Sidak's Multiple Comparisons Test, (**k**) Chi-square test.

Figure 5. Cnn localization in the dendrite arbor

(**a**) GFP::Cnn localizes in discrete puncta along dendrite branches, at branch points (green arrowheads), and at branch tips (magenta arrowheads). Scale bar: 7.5µm.

(**b**-**e**) In *plp* mutants general localization of Cnn throughout the dendrite arbor (**c**, **d**) is not changed; however (**e**) branchpoints show an increased density of puncta (n (neurons)=8, 7). Cnn occupancy is defined in (**c**, **d**) as the number of foci/number of positions; in (**e**) as the number of puncta at a branchpoint where at least one foci is present /number of branchpoint where at least one foci is present.

(**f**) GFP::Cnn localizes adjacent to ManII::Ch2-labelled dendrite Golgi outposts.

Scale bar 10µm. Data drawn as scatter plots with the mean ±SEM. i –magnification of the signals, ii –path of the arbor. Abbreviation: p - proximal.

Statistical test used: (**c**, **d**, **e**) t-test.

Figure 6. Cnn and Ab recruit microtubule nucleation to Golgi outposts

(**a**, **b**) The position of the formation of an EB1::GFP comet is marked with an arrowhead; in (**a**) this is a Golgi outpost. The direction of comet movement is marked with an arrow. The positions of the comets marking the end of the polymerizing microtubule is marked with asterisks, the same color asterisk indicates the successive movement of one comet. (**a**) Multiple comets leave a Golgi outpost in the same direction. (**b**) Multiple comets form at a position where no outpost is present and leave in opposite directions.

(**c**) When Golgi outposts emit multiple successive EB1::GFP comets, they travel in the same direction. At non-Golgi positions emitting multiple EB1::GFP particles successive comets often travelled in opposite directions (n(comets)=22, 40).

(**d**) The proportion of EB1::GFP comets that initiate at Golgi (n(comets)=50, 49,48).

(**e**) The number of microtubule nucleation events is not reduced in the termini of *cnn* or *ab* mutants. n (neurons)=9, 9, 9).

Data drawn as scatter plots with the mean ±SEM.

Statistical tests used (**c**) Fisher's exact test, (**d**) Fisher's exact test with Bonferroni correction, (**e**) one-way ANOVA.

Figure 7. Cnn controls the polarity of dendrite microtubule polymerization and the extension of termini

(**a**) Time lapse images of the growing class I primary dendrite (st17). The comet is marked with an asterisk, and forms in the first frame shown formation, the direction of movement marked with an arrow. The length of the dendrite terminal branch at the beginning and end of each series is marked with vertical bars. Microtubule growth, as represented by the EB1::GFP comets drives branch extension. This extension is represented by the pink extension to the green and blue bars, respectively. Images taken every 4 seconds are shown. Scale bar: 1µm.

(**b**) The black bars show the proportion of comets moving in anterograde direction in the terminal dendrites increases in *cnn* and *ab* mutants (n(comets)=249, 267, 219). (**c**) Furthermore it shows a synergistic increase when *cnn* levels were simultaneously reduced in the hypomorphic *ab* background (n=124, 150, 252, 91).

(**d**) The increase in anterograde comets is suppressed by simultaneous removal of wac (n=332, 150, 248, 275). The blue bars show the proportion of comets involved in branch initiation and branch extension events correlated with microtubule invasion (n (comets)= **b** 199, 300, 348; **c** 178, 305, 477, 273; **d** 306, 191, 174, 233). (**e**) The increase in dendrite branch number in *cnn* is suppressed by simultaneous removal of *wac* (n (neurons) = 12, 21, 28, 26).

Data shown as percentages or as scatter plots with the mean ±SEM.

Statistical tests used: (b-d) Fisher's exact test with Bonferroni correction, (c) two-way ANOVA.

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