Cortactin binding protein 2 increases microtubule stability and regulates dendritic arborization

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Running head: CTTNBP2 stabilizes microtubules.
Summary

Neurons are characterized by subcellular compartments such as axons, dendrites and synapses that have highly specialized morphologies and biochemical specificities. Cortactin binding protein 2 (CTTNBP2), a neuron-specific F-actin regulator, has been shown to play a role in the regulation of dendritic spine formation and maintenance. Here, we show that in addition to F-actin, CTTNBP2 also associates with microtubules before mature dendritic spines formed. The association of CTTNBP2 and microtubules induced the formation of microtubule bundles. Although the middle (Mid) region of CTTNBP2 was sufficient for association with microtubules, for microtubule bundling, the N-terminal region containing the coiled coil motifs (NCC) mediating the dimerization or oligomerization of CTTNBP2 is also required. Our study indicates that CTTNBP2 proteins form a dimer or oligomer and bring multiple microtubule filaments together to form bundles. In cultured hippocampal neurons, knockdown of CTTNBP2 or expression of the Mid or NCC domain alone reduced the acetylation levels of microtubules and impaired dendritic arborization. The study suggests that CTTNBP2 influences both the F-actin and microtubule cytoskeletons and regulates dendritic spine formation and dendritic arborization.
Introduction

Neurons are highly differentiated cells composed of specialized subcellular compartments (i.e., axons, dendrites and synapses) that are supported by different cytoskeletons. For instance, F-actin cytoskeletons are the major cytoskeletons determining the morphology of dendritic spines, the locations of excitatory synapses in mammals (Racz and Weinberg, 2013); microtubules on the other hand are mainly present in dendritic and axonal shafts, although they also transiently invade dendritic spines (Gu et al., 2008; Hoogenraad and Bradke, 2009; Hu et al., 2008; Jaworski et al., 2009; Luo, 2002). Because axons, dendrites and dendritic spines are all neuron-specific subcellular structures, it is reasonable to speculate that there are neuron-specific regulators that control the behaviors of the F-actin and microtubule cytoskeletons of these neuron-specific compartments and are involved in their formation, maintenance and regulation. Cortactin binding protein 2 (CTTNBP2), a neuron-specific protein (Chen et al., 2012; Chen and Hsueh, 2012; Ohoka and Takai, 1998), has been shown to play such a role in dendritic spine formation.

CTTNBP2, also known as CortBP2 (Cheung et al., 2001) or CBP90 (Ohoka and Takai, 1998), interacts with the SH3 domain of cortactin via its C-terminal proline-rich domain (Chen and Hsueh, 2012; Ohoka and Takai, 1998). Cortactin is widely expressed in various cells, including neurons. In mature neurons, cortactin is highly enriched at dendritic spines, where it promotes F-actin branching and bundle formation and thus enlarges the dendritic spine heads (Hering and Sheng, 2003; Huang et al., 1997; Weaver et al., 2001). CTTNBP2 is also highly concentrated in dendritic spines (Chen and Hsueh, 2012). The neuron-specific expression of CTTNBP2 suggests that the interaction between CTTNBP2 and cortactin likely controls the neuron-specific function of cortactin. Indeed, CTTNBP2 is critical for determining the mobility of cortactin in dendritic spines and thus controls dendritic spine formation and maintenance (Chen and Hsueh, 2012). In addition to cortactin, CTTNBP2 also binds striatin and zinedin, the regulatory B subunits of protein phosphatase 2A (PP2A) in neurons via the interaction between the N-terminal coiled-coil (NCC) domains of both CTTNBP2 and striatin/zinedin (Chen et al., 2012). Similar to cortactin, the dendritic spine distribution of striatin and zinedin is also controlled by CTTNBP2 (Chen et al., 2012). CTTNBP2, therefore, regulates dendritic spine formation and signaling through interaction with cortactin and the PP2A complex.
While analyzing the expression pattern of CTTNBP2 in COS cells, we noticed that in addition to being present in the cell cortex where CTTNBP2 colocalizes with cortactin and F-actin cytoskeletons, CTTNBP2 is also associated with some filamentous structures (Chen et al., 2012; Chen and Hsueh, 2012). In this study, we identified these filamentous structures as microtubules and found that CTTNBP2 also interacts with microtubules and increases microtubule stability. Our study suggests that in addition to associating with F-actin cytoskeletons, CTTNBP2 also regulates neuronal morphology through controlling microtubule stability.

Results

CTTNBP2 associates with microtubules

Using a COS cell expression system, our previous studies showed that CTTNBP2 colocalizes with cortactin and F-actin at the cell cortex (Chen et al., 2012; Chen and Hsueh, 2012). However, we noticed that in addition to the cell cortex, CTTNBP2 proteins were also present in some filamentous structures which were not F-actin-positive (Supplementary material Fig. S1A). The percentage of cells possessing CTTNBP2 filaments varied from batch to batch, ranging from ~30% to ~80% of CTTNBP2-transfected cells. Although there was variability in the percentage, such cells were always present in CTTNBP2-expressing cultures. We were therefore curious about what such structures were. Using double immunostaining with tubulin antibody, confocal analysis indicated that CTTNBP2-positive filamentous structures were colocalized with microtubules (Supplementary Material Fig. S1B). We also used three-dimensional structured illumination microscopy (3D-SIM) to further investigate the interaction of CTTNBP2 and microtubules. It was clear that CTTNBP2 attaches to microtubules and forms filamentous structures along microtubules in transfected COS cells (Supplementary Material Fig. S1C).

To confirm the association of CTTNBP2 with microtubules, we performed a nocodazole treatment/washout experiment using CTTNBP2 transfected COS cells. Nocodazole is an agent commonly used to induce microtubule depolymerization. If the CTTNBP2 associated filaments are indeed microtubules, nocodazole treatment is expected to disrupt the filamentous structures. In cells treated with vehicle, around 71% of
CTTNBP2-expressing cells possessed CTTNBP2-positive filaments, which were colocalized with microtubules (Fig. 1A,B). After nocodazole treatment for 2 h, microtubule filaments were disrupted in COS cells, meanwhile CTTNBP2 filamentous structures also disappeared (Fig. 1A,B). After washout of nocodazole for 2 h, microtubules reassembled into a filamentous network (Fig. 1A). CTTNBP2-positive filaments also reoccurred and associated with microtubules in around 63% of transfected cells (Fig. 1A,B). Taken together, these cell biology analyses suggest the association of CTTNBP2 with microtubules.

We also performed a biochemical study – microtubule binding protein spin-down assay – to investigate the association between CTTNBP2 and microtubules. In the assay, purified tubulins formed microtubules and were concentrated in the pellets after polymerization and sedimentation. CTTNBP2 was co-fractionated with microtubules in the pellets (Fig. 1C,D), confirming the association of CTTNBP2 with microtubules. In the absence of microtubules, CTTNBP2 was not noticeably precipitated by centrifugation (Fig. 1D) supporting the specificity of the presence of CTTNBP2 in the microtubule fraction.

The middle (Mid) region of CTTNBP2 is the microtubule interacting domain

We then wondered which region of CTTNBP2 is involved in the association with microtubules. A series of CTTNBP2 fragments (Fig. 1C) were constructed and expressed in COS cells. After removing endogenous microtubules, the COS cell lysates were then applied to microtubule binding protein spin-down assay. We found that in addition to the full-length CTTNBP2, the fragment containing the Mid domain alone was sufficient for microtubule association (Fig. 1C,D). Although the NCC fragment was also precipitated in the microtubule pellets, the presence of the NCC in the pellet was likely non-specific, because the solubility of the NCC did not seem comparable to other fragments. It was also present in a noticeable amount in the pellets even in the absence of microtubules (Fig. 1D). We thus suggest that the Mid domain is the major microtubule interacting region of CTTNBP2.

In addition to using crude COS cell extracts for microtubule binding assay, we also expressed and purified glutathione S-transferase (GST)-fusion proteins containing the NCC, Mid or P-rich domain of CTTNBP2 from bacteria for the microtubule binding
experiment. The results showed that similar to the NCC and P-rich domain-fusion proteins and GST alone, the GST-Mid fusion proteins were not significantly brought down by microtubule filaments in the spin-down assay (Fig. 1E). This suggests that the association between the Mid domain and microtubules may not be direct and that an unknown factor in COS cells regulates the association of the Mid domain of CTTNBP2 and microtubule filaments.

**Cell-substratum interaction influences the association of CTTNBP2 with cytoskeletons**

Because we consistently found two distinct distributions of CTTNBP2 in COS cells, it seemed likely that an intrinsic factor in the culture, such as the status of cell growth, controls the distribution of CTTNBP2. To explore this possibility, we used a replating experiment to investigate the role of cell-substratum interaction in the regulation of the association of CTTNBP2 with cytoskeletons. Twenty-four hours after transfection with HA-tagged CTTNBP2, COS cells were harvested and replated on new coverslips for 1, 2 and 4 hours. We found that at 1 hour after replating, in ~90% of transfected cells, CTTNBP2 tended to form patches and distribute into the cell cortex, where CTTNBP2 colocalized very well with F-actin cytoskeletons (Fig. 2A). At this stage, microtubule filaments were clearly presented in COS cells. However, they did not show noticeable colocalization with CTTNBP2 (Fig. 2A). The results of quantification showed that at this stage, ~37% of CTTNBP2-positive pixels were colocalized with the signals of F-actin cytoskeletons, while only 8% of CTTNBP2-positive pixels were overlapped with microtubule filaments (Fig. 2B). One hour later, the association of CTTNBP2 and microtubules became clearer. We found two populations of CTTNBP2-expressing cells at this stage. The first type of cells (~40% of transfected cells) still had prominent cell cortex distribution of CTTNBP2. However, in this type of cells, the degree of colocalization of CTTNBP2 and microtubules was already noticeable (Fig. 2A). In the second type of cells (~60% of transfected cells), CTTNBP2 displayed a filamentous structure and showed fairly good colocalization with microtubules (Fig. 2A). Consequently, the colocalization coefficient of CTTNBP2 and microtubules was increased to ~40%, while the colocalization of CTTNBP2 and F-actin was reduced to ~20% at two hours after replating.
At 4 hours after replating, the filamentous CTTNBP2 was even more promising (Fig. 2A). The colocalization coefficient of CTTNBP2 and F-actin noticeably dropped to ~10% (Fig. 2B). Taken together, the results of replating experiment suggest that the cell-substratum interaction regulates the associations of CTTNBP2 with different cytoskeletons. When the cell-substratum interaction is more stable, the association of CTTNBP2 with microtubules is stronger.

CTTNBP2 associates with microtubules in cultured hippocampal neurons

Because CTTNBP2 is specifically expressed in neurons, we then examined the distribution of endogenous CTTNBP2 in neurons. Our previous studies showed that in mature neurons, CTTNBP2 resides stably in dendritic spines, which are mainly supported by F-actin (Chen et al., 2012; Chen and Hsueh, 2012). We speculated that CTTNBP2 associates with microtubules along dendrites before dendritic spines form. To explore this possibility, we first examined the protein expression of CTTNBP2 before dendritic spine formation in our neuronal cultures. The results of immunoblotting indicated that in addition to at 18 DIV, when dendritic spines typically form under our culture conditions, CTTNBP2 proteins were also expressed in immature neuronal cultures at 1, 7, and 14 DIV (Fig. 3A). Immunostaining was performed and analyzed by both confocal and 3D-SIM imaging to examine the distribution of CTTNBP2 in cultured hippocampal neurons. At 14 DIV, when mature dendritic spines had not yet formed, CTTNBP2 proteins already formed patches and attached to microtubule bundles along dendrites (Fig. 2B). We also found F-actin puncta in the dendritic shafts. However, F-actin and CTTNBP2 only partially overlapped each other (Fig. 2B). When the distributions of CTTNBP2 at 14 and 18 DIV were directly compared (Fig. 3C), it was clear that at 18 DIV, both CTTNBP2 and F-actin tended to move away from microtubule bundles in the dendritic shafts and colocalized with each other pretty well (Fig. 3C, right panel confocal images). However, at 14 DIV, though both CTTNBP2 and F-actin formed patches and attached to microtubules, the colocalization of CTTNBP2 and F-actin was partial (Fig. 3C, left panel confocal images). The 3D-SIM further illustrated that CTTNBP2 had a close interaction with microtubules at 14 DIV but not at 18 DIV (Fig. 3B, C).

We further quantified the distribution of CTTNBP2 in dendritic spines and
dendritic shafts. A GFP construct was transfected into cultured hippocampal neurons at 8, 12 and 14 DIV to outline the neuronal morphology. Immunostaining using GFP and CTTNBP2 antibodies was performed at 14, 18 and 20 DIV. The relative intensity of CTTNBP2 in dendritic shafts was gradually reduced during dendritic spine maturation, while the dendritic spine intensity of CTTNBP2 was increased. For dendritic spines, the change from 14 DIV to 18 DIV was particularly noticeable (Fig. 3D,E).

In conclusion, these data suggest that the association of CTTNBP with the F-actin and microtubule cytoskeletons is developmentally regulated in neurons.

CTTNBP2 regulates microtubule organization and bundle formation

Next we investigated the function of the association of CTTNBP2 with microtubules. We noticed that in COS cells, CTTNBP2 expression changed the organization and structure of microtubules. In untransfected cells, microtubule filaments form a radial pattern extending from the microtubule organization center to the cell edge (Figs 1A,4A). This pattern was disrupted by CTTNBP2 expression. The microtubules tended to circle round and form thicker bundles in CTTNBP2-expressing cells (Figs 1A,4A). We quantified the percentage of cells containing microtubule bundles. In the presence of CTTNBP2, more than half of the transfected cells contained microtubule bundles (mean ± s.d.: 55.7 ± 8.1 %, total 123 cells collected from three experiments), a result that was rarely found in GFP expressing cells (mean ± s.d.: 7.4 ± 4.2%, total 120 cells collected from three experiments). These results suggest that CTTNBP2 is able to influence microtubule organization. To further investigate this point, we performed transmission electron microscopy (TEM). Indeed, we found that microtubule organization is very different in CTTNBP2-expressing and GFP-transfected cells (Fig. 4B). In CTTNBP2-expressing COS cells, microtubules tended to align and form tight bundles (Fig. 4B, right panel, arrows). This kind of microtubule array was never found in cells transfected with GFP control (Fig. 4B, left panel). The TEM results, therefore, further indicate that CTTNBP2 promoted the formation of microtubule bundles.

We then investigated whether cortactin influences the microtubule bundling activity of CTTNBP2. In COS cells, cortactin is typically localized to the cell cortex (Fig 4C, upper panel). Expression of cortactin knockdown construct Cort300 (Hering and
Sheng, 2003) noticeably reduced the protein levels of endogenous cortactin (Fig. 4C,D). CTTNBP2 still associated with microtubules and promoted microtubule bundling when the protein levels of cortactin were reduced (Fig. 4C,E). The percentages of cells containing microtubule bundles were comparable between vector control and Cort300 (Fig. 4E). This result suggests that cortactin is not required for the microtubule bundling activity of CTTNBP2. To further confirm this point, the CTTNBP2 PA1 mutant that lacks efficient interaction with cortactin (Chen and Hsueh, 2012) was expressed in COS cells. Similar to the wild-type proteins, the PA1 mutant proteins still effectively promoted microtubule bundling (Supplemental Material Fig. S1D,E), further indicating that the interaction with cortactin is not critical for the microtubule bundling activity of CTTNBP2. Note that cortactin was recruited to the microtubule filaments in the presence of CTTNBP2 (Fig. 4C, middle panel), though cortactin is not required for the microtubule association of CTTNBP2. It, therefore, seems likely that CTTNBP2 may act as linker to bring microtubule and cortactin together.

Oligomerization of CTTNBP2 is required for microtubule bundling

Since the Mid domain alone is sufficient for microtubule association (Fig. 1C,D), we also examined whether the Mid domain alone is sufficient for microtubule bundling. When the Mid domain was expressed in COS cells, the radial microtubule pattern was disrupted; however, the pattern was not similar to that of full-length CTTNBP2 (Fig. 4A). The global microtubule signals were reduced. And also, there was almost no thicker microtubule bundle in the Mid domain-expressing cells (Fig. 4A, mean ± s.d.: 1.5 ± 1.3%, total 123 cells from three experiments). These data suggest that the binding to microtubules by the Mid domain is not sufficient for microtubule bundling and that another region of CTTNBP2 is required for microtubule bundling. Previously, we showed that CTTNBP2 is able to form hetero-oligomers with striatins via the NCC domain (Chen et al., 2012). It therefore seemed possible that to promote microtubule bundling, CTTNBP2 forms a homo-oligomer or at least a homodimer via a region outside of the Mid domain. Thus, a CTTNBP2 dimer or oligomer would be able to bind two or more microtubule filaments and promote bundling. The large puncta pattern of CTTNBP2 along dendritic microtubules (Fig. 3B,C) also favors oligomeriation of CTTNBP2. To examine the possibility of the
oligomeriation of CTTNBP2, we first performed a co-immunoprecipitation experiment to investigate the homophilic interaction of CTTNBP2. Myc- and HA-tagged full-length CTTNBP2 (the schematic structure of CTTNBP2 construct is shown in Fig. 5A) were cotransfected into COS cells. The cell lysates were then immunoprecipitated with Myc tag antibody. We found that Myc tag antibody not only precipitated Myc-tagged CTTNBP2 from the lysates but also brought down HA-tagged CTTNBP2 (Fig. 5B). The co-immunoprecipitation was specific because Myc tag antibody did not precipitate HA-tagged CTTNBP2 in the absence of Myc-tagged CTTNBP2 (Fig. 5B). These results support the oligomerization of CTTNBP2.

We then identified the oligomerization domain of CTTNBP2 by investigating the interaction between HA-tagged full-length CTTNBP2 and a series of Myc-tagged domain deletion constructs of CTTNBP2. The construct NCCM, missing the C-terminal proline-rich domain, was still able to interact with full-length CTTNBP2 (Fig. 5B), suggesting that the proline-rich domain is not involved in the oligomerization of CTTNBP2. The Mid domain alone did not bring down full-length CTTNBP2 (Fig. 5B), also suggesting that the Mid domain is not the oligomerization domain. The NCC construct containing the extreme N-terminal region and the following coiled-coil domain was co-immunoprecipitated with full-length CTTNBP2 (Fig. 5B). These data indicate that the NCC region is the oligomerization domain.

To further characterize these domain deletion constructs, we next examined the subcellular distribution of these constructs in COS cells. The NCC construct tended to form aggregates and did not show obvious colocalization with microtubules in COS cells (Supplemental Material Fig. S2). This is consistent with the results of biochemical study that showed that the NCC fragment had a lower solubility (Fig. 1D). The radiation pattern of microtubules was preserved in NCC expressing cells (Supplemental Material Fig. S2). The NCCM construct also formed aggregates in COS cells; however, a fraction of NCCM was colocalized with microtubules (Supplemental Material Fig. S2). We noticed that similar to the full-length CTTNBP2, the radiation pattern of microtubules was also missing in NCCM-expressing COS cells (Supplemental Fig. S2). The proline-rich domain alone was evenly distributed in cells and did not noticeably influence the microtubule organization in COS cells (Supplemental Material Fig. S2). The results of immunostaining
also favor the hypothesis that the region containing the NCC is sufficient to induce self
aggregation and that the presence of the Mid domain may bring the oligomers to associate
with microtubule filaments.

Based on the results above, it is reasonable to hypothesize that oligomerization
through the NCC domain allows CTTNBP2 oligomers to have multiple binding sites for
microtubule filaments and thus promote microtubule bundling. If this hypothesis is correct,
disruption of CTTNBP2 oligomerization would be expected to disrupt the function of
CTTNBP2 in promoting microtubule bundling. To investigate this possibility, CTTNBP2
was cotransfected with different CTTNBP2 domains into COS cells. In the presence of the
NCC domain, the percentage of cells containing microtubule bundles dropped to ~10%
(Fig.5D). When the NCCM domain was cotransfected with CTTNBP2, NCCM was
colocalized with full-length CTTNBP2 and distributed along the microtubules. It even
further promoted microtubule bundling in COS cells (Fig. 5C,D). The proline-rich domain
alone had no effect on CTTNBP2-dependent microtubule reorganization (Fig. 5C,D). For
the Mid domain, although it tended to reduce microtubule bundling induced by full-length
CTTNBP2, the difference was not significant. It also did not impair the filament formation
of CTTNBP2 (Fig. 5C,D). The data suggest that the Mid domain cannot efficiently inhibit
the association of CTTNBP2 with microtubules in COS cells. Perhaps, this is because the
full-length CTTNBP2 forms at least a dimer through the NCC region (Fig. 5B). In COS
cells, both CTTNBP2 and the Mid fragment were overexpressed. The monovalent Mid
domain may not efficiently compete with bivalent full-length CTTNBP2.

Taken together, these cotransfection experiments also suggest that the NCC domain
is involved in oligomerization of CTTNBP2 and that the oligomerization of CTTNBP2 via
the NCC domain is also required for microtubule bundling.

CTTNBP2 regulates microtubule stability in COS cells

Since microtubule bundling is highly associated with increased microtubule stability (Fong
et al., 2013; Kanai et al., 1992; Kanai et al., 1989) and since CTTNBP2 expression induced
formation of thick microtubule bundles, it is likely that microtubule stability is increased in
the presence of CTTNBP2. To address this question, two experiments were performed.
First we performed immunostaining to monitor the acetylation and tyrosination of
microtubules. Tubulin acetylation indicates stable microtubules; in contrast, tyrosinated tubulin suggests unstable microtubules (Fukushima et al., 2009; Garnham and Roll-Mecak, 2012; Takemura et al., 1992). Compared with neighboring untransfected cells and GFP transfected cells, the tubulin acetylation levels in CTTNBP2-expressing cells were noticeably increased, while the tubulin tyrosination levels were decreased (Fig. 6A,B). In contrast, expression of the Mid domain reduced the tubulin acetylation levels, though it did not noticeably influence the levels of tubulin tyrosination compared with the GFP control (Fig. 6A,B). These results indicate that the presence of CTTNBP2 influences the posttranslational modification of microtubules.

Second we examined the sensitivity of microtubules to nocodazole. Nocodazole was added to transfected COS cells at final concentrations of 0, 1, 4, 10 μM for 30 min. In cells transfected with GFP control, 10 μM nocodazole disrupted microtubule filaments in almost all the cells (Fig. 6C,D). When the concentration of nocodazole was reduced to 4 or 1 μM, ~10 or 60% of GFP transfected cells, respectively, still processed microtubule filaments (Fig. 6D). In CTTNBP2 transfected cells, nocodazole had no obvious disruptive effect on microtubules at the concentration of 1 μM. Even at concentrations of 4 and 10 μM, ~60% of CTTNBP2 expressing cells still contained microtubule filaments (Fig. 6C,D), suggesting that the presence of CTTNBP2 resulted in resistance to nocodazole treatment. In contrast, the Mid domain-expressing cells were highly sensitive to nocodazole (Fig. 6C,D). One micromolar of nocodazole was sufficient to disassemble microtubules in more than 95% of the Mid domain-expressing cells (Fig. 6D). These data also suggest that CTTNBP2 expression increased microtubule stability, while the Mid domain alone reduced microtubule stability.

The NCCM fragment of CTTNBP2 is sufficient for microtubule stabilization

The aforementioned data suggested that the interaction with cortactin is not involved in the microtubule bundling mediated by CTTNBP2. The NCC domain mediating oligomerization and the Mid domain interacting with microtubules are required for microtubule bundling, which then increases the microtubule stability. Along these lines, we expected that the NCCM fragment is sufficient for microtubule bundling and stabilization. To examine this possibility, we investigated the function of the NCCM fragment in
regulation of tubulin acetylation. Indeed, the tubulin acetylation level was higher in the NCCM transfected cells compared with cells transfected with GFP (Supplemental Material Fig. S3A,B). Besides, expression of the NCCM fragment was also sufficient for increase of nocodazole resistance. More than 40% of the NCCM expressing COS cells still processed microtubule filaments after 10 μM nocodazole treatment for 30 minutes (Supplemental Material Fig. S3C,D). These data indicate that the NCCM fragment is sufficient for microtubule stabilization.

CTTNBP2 influences tubulin acetylation in cultured hippocampal neurons

We then wondered whether CTTNBP2 is able to regulate posttranslational modification of tubulin in cultured hippocampal neurons. Cultured hippocampal neurons were transfected with a construct expressing artificial miRNA against CTTNBP2 (Chen and Hsueh, 2012) at 8 DIV and subjected to immunostaining with acetylated tubulin antibodies at 14 DIV, when CTTNBP2 associated with microtubules in cultured hippocampal neurons (Fig. 3B,C). The quantitative analysis indicated that the acetylation levels of tubulins in dendrites were reduced in CTTNBP2 knockdown neurons (Fig. 7A,B). Similarly, expression of the Mid or NCC domain also reduced the tubulin acetylation (Fig. 7C,D). These results suggest that tubulin acetylation in neurons was influenced by CTTNBP2.

Disruption of CTTNBP2 function impairs dendritic arborization

Because microtubules make up most of the cytoskeleton supporting dendrites, the influence of CTTNBP2 on microtubule stability in dendrites suggests that it likely controls dendritic arborization. To investigate this possibility, we investigated whether knockdown of endogenous CTTNBP2 or overexpression of the Mid and NCC fragments has a negative effect on dendritic growth in cultured hippocampal neurons. Compared with vector control, expression of CTTNBP2 miRNA knockdown construct (BP2-KD) obviously reduced the protein levels of endogenous CTTNBP2 (Supplemental Material Fig. S4A). Moreover, expression of BP2-KD impaired dendritic arborization as shown by Sholl analysis that showed that CTTNBP2 knockdown neurons had obviously fewer intersection numbers (Fig. 8A,B,C). This indicates that CTTNBP2-deficient neurons have less complex dendritic arbors. To rule out the off-target effect of BP2-KD, the CTTNBP2 silent mutant
(WT-res) resistant to BP2-KD was cotransfected with BP2-KD into cultured neurons. We found that WT-res effectively rescued the defects of dendritic arborization induced by BP2-KD (Fig. 8A,C), confirming the specificity of BP2-KD. Besides WT-res, the CTTNBP2 PA1 mutant (PA1-res) resistant to BP2-KD was also able to rescue the defects (Fig. 8A,C). This is consistent with the observation that the interaction between CTTNBP2 and cortactin was not required for microtubule stabilization promoted by CTTNBP2. We also found that overexpression of CTTNBP2 did not further induce dendritic growth as dendritic arborization in neurons transfected with CTTNBP2 was comparable to those transfected with vector control (Supplemental Material Fig. S4B,C).

The negative effect of the Mid and NCC fragments on dendritic arborization was also investigated. As expected, overexpression of the Mid and NCC fragments noticeably impaired dendritic arborization (Fig. 8D,E). These results further indicate that microtubule stabilization controlled by CTTNBP2 is involved in the regulation of dendritic arborization.

Discussion

CTTNBP2 was originally identified as a cortactin binding partner (Ohoka and Takai, 1998) that can control the synaptic distribution of cortactin (Chen and Hsueh, 2012). In this report, we further show that CTTNBP2 also associates with microtubules and regulates microtubule stability. CTTNBP2 uses different protein domains to achieve these two functions. The C-terminal proline-rich domain of CTTNBP2 interacts with the SH3 domain of cortactin. To associate with microtubules, the Mid domain alone is sufficient. The analysis of the amino acid sequence did not reveal any known protein motif in the Mid domain of CTTNBP2. The microtubule binding protein spin-down assay further indicated that an unknown factor is involved in the interaction between CTTNBP2 and microtubules. Although it is unclear how the Mid domain associates with microtubules, our data suggest that to promote microtubule bundling and increase microtubule stability, both the NCC and the Mid domain are required. Because CTTNBP2 forms at least a dimer or oligomer via the NCC domain, the CTTNBP2 dimer or oligomer can then bind multiple microtubule molecules via the unknown mediator. Through this physical link, CTTNBP2 may then induce bundle formation of microtubules and increase the stability of microtubules. It is
interesting that expression of the Mid domain actually has a dominant negative effect on maintenance of microtubule filaments in COS cells. Perhaps, the Mid domain-associating protein that directly interacts with microtubules can also be recognized by other microtubule regulators in COS cells. The presence of the Mid domain can then compete with the endogenous microtubule regulators in COS cells and destabilize the microtubule filaments.

In this study, our data suggest that the associations of CTTNBP2 with microtubules and F-actin are regulated by cell-substratum interaction in COS cells and the developmental stage in neurons. When the interaction between COS cells and substratum had not been stabilized (one hour after replating), CTTNBP2 entered the cell cortex and colocalized with F-actin. While COS cells establish stable interaction with the substratum (such as at 4 hours after replating), CTTNBP2 prefers to associate with microtubules. In cultured neurons, when mature spines form, CTTNBP2 targets to dendritic spines and colocalizes with F-actin cytoskeletons. Before mature spines form, CTTNBP2 associates with microtubules. Although it is unclear what signal regulates this partner choice, our results clearly showed that the interaction with cortactin does not play a critical role in the regulation of microtubule association of CTTNBP2. Thus these two associations are separated and independent. However, we cannot rule out the possibility that CTTNBP2 can function as a bridge to link microtubules and F-actin under specific circumstances. More investigations are needed to examine which signaling pathway regulates the association of CTTNBP2 with different cytoskeletons and whether CTTNBP2 can act as a bridge to link these two cytoskeletons together.

We previously showed that CTTNBP2 controls dendritic spine formation and maintenance (Chen and Hsueh, 2012). The negative effect of CTTNBP2 knockdown on dendritic spine density was rescued by the CTTNBP2 silent mutant resistant to CTTNBP2 miRNA, but not the CTTNBP2 PA1 mutant without the ability to interact with cortactin (Chen and Hsueh, 2012). These data suggest that cortactin is critical for CTTNBP2-dependent dendritic spine formation (Chen and Hsueh, 2012). Here, we report a second function of CTTNBP2 in neuronal morphogenesis, which is to regulate dendritic arborization. This second function is obviously independent of cortactin binding, because the PA1 mutant still effectively rescued the defects caused by CTTNBP2 knockdown.
Besides, the previous study also showed that knockdown of cortactin does not influence dendritic arborization (Jaworski et al., 2005). Our data showed that overexpression of the NCC or Mid domain impaired the dendritic arborization. Since the NCC domain is required for dimerization or oligomerization of CTTNBP2 and the Mid domain is involved in microtubule association, CTTNBP2 dimers or oligomers likely interact with an unknown microtubule binding protein(s) and promote bundle formation of microtubules. The bundling mediated by CTTNBP2 then increases microtubule stability and thus controls dendritic arborization. Indeed, the stability of microtubules, the major cytoskeleton to support dendrite morphology, has been shown to be crucial for dendrite morphology (Chen et al., 2011; Chen et al., 2013; Ori-McKenney et al., 2012; Stewart et al., 2012), although in the previous studies, stabilization of microtubules was not via microtubule bundling.

Because CTTNBP2 is specifically expressed in neurons (Chen et al., 2012; Ohoka and Takai, 1998), it may play a unique role in controlling neuronal morphology. Interestingly, recent human genetic studies identified several missense mutations in the CTTNBP2 gene in patients with autism spectrum disorders (Iossifov et al., 2012; Parikshak et al., 2013). Because autism spectrum disorders are recognized as neurodevelopmental disorders, our study revealing the role of CTTNBP2 in regulating neuronal morphology may also potentially provide a link between CTTNBP2 and psychiatric disorders.

Materials and Methods
Antibodies and reagents
The following antibodies were used in this study: mouse monoclonal anti-Myc (9B11; Cell Signaling Technology, Danvers, MA, USA); rabbit polyclonal anti-Myc (06-549, Millipore, Billerica, MA, USA); chicken polyclonal anti-GFP (ab13970, Abcam, Cambridge, MA, USA); rat monoclonal anti-HA (3F10, Roche, Indianapolis, IN, USA); mouse monoclonal anti-β-tubulin, α-tubulin, acetylated tubulin and tyrosinated tubulin (TUB 2.1, B-5-1-2, 6-11B-1 and TUB-1A2, respectively; Sigma-Aldrich, St. Louis, Missouri, USA); rabbit monoclonal anti-Tuj1 (TUJ 1-15-79, Emeryville, CA, USA); rabbit polyclonal anti-cortactin (H-191, Santa Cruz Biotechnology, Texas, USA). The rabbit polyclonal anti-CTTNBP2 antibody was generated as previously described (Chen and Hsueh, 2012).
Animals

All animal experiments were performed with the approval of the Academia Sinica Institutional Animal Care and Utilization Committee. For primary culture, pregnant rats were sacrificed by CO₂ inhalation; E18–E19 fetal pups were then isolated and sacrificed by decapitation.

Plasmids

Myc-tagged CTTNBP2, BP2-miR and Ctrl-miR were constructed as previously described (Chen and Hsueh, 2012). Full-length or partial cDNA sequences corresponding to different regions of CTTNBP2 (NCC, 1-259aa; CC, 118-259aa; CCM, 118-522aa; Mid, 260-522aa; P-rich, 523-625aa) were amplified and cloned into vectors pGW1-CMV-Myc, pGW1-CMV-HA or pGEX-4T-1 in order to be expressed in COS cells or bacteria. The cortactin knockdown construct Cort300 was a generous gift from Dr. Morgan Sheng.

In vitro microtubule binding protein spin-down assay

COS cells expressing CTTNBP2 fragments were lysed in 50 mM HEPES (pH 8), 320 mM sucrose, and 1% Triton, then centrifuged at 16,000 × g for 30 min to remove insoluble debris. Prior to microtubule binding assay, endogenous microtubules were cleared by incubation with 20 μM taxol at 35°C for 30 min and centrifugation onto the microtubule-free supernatant and were kept for analysis. Various GST-fusion proteins were purified from bacteria and also subjected to spin-down assay. The microtubule binding assay was performed according to the manufacturer’s instructions (BK029, Cytoskeleton, Denver, CO). In brief, purified tubulin was incubated in General Tubulin Buffer at 35°C for 20 min. Taxol (20 μM) was then added to stabilize the microtubules. The microtubule-free cell lysate was then incubated with or without microtubules in General Tubulin Buffer and 20 μM taxol at room temperature for 30 min. Samples were centrifuged onto a 100-μL cushion buffer (60% glycerol in general tubulin buffer [80 mM PIPES, pH 7, 2 mM MgCl₂, 0.5 mM EGTA, and 1 mM GTP]) at 100,000 × g for 40 min at room temperature. The supernatant and pellet were collected and analyzed by immunoblotting. Microtubule polymers and associated proteins were present in the pellet;
free tubulins and non-associated proteins were present in the supernatant.

**Neuronal cultures and transfection**

Rat hippocampal neurons from embryonic day 18-19 embryos were dissociated and cultured as previously described (Chen and Hsueh, 2012). The transfection of neurons was performed using the calcium phosphate precipitation method.

**COS cell culture and transfection**

COS-1 cells were originally obtained from the Bioresource Collection and Research Center, Taiwan, and maintained in DMEM with 10% fetal bovine serum. The transfection of COS cells was conducted with Lipo2000 reagent according to manufacturer’s instructions (Invitrogen, Taipei, Taiwan).

**Transmission Electron Microscopy (TEM)**

Cells were seeded on ACLAR Embedding Film at ~90% confluence. After rinsing with wash buffer (0.1 M cacodylate buffer, pH 7.2, 4% sucrose and 0.05% CaCl₂) at room temperature, cells were fixed with fixation solution (0.1 M cacodylate buffer, pH 7.2, 2.5% glutaraldehyde and 1% tannic acid) at 37°C. After extensive rinsing with wash buffer, cells were post-fixed in 1% OsO₄ solution buffered with 0.1 M cacodylate buffer, pH 7.2, at room temperature followed by staining with 1% uranyl acetate at room temperature. After dehydration with graded alcohol, cells were filtrated with a series of solutions as follows: (1) EtOH:Spurr’s Resin (Electron Microscopy Sciences, Hatfield, PA) = 1:1 for 30 min; (2) EtOH:Spurr’s Resin = 1:2 for 40 min; (3) pure Spurr’s Resin for 1 h. Samples were then polymerized at 70°C for 20 h. After slicing, the images of ultrastructure were captured by Tecnai G2 Spirit TWIN electron microscope (FEI) equipped with a Gatan CCD Camera (794.10.BP2 MultiScan) and acquisition software DigitalMicrograph (Gatan).

**Super-resolution microscopy and 3D-reconstruction**

Super-resolution microscopy was performed using an Elyra PS.1 microscope (Carl Zeiss) equipped with 63X/NA 1.4 oil (Plan-Apochromat; Carl Zeiss) objective lens and iXon 885 EMCCD (Andor Technology) at room temperature. The Z series was set at an interval of
0.11 μm. The 3D structure illumination was processed using Zen 2011 software at the following settings: 488, 555, and 647 channels were aligned in axes x, y and z using 100-nm and 500-nm TetraSpec Fluorescent Microspheres (Invitrogen); noise filter parameter value, -3; and SR frequency weighting value, +1. The 3D-reconstruction modeling was further conducted using Imaris image analysis software.

Immunostaining and morphometry

Cells were fixed with 4% paraformaldehyde and 4% sucrose in PBS, followed by permeabilization with 0.2% Triton X-100 in PBS. After blocking with 10% bovine serum albumin, the cells were incubated with primary antibodies diluted in PBS containing 3% bovine serum albumin at 4°C overnight. Following PBS washes, the cells were incubated with secondary antibodies conjugated with Alexa Fluor 488, 555, and/or 647 (Invitrogen) for 2 h. The images were acquired at room temperature using a confocal microscope (LSM700; Carl Zeiss, Oberkochen, Germany) equipped with a 10X/NA 0.3 or 63X/NA 1.4 oil (Plan-Apochromat; Carl Zeiss) objective lens and Zen 2009 (Carl Zeiss) acquisition and analysis software or an upright microscope (Microscope Axio Imager.M2, Carl Zeiss) equipped with a 63X/NA 1.4 oil (Plan-Apochromat; Carl Zeiss) objective lens, EMCCD camera Rolera EM-C² (QImaging, Surrey, BC) and Zen 2011 program (Carl Zeiss) acquisition software. The Z-series images were then projected for analysis. Some of the image acquisition and quantitation were blindly performed to minimize the effect of bias. For publication, the images were processed using Photoshop without any modification or with minimized adjustment of brightness and/or contrast applied to the entire images.

Replating of CTTNBP2 transfected cells

After incubation overnight, HA-tagged CTTNBP2 transfected COS cells were trypsinized and replated in a new 12-well dish (1 × 10^5 cell/well). One, two and four hours after replating, cells were fixed for immunostaining using HA and α-tubulin antibodies and Alexa 647-phalloidin. Images were then analyzed using a microscope.

Image analysis and quantification

All the quantification analyses of the fluorescent images were performed using either
ImageJ (v.14.7, NIH) or Zen 2009 analysis software (Carl Zeiss). For dendritic shaft and spine distribution of CTTNBP2, the fluorescent intensities of CTTNBP2 in the dendritic shafts and spines were measured to determine the enrichment coefficient of dendritic shafts and spines as follows: the average intensity at dendritic shaft or spine/the average of total intensity containing both dendritic spine and dendritic shaft. To quantify the colocalization of CTTNBP2 with microtubules and F-actin, Zen 2009 analysis software was used with the threshold set at higher than 100 for each channel. The coefficient indicated the percentage of CTTNBP2-positive pixels colocalized with microtubules or F-actin: the coefficient = the number of pixels with both CTTNBP2 and F-actin or MT signals/total number of pixels with CTTNBP2 signals. To define microtubule bundles, since microtubule thickness was usually about 0.5 μm in confocal images of untransfected or control vector transfected cells, cells containing at least 2 microtubule bundles with > 1.5 μm in thickness and > 5 μm in length were recognized to have microtubule bundles. Because the centrosome has a microtubule aggregate, this measurement was only performed outside the centrosome. For CTTNBP2 filaments, cells should contain at least 3 CTTNBP2 filaments and each filament was longer than 5 μm. To define cells with a microtubule network, cells had to contain at least 10 microtubule filaments and each filament had to be longer than 10 μm. The thickness of microtubules was not taken into consideration.

**Statistical analysis**

All the data were processed using Prism version 5.03. For Figure 4D, 4E, 5D (all of the columns were compared with the GFP Ctrl), 7B, S1E, S3B, and S4A Student’s t test was used. For Figures 3E, 6B, and 7D the data were analyzed by one-way ANOVA with the Bonferroni test. For Figure 8C, 8E, and S4C the data were analyzed by two-way ANOVA. Mean plus s.e.m or s.d. and n number are presented in all the figures or figure legends. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ns., not significant.

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Competing interests
The authors have no conflicting financial interests.

References


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**Figure legends**

**Fig. 1.** CTTNBP2 associates with microtubules through the Middle (Mid) domain.

(A,B) Nocodazole treatment disrupts the filamentous structure of CTTNBP2 in COS cells. (A) COS cells expressing HA-tagged CTTNBP2 (BP2) were treated with DMSO (control) or 10 μM nocodazole (Noc) for 2 h. In the washout group (Noc/washout), cells were treated with 10 μM nocodazole for 2 h followed by incubation in fresh culture medium for 2 h. Cells were then fixed and stained with anti-HA and anti-tubulin antibodies. Arrows point to microtubule organization centers, which were missing in CTTNBP2 expressing cells. Scale bars, 20 μm. (B) Quantification of (A). Cells were randomly collected from three independent experiments. The total number of counted transfected cells and the number of cells containing CTTNBP2 filaments are indicated. (C,D) Mapping of the microtubule-associating domain of CTTNBP2. (C) Schematic domain structure and deletion constructs of CTTNBP2. Microtubule (MT) binding ability was also summarized based on the results in (D). (D) Microtubule binding protein spin-down assay showing that the Mid domain is involved in the association with microtubules. Different Myc-tagged deletion constructs of CTTNBP2 were expressed in COS cells and subjected to in vitro microtubule spin-down assay followed by immunoblotting with Myc and tubulin antibodies. (E) MT binding protein spin-down assay using purified GST fusion proteins. Purified GST fusion proteins did not noticeably associate with MT in vitro. In (D,E), the Myc or GST-tagged proteins (arrow) and purified tubulin (arrowhead) are indicated. P, pellet; S, supernatant.

**Fig. 2.** Cell-substratum interaction regulates the association of CTTNBP2 with different cytoskeletons.

(A) Subcellular distribution of HA-CTTNBP2 in replated COS cells. At 1, 2 and 4 h after replating, HA-CTTNBP2 transfected cells were immunostained to examine the distribution of CTTNBP2, microtubules and F-actin. CTTNBP2 preferred to interact with F-actin 1 h after replating and shifted to microtubules when cells were cultured for a longer period of time. (B) Quantification of colocalization between CTTNBP2 and microtubules and F-actin in (A). Twenty cells collected from two independent experiments were quantified. Data represent mean plus s.d.. Scale bar, 10 μm.
Fig. 3. **CTTNBP2 has differential subcellular distribution in premature and mature cultured hippocampal neurons.** (A) CTTNBP2 is expressed in cultured hippocampal neurons at 1, 7, 14 and 18 days in vitro (DIV). α-tubulin was used as a loading control. Note that mature spines typically form at 18 DIV in our system. (B,C) Distribution of endogenous CTTNBP2, F-actin, and microtubules in cultured hippocampal neurons at 14 and 18 DIV. (B) Confocal and structured illumination microscopy (SIM) images of endogenous CTTNBP2 and α-tubulin and F-actin (viewed by phalloidin) at 14 DIV. 3D-SIM, image of SIM after 3D reconstruction with Imaris software. (C) Direct comparison of neurons at 14 and 18 DIV. The same cells were analyzed using both confocal and SIM imaging. In (B,C), the confocal images revealed whether the proteins were colocalized; the SIM-3D images revealed the direct interaction between different proteins. The 3D images at the lowest panel are the enlargements corresponding to the insets in SIM single plane images. CTTNBP2 had a closer interaction with microtubules at 14 DIV and was colocalized with F-actin at 18 DIV. (D) Dendritic spine distribution of endogenous CTTNBP2 at 14, 18 and 20 DIV. The yellow lines outline the morphology of dendritic shafts and spines. (E) Quantification of the CTTNBP2 protein levels in dendritic shafts and spines at 14, 18 and 20 DIV. Data represent mean plus s.e.m.. Scale bar, (B) 3 μm; (C) upper 5 μm, lower 1 μm; (D) 5 μm.

Fig. 4. **CTTNBP2 expression induces microtubule bundle formation in a cortactin independent manner.** (A,B) CTTNBP2 induces microtubule bundle formation. COS cells were transfected with GFP control, Myc-tagged full-length CTTNBP2 and the Mid domain. Cells were immunostained with α-tubulin and GFP or Myc tag antibodies. (A) Confocal images. (B) TEM images. The lower magnification images are shown in the upper panel. Two enlargements containing microtubules are shown in the middle panel. Higher magnification images of the middle images are included in the lower panel. (C-E) Cotactin is not involved in CTTNBP2-induced microtubule bundling. In (C), the upper panel shows an untransfected cell. Middle and lower panels show cells cotransfected with HA-tagged CTTNBP2 and vector control or cortactin knockdown construct Cort300. Immunostaining using α-tubulin, cortactin and GFP or HA-tag antibodies, as indicated. (D) Quantification
of cortactin knockdown efficiency. (E) Quantification of the effect of cortactin knockdown on MT bundling induced by CTTNBP2. Data represent mean plus s.d.. Cells were randomly collected from three independent experiments. Scale bars, (A) 20 μm; (B) top, 1 μm; middle and bottom, 0.2 μm. (C) 10 μm.

Fig. 5. Oligomerization of CTTNBP2 is required for microtubule bundling. (A) Schematic structures of full-length CTTNBP2 and domain deletion mutants. HA or Myc tag was added to the N-terminal end of the constructs as indicated. (B) The NCC and NCCM fragments are coimmunoprecipitated with CTTNBP2. The various constructs were cotransfected into COS cells as indicated. Immunoprecipitation was performed using Myc tag antibody and immunoblotting was carried out with HA or Myc tag antibody. (C) The dominant negative effect of the NCC fragment on MT bundling induced by CTTNBP2. HA-tagged full-length CTTNBP2 was cotransfected with Myc-tagged domain deletion constructs of CTTNBP2. Cells were then immunostained with HA tag, α-tubulin and GFP or Myc tag antibodies as indicated. (D) The percentages of cells containing CTTNBP2 filaments and MT bundles were shown. The NCC domain showed a noticeable negative effect on microtubule bundling and CTTNBP2 filament formation. Data represent mean plus s.d. of three independent experiments (cell number: GFP = 64, NCC = 54, Mid = 48, NCCM = 56, P-rich = 56). Scale bar, (C) 10 μm.

Fig. 6. CTTNBP2 promotes microtubule stability. (A,B) CTTNBP2, but not the Mid domain, increases acetylation and decreases tyrosination of tubulins in COS cells. COS cells were transfected with the constructs and subjected to immunostaining with antibodies as indicated. (A) Confocal images. (B) Quantification of (A). The total cell numbers for each experiment are indicated. Data represent mean plus s.e.m.. (C,D) CTTNBP2-expressing cells are resistant to nocodazole treatment. COS cells were transfected with the indicated plasmids and treated with 0, 1, 4, and 10 μM nocodazole for 30 min. Immunostaining was then performed with α-tubulin and Myc tag or GFP antibodies. Only the representative images of cells treated with vehicle control and 10 μM nocodazole are shown. (D) Quantitative results of the effect of nocodazole on microtubule filaments. The percentages of transfected cells containing microtubule filaments are shown.
Data represent mean ± s.d. of three independent experiments. For each point, 30-40 transfected cells were analyzed. For some groups, the error bars are too small to be seen. Scale bar, 10 μm

**Fig. 7. CTTNBP2 controls tubulin acetylation in cultured hippocampal neurons.** Rat hippocampal neurons were transfected with the various plasmids as indicated at 8 DIV and were harvested for immunostaining with antibodies as indicated at 14 DIV. (A,B) Knockdown of CTTNBP2 reduces tubulin acetylation. (C,D) Expression of the NCC and Mid domains decreases tubulin acetylation. Neurons were collected from three independent experiments. The numbers of analyzed dendrites (n) are indicated. Data represent mean plus s.e.m.. Scale bar, 10 μm.

**Fig. 8. CTTNBP2 regulates dendritic arborization.** (A) Knockdown of CTTNBP2 impairs dendritic arborization. Representative images of neurons transfected with various constructs as indicated are shown. Transfection was conducted at 8 DIV and immunostaining was carried out at 14 DIV. Both WT silent mutant resistant to BP2-KD and PA1 mutant resistant to BP2-KD effectively rescue the impairment induced by BP2-KD. (B) Schematic figure of Sholl analysis with radii increasing at 10 μm intervals. (C) Quantification of the intersection number from Sholl analysis in (A). (D) Overexpression of the NCC and Mid domains reduces dendritic arbors of hippocampal neurons. Representative images of neurons transfected with constructs as indicated. (E) Quantification of the intersection number from Sholl analysis in (D). Data were collected from three independent experiments. Sample sizes (n) are indicated. Scale bar, 100 μm.
Figure 2

A

1 hr

HA-BP2 α-tubulin Phalloidin

2 hr

HA-BP2 α-tubulin Phalloidin

4 hr

HA-BP2 α-tubulin Phalloidin

B

Colocalization coefficient (%)

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<th>1 hr</th>
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Figure 8

A

EmGFP+GW1

BP2-KD+GW1

BP2-KD+Myc-WT res

BP2-KD+PA1 res

B

C

Distance to soma (µm)

Intersection No.

EmGFP+GW1 (n=52)

BP2-KD+GW1 (n=50)

BP2-KD+WT-res (n=48)

BP2-KD+PA1-res (n=48)

D

GFP+GW1

GFP+Myc-Mid

GFP+Myc-NCC

E

***

GFP+GW1 (n=57)

GFP+Myc-Mid (n=59)

GFP+Myc-NCC (n=63)

Intersection No.