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Microtubule severing at crossover sites by katanin generates ordered cortical microtubule arrays in Arabidopsis

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Timely severing of microtubules at crossover sites by katanin is essential for generating ordered cortical microtubule arrays in *Arabidopsis*

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Running title: Role of severing in CMT array organization

SUMMARY

The noncentrosomal cortical microtubules (CMTs) of land plants form highly ordered parallel arrays that mediate cell morphogenesis by orienting cellulose deposition [1-3]. Since new CMTs initiate from dispersed cortical sites at random orientations [4], parallel array organization is hypothesized to require selective pruning of CMTs that are not in the dominant orientation. Severing of CMTs at crossover sites is proposed to be a potential pruning mechanism [5], however the parameters and molecular mechanisms underlying this activity are unknown. Here, using live-cell imaging, we show that severing preferentially targets the overlying CMTs at crossover sites and leads to their depolymerization about 85% of the time. In addition, the probability of severing has a sigmoidal relationship to the crossover dwell time, indicating a strong bias for longer-lived crossover sites to be severed. We found that severing at CMT crossover sites was completely abolished in the *Arabidopsis katanin* mutant. Consistent with this finding, GFP-tagged katanin driven by its native promoter localizes at sites of CMT crossover prior to severing. Furthermore, array recovery experiments showed that CMTs fail to become ordered in the *katanin* mutant. We conclude that katanin is solely responsible for severing at CMT crossover sites and that this activity is essential to generate ordered CMT arrays.

HIGHLIGHTS

- Severing predominantly leads to depolymerization of the overlying CMT at crossover sites.
- Severing probability increases nonlinearly with crossover time.
- Katanin localizes to crossover sites and is required for severing.
- Loss of katanin activity prevents the formation of coaligned CMT arrays.

RESULTS

Severing targets overlying CMTs and leads to their depolymerization

In this study, we used an *Arabidopsis* marker line co-expressing EB1b-mCherry and GFP-TUB6 to image CMTs in living cells. The EB1b-mCherry marker specifically labels growing plus-ends of CMTs and the GFP-TUB6 marker labels the entire microtubule lattice. The combination of these two markers allows us to easily track the dynamics of individual microtubules within the crowded CMT array.

As reported by Wightman and Turner [5], we found that CMTs are severed at intersections that are generated when growing CMT plus-ends cross over preexisting CMTs (Figure 1A; Movie S1). Here we refer to the crossing CMT as the "overlying" CMT and the preexisting CMT as the "underlying" CMT. In hypocotyl cells with transverse arrays, the mean severing frequency is $4.5 \pm 1.6 \times 10^{-3}$ events/μm^2/min (n = 16 cells). To understand the parameters that underlie this severing activity, we determined the relationship of severing to crossover angle and configuration of underlying CMTs. We found that severing probability is relatively constant for crossover angles ranging from 50° to 90° (Figure 1B), over which about 94% of crossover events occur. In addition, severing probability was not correlated to whether the crossover site consisted of a single underlying CMT, a bundle of CMTs or multiple intersecting CMTs (Figure 1C). Together, these data show that severing at CMT crossover sites is insensitive to crossover geometry.

Interestingly, we observed that severing specifically targets the overlying CMTs regardless of the crossover geometry (Figure 1D). As the majority of overlying CMTs in a transverse array are the discordant ones (Movie S1), they are the primary targets of severing. In addition, we found that the lagging part of severed CMTs completely depolymerize about 85% of the time (Figure 1E; Movie S1). In the remaining 15% of the time, the lagging part of severed CMTs starts to depolymerize and subsequently transitions to growth (Movie S2). Since the dominant outcome is microtubule depolymerization, severing works to eliminate unaligned CMTs.

Severing probability varies nonlinearly with crossover time

To examine the temporal relationship between crossover site formation and severing, we measured the time interval between creation of crossover sites and detectable severing

activity. In hypocotyl cells with transverse CMT arrays, it takes an average of 41 ± 14 sec for a crossover site to get severed (Figure 2A). Because CMTs are highly dynamic, most crossover sites in transverse arrays are short-lived, with an average lifetime of 31 ± 18 sec (Figure 2A). From these data, we calculated the severing probability as a function of crossover time and found a sigmoidal relationship (Figure 2B). This finding indicates that crossover sites in a transverse array must persist longer than about 15 sec to become available for severing. Severing probability sharply rises beyond this threshold time and then plateaus around 50%. Thus, while long-lived crossover sites are far more likely to get severed than short-lived ones, not all long-lived crossover sites get severed.

Unlike the transverse arrays in hypocotyl cells, CMTs in leaf pavement cells form net-like arrays with relatively little microtubule coalignment. The severing frequency in pavement cells has been reported to be lower than in petiole cells containing transverse arrays [5]. Similarly, we found the mean severing frequency in pavement cells to be $4.9 \pm 1.9 \times 10^{-5}$ events/μm^2/min (n = 12 cells); about 100-fold lower than in hypocotyl cells. To investigate the basis for this difference, we compared the time to severing between hypocotyl and pavement cells. We found that it takes an average of 109 ± 80 sec for a crossover site to get severed in pavement cells (Figure 2C), which is about 3-fold longer than in hypocotyl cells. In addition, severing time varies greatly in leaf pavement cells compared to hypocotyl cells. Together, these data show that severing is slower and not as tightly controlled in net-like arrays compared to transverse arrays. In contrast to this difference, the CMT plus-end dynamics and nucleation frequency are largely similar between pavement and hypocotyl cells (Table S1). Therefore, the difference in array organization between these cell types is likely largely due to the observed difference in severing time.

Severing at crossover sites is abolished in the *ktn1-2* mutant

To determine if the microtubule severing protein katanin is responsible for severing CMTs at crossover sites, we introduced our EB1b-mCherry;GFP-TUB6 dual-fluorescence marker into the *ktn1-2* mutant. The *ktn1-2* mutant harbors a T-DNA insertion in the fifth exon of *KTN* and is null for katanin [6]. Live-cell imaging experiments showed that severing at CMT crossover sites was abolished in hypocotyl and leaf pavement cells in the *ktn1-2* mutant

(Movie S3). Of 1030 crossover events that we observed in the *ktn1-2* mutant, none were severed. Therefore, katanin is solely responsible for severing at CMT crossover sites.

To visualize katanin localization in living cells, we introduced GFP-tagged *Arabidopsis* katanin (KTN-GFP) driven by its native promoter into wild-type plants expressing the RFP-TUB6 microtubule marker. The mean severing frequency in transverse arrays of hypocotyl cells in these plants is $3.8 \pm 2.1 \times 10^{-3}$ events/μm^2/min (n = 5 cells), which is not significantly different from control plants ($4.5 \pm 1.6 \times 10^{-3}$ events/μm^2/min). Therefore, expression of the KTN-GFP marker does not appear to perturb severing activity. Two-color, live-cell imaging revealed a punctate pattern of KTN-GFP localization, with individual puncta appearing and disappearing along CMTs (Figure 3A, 3B and Movie S4). About 70% of these puncta localize to CMT nucleation and crossover sites, while the remaining 30% localize along the sidewalls of CMTs (Figure 3C). The localization of KTN-GFP to nucleation sites confirms previous observations [6]. At crossover sites, the dwell time of KTN-GFP puncta correlates with severing activity (Figure 3C). Puncta along CMT sidewalls and at crossover sites that do not get severed persisted for a mean duration of 19.8 ± 11.8 and 14.1 ± 8.1 sec, respectively. In contrast, puncta at CMT nucleation and crossover sites that did get severed lasted for a mean time of 43.5 ± 20.4 and 36.3 ± 14.5 sec, respectively. Importantly, 96% of crossover sites that get severed are associated with KTN-GFP signal prior to severing (51 out of 53 severing events) (Figure 3B), consistent with our finding that katanin is responsible for severing CMTs at crossover sites.

Katanin-mediated severing is necessary to generate coaligned CMT arrays

In *katanin* mutants, the hypocotyl cells contain net-like arrays [7-9], indicating that severing by katanin is essential to organize CMTs into parallel arrays. However, these observations were conducted at steady-state conditions, making it difficult to determine if severing is required to create parallel CMT organization or to maintain this organization. To distinguish between these possibilities, we incubated *Arabidopsis* seedlings at -5°C for 5 min to depolymerize CMTs and then transferred the seedlings to room temperature to allow CMTs to recover and reorganize into arrays. Immediately after cold treatment, CMTs appear as short filaments (Figure 4). In wild-type hypocotyl cells, CMTs start to organize into parallel arrays within 40 min after transfer to room temperature, as evidenced by the emergence of

clear dominant CMT orientations (Figure 4A). In contrast, CMTs do not form parallel arrays in *ktn1-2* hypocotyl cells and remain disorganized even after 1-2 hours at room temperature (Figure 4B). It is important to note that CMT recovery after cold-treatment is not affected in the *ktn1-2* mutant. Indeed, CMTs in the *ktn1-2* mutant rapidly polymerize upon transfer to room temperature (Figure 4B). Consistent with this observation, CMT plus-end dynamics and nucleation frequency are comparable between *ktn1-2* and wild-type hypocotyl cells, with the exception of a small decrease in plus-end growth rate and dynamicity in the *ktn1-2* mutant (Table S1). Based on these data, we conclude that severing by katanin is necessary to create coaligned CMT arrays.

DISCUSSION

The parallel organization of CMTs is vital for directional cell expansion and consequently for plant growth and development. How this organization is generated in the absence of a dedicated microtubule-organizing center remains a major open question. In this study, we show how severing at crossover sites contributes to the formation of parallel CMT arrays and reveal katanin to be the enzyme responsible for this activity.

Computer simulation studies of CMT organization show that elimination of discordant CMTs is important for generating ordered arrays [10-12]. In these simulations, CMT catastrophe following steep-angle collisions was found to be sufficient to remove CMTs that were not in the dominant orientation. Here, we show that severing at crossover sites represents another important mechanism for removing unaligned CMTs. In animal cells, severing of microtubules has been described to either amplify microtubule numbers by creating more plus-ends for microtubule growth [13-15] or induce microtubule depolymerization from the plus-end [16, 17]. Based on our finding that 85% of crossover-based severing leads to depolymerization of the newly created plus-end, we propose that severing primarily serves a microtubule pruning function in the CMT array.

For severing to work as an effective pruning mechanism, it must specifically target the overlying CMT at crossover sites. Otherwise, severing would disrupt any existing CMT organization created by activities such as bundling [18] and parallel-form nucleation [19]. We found that the katanin-based severing mechanism meets this specification by preferentially cutting overlying CMTs. *In vitro,* katanin severs along the length of microtubules [20, 21]. What makes overlying CMTs at crossover sites a preferred substrate for katanin *in vivo* is unknown. One possibility is that crossover results in a bend in the overlying CMT at the crossover site, which might mimic microtubule lattice defects and directly attract katanin binding. We note that kinks or discontinuities in the microtubule lattice have been observed to localize metazoan katanin to these sites [22, 23], so it is possible that *Arabidopsis* katanin behaves similarly. Alternatively, some other protein might specifically bind to the bent overlying CMT site and recruit katanin for severing. In animals, the regulatory p80 subunit targets the catalytic p60 subunit of katanin to the centrosome and spindle poles and stimulates severing activity [24, 25]. *Arabidopsis* encodes for four p80 subunits [26]; one or more of these might work to target katanin to crossover sites.

Our severing time analysis revealed that crossover sites must persist for at least 15 sec for severing to occur. This likely reflects the minimum time needed to recruit katanin to these sites. However, it is important to note that katanin binding *per se* is not sufficient for severing activity. Evidence for this comes from our observation that short dwell times of KTN-GFP do not correlate with severing. Rather, severing is associated with three-fold longer dwell time of KTN-GFP, suggesting that stable binding of katanin is necessary for its catalytic activity. Our observation that crossover sites are severed about three-times faster in well-ordered transverse arrays than in net-like arrays also suggests that regulation of severing time is likely to be an important mechanism for controlling severing frequency and consequently the degree of parallel CMT organization. The molecular mechanisms that regulate severing time need to be explored in future work. As oligomerization of katanin into a ring-shaped hexamer correlates to severing activity [27, 28], its regulation represents one potential mechanism to control severing time. Alternatively, the concentration, targeting and/or activation of katanin might be modulated to control the rate of severing. Recently, genetic studies identified RIC1 (ROP-interactive CRIB-motif containing protein 1) as a factor that stimulates katanin-mediated severing at branch-form nucleation sites [29]. It will be interesting to determine whether RIC1 performs a similar function at crossover sites and to dissect the mechanism for this activity.

We found that not all crossover sites get severed even at longer lifetimes. Part of the reason for this might be that katanin binds only transiently to some crossover sites and fails to sever. In addition, about 15% of severed CMTs show rescue. Based on the knowledge that induced overexpression of katanin in *Arabidopsis* plants leads to short CMTs and fragmented arrays [30], we speculate that these characteristics allow cells to deploy severing to facilitate CMT organization while avoiding array dismantling due to excessive CMT breakdown.

Our array recovery experiments demonstrate that katanin activity is essential for the formation of parallel CMT arrays. Given that katanin localizes to CMT nucleation and crossover sites, and that severing activity is lost at both of these sites in the *ktn1-2* mutant, we propose that katanin contributes to CMT organization in at least two ways. First, by severing at crossover sites, katanin mediates elimination of unaligned CMTs. A corollary of this proposition is that coaligned CMTs, which do not crossover by definition, are not severed and therefore persist longer than unaligned CMTs. Second, by severing at nucleation sites,

katanin enables polymer treadmilling and removal of unaligned portions of bundled CMTs by depolymerization from the free minus-end [31]. Together, these activities work to generate parallel CMT organization.

SUPPLEMENTAL INFORMATION

Supplemental information includes Supplemental Experimental Procedures, one table, and four movies and can be found with this article online.

ACKNOWLEDGMENTS

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FIGURE LEGENDS

Figure1. Severing at crossover sites targets the overlying CMTs and leads to their depolymerization.

(A) An example of CMT severing at a crossover site. The white arrowhead tracks the plus-end of the CMT of interest. The purple star marks the crossover site where severing takes place. The yellow arrow tracks the back end of the severed CMT, which rapidly depolymerizes. Numbers indicate time in seconds. Scale bar = 3μm.

(B) Bar graph of the relative severing frequency as a function of the angle between the crossing CMTs. The severing frequency data is shown for 10° bins of crossover angles. N = 206 severing events in transverse arrays of hypocotyl cells from 5 independent plants.

(C) Bar graph of the relative severing frequency at different crossover configurations. The single, bundle and multiple CMT configurations are shown at the top of each bar. N = 227 severing events in transverse arrays of hypocotyl cells from 6 independent plants.

(D) Percentages of overlying and underlying CMTs that are severed at crossover sites. N = 154 severing events in transverse arrays of hypocotyl cells from 4 independent plants.

(E) Percentages of rescue and catastrophe of the lagging halves of CMTs following severing. N = 151 severing events in transverse arrays of hypocotyl cells from 4 independent plants.

Figure 2. Severing at crossover sites occurs in a time-dependent manner.

(A) Frequency distribution of the lifetime of crossover sites that are severed (red curve) or not severed (green curve) in transverse arrays of hypocotyl cells. The average severing time is 41 ± 14 sec (SD), while the average lifetime of unsevered crossover sites is 31 ± 18 sec (SD). N = 770 total crossover events.

(B) Severing probability as a function of crossover time. The data are best fit by a sigmoidal curve (R^2 = 0.93).

(C) Frequency distributions of severing time in transverse (red bars) and net-like (blue bars) CMT arrays. The average severing time is 109 ± 80 sec (SD) (N = 65) for net-like arrays.

Figure 3. KTN-GFP localizes to crossover sites prior to severing activity.

(A) Image showing KTN-GFP localization in a wild-type hypocotyl cell co-expressing RFP-TUB6. Examples of KTN-GFP puncta localized to CMT sidewalls, nucleation sites and crossover sites are labeled by white, yellow and magenta arrowheads, respectively. Scale bar = 6μm. (B) Image sequence showing localization of KTN-GFP to a CMT crossover site that gets severed. The white arrowhead tracks the plus-end of the CMT of interest. The purple arrowhead marks the crossover site that accumulates katanin and subsequently gets cut. The white arrow tracks the depolymerizing cut end. Numbers indicate time in seconds. Scale bar = 3μm. (C) The mean ± SD of the dwell time of KTN-GFP along CMT sidewalls, nucleation sites, crossover sites that do not get severed and crossover sites that get severed, respectively. The number of observed events is shown in parentheses.

Figure 4. Loss of katanin prevents the formation of ordered arrays.
Recovery pattern of CMT arrays following their cold-induced depolymerization in wild-type (A) and *ktn1-2* mutant (B). The first image following cold treatment represents 0 min. Over time, the CMTs become coaligned in wild-type cells but not in the *ktn1-2* mutant. The accompanying plots show the angular distribution of CMTs over time in two representative cells (marked in the final image). The green and red traces show the angular distributions at the first and last time point respectively. In wild-type, a clear dominant peak arises with time, indicative of parallel CMT organization. In *ktn1-2*, while the red trace is higher due to increased CMT number over time, there is no predominant peak. Scale bar = 6μm.

Figure 1

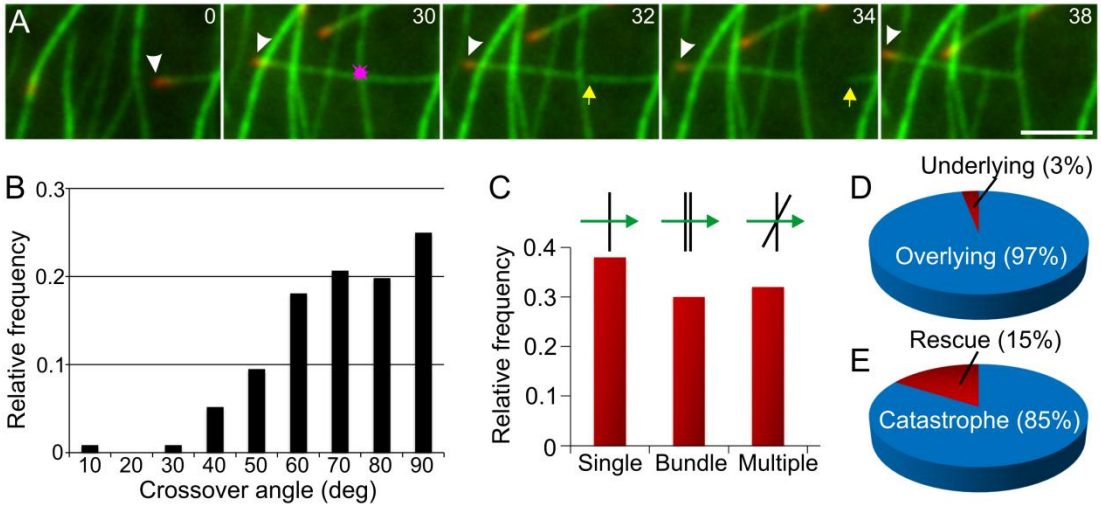

Figure 2

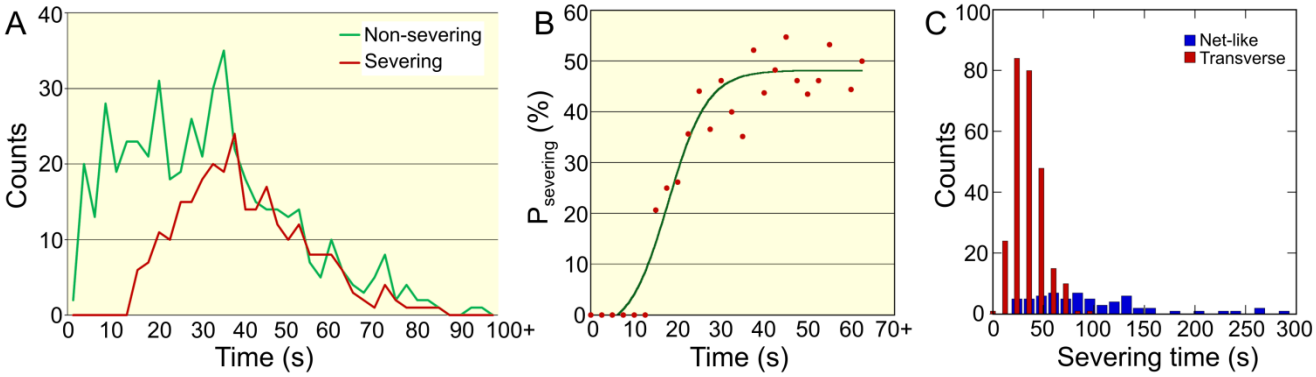

Figure 3

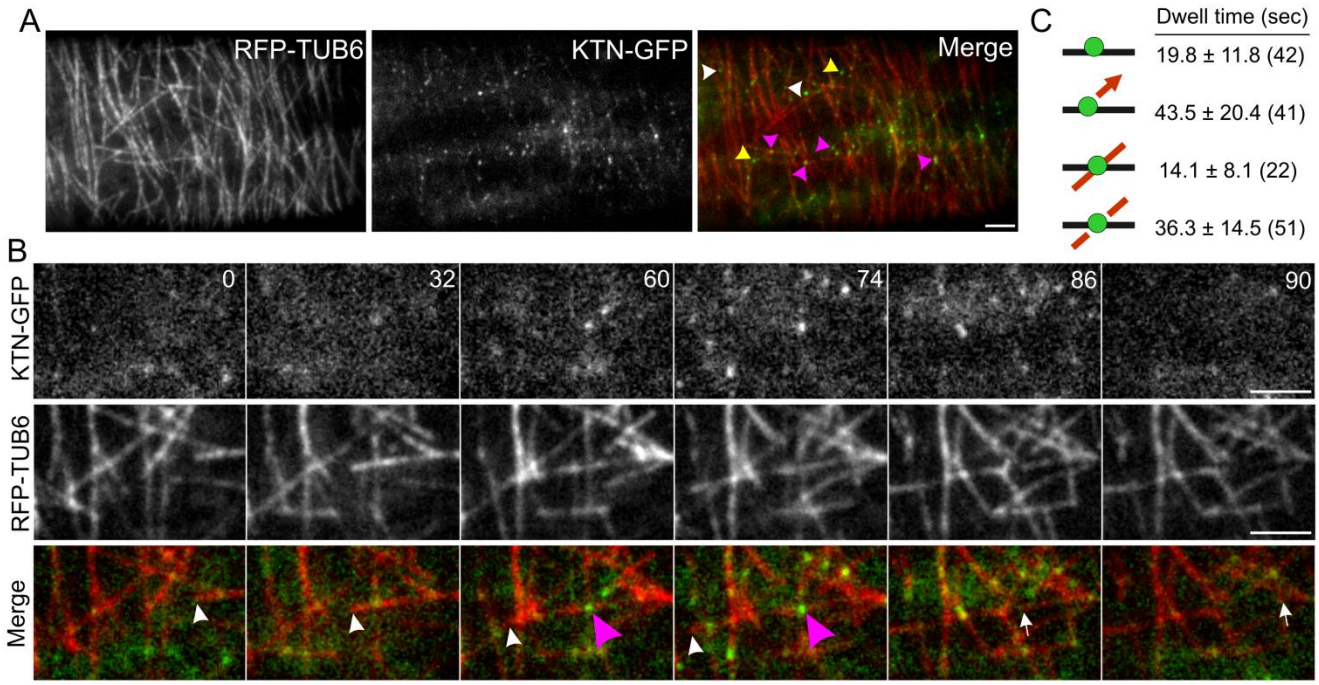

Figure 4