## [Figure 1.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/EWaePsL0gKxOvJLJOhgfX4wBgaVLfBgRBQKLmlxVVKi0sA?e=KFGmwB) and [Figure S1.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/ESdSnZpVpFNLhCkaPSSWXRUBesGkOh7csIoxEHew6gBpRg?e=sUHFwl)

[Figure 2.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/EV30O21kZ6tFhyKsQAAYPhAB8dI6dJE2kxdOLXfkTPaX2w?e=EAPXYg) and [Figure S2.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/ETAjPa_NS3NKnozbrim2cR4BcnBUWINPhkOUb3zvOp6nvA?e=8BA1ce)

## [Figure 3.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/EbcsONwL5UZOmxhyEkhmVlcBpX0g6eM6X_PN9DA5oOn85A?e=WuWUbf) and [Figure S3.docx](https://avcr-my.sharepoint.com/:w:/g/personal/krattenmacherj_ibt_cas_cz/EbjFMaA9ZyRFiYSdd1DyrsIB7QvgYqGGtqEbxhxPKDDpIg?e=qBEsag)

## Abstract

We also show that sweeping of diffusive microtubule crosslinkers occurs during microtubule depolymerization, analogous to previously-shown sweeping due to microtubule sliding.

## Introduction

Spatial regulation of microtubule (MT) organization and dynamics is critical for the assembly of MT-based structures such as the mitotic spindle (Nédélec et al. 2003). The stabilization of MTs underlies mechanisms of spindle assembly, chromosome segregation, cytokinesis, and polarization of interphase arrays in many cell types.

The fission yeast Schizosaccharomyces pombe serves as a simple model cell for studying MT dynamics and organization. These cells exhibit two types of structures that contain bundles of stable overlapping anti-parallel MTs: the interphase MT bundles and the mitotic spindle. One important factor for assembly and maintenance of these overlapping MT arrays is a conserved, diffusive MT-bundling protein, ase1p ((Löiodice 2005, Yamashita 2005). Ase1p, and its human (PRC1) and plant (MAP-65) orthologues, beside crosslinking MTs, is involved in the regulation of spindle elongation and serves as a complex regulatory platform for the recruitment of other midzone proteins at the spindle midzone (She et al. 2019). Ase1Δ mutants thus, while being viable, exhibit interphase MTs with reduced bundling and mitotic spindles that often fall apart in anaphase.

An important characteristic common to the Ase1/MAP65/PRC1 family is autonomous and preferential binding to MTs bundled in antiparallel fashion (She et al. 2019). This allows for the precise recruitment of other midzone proteins, but it is also known to have direct effects microtubule dynamics. In-vitro experiments have shown that MAP65-1, when bundling microtubules, promotes microtubule rescues (Stoppin-Mellet et al. 2013). Based on the modeling of their observed bundle dynamics, (Stoppin-Mellet et al. 2013) predicted MAP65-1 crosslinks in parallel microtubules to have less effect on microtubule dynamics. This would be in line with evidence showing that MAP65-1, like Ase1 (Janson et al. 2007) and PRC1 (Subramanian et al. 2010), preferentially binds to antiparallel overlaps (Gaillard et al. 2008). However, direct experimental evidence for whether microtubules bundled in parallel fashion are affected differently than microtubules bundled in antiparallel fashion is still outstanding. We here present the results of *in-vitro* assays which show that the depolymerization of microtubules in parallel bundles is indeed affected differently by Ase1 than those of microtubules in antiparallel overlaps.

In our assays, we also observed Ase1 to directly have an impact on the depolymerization of single microtubules. Furthermore, we observed Ase1 accumulating at the tip of depolymerizing microtubules. These findings indicate that Ase1 exhibits either biased diffusion or is being moved by powerstrokes performed by the bending protofilaments at the depolymerizing microtubule end, similar to the Dam1 complex and the Ndc80 complex which link kinetochores to microtubules (Gardner and Odde 2008, Powers et al. 2009, Grishchuk 2017).

## Figure 1

To study the interactions between diffusible MT crosslinkers and depolymerizing MT tips, we employed an *in vitro* assay: We performed total internal reflection (TIRF) time-lapse imaging of immobilized, GMPCPP-stabilized microtubule seeds under the presence of Ase1-neon, free tubulin, GTP, and a crowding agent in a buffered solution (Methods). With this, we observed dynamic, Ase1-decorated MT extensions growing from the MT seeds. These extensions bundled, or at higher angles of incidence, crossed, with other MTs they encountered. As has been reported before (Janson et al. 2007), we observed MTs antiparallel bundles to form readily even at large angles of incidence (up to 40°), while parallel bundles only formed at angles below 20° (Figure S1A). Due to this flexibility, and the high growth rates of MT plus ends, we often observed two growing plus ends passing each other, forming a “midzone” of crosslinked dynamic extensions. We recorded the lifetime of these *in-vitro* midzones, and compared them to the lifetime of dynamic extensions being crosslinked in a parallel orientation. Our results show that such “midzones” have a higher lifetime than parallel bundles (Figure 1A-E). This has been the case for both our experimental conditions: (1) At 42nM Ase1 in solution, where the Ase1 density on parallel bundles was much lower than on antiparallel bundles (as has been reported before, Ase1 has a higher affinity for antiparallel bundles (Janson et al. 2007)), and (2) at 420nM Ase1 in solution, where antiparallel and parallel bundles exhibited similar Ase1 densities, around two times the density of single MTs (Figure 1F). It bears noting that, due to the low growth rate of minus ends, we very rarely observed “midzones” established by two minus ends growing past each other, and we did not attempt to quantify the lifetime of the ovserved instances.

## Figure 2

The kymographs shown in Figure 1A-D already suggest that the prolonged lifetime of antiparallel bundles, in addition to the fact that two plus ends growing in opposite directions quickly created long regions of MT overlap, could partly be explicable by an increased rescue rate. A quantitative analysis of the microtubule dynamics which occurred in our assay confirms this: While growth rates and catastrophe rates were not strikingly different between antiparallel bundles, parallel bundles or single MTs, antiparallel bundles displayed greatly increased rescue frequencies at both 42nM and 420nM Ase1 in solution (Figure 2). These observations are in agreement with an earlier MAP65 study which compared single MT dynamics to MT dynamics within bundles (Stoppin-Mellet et al. 2013). However, the essay by (Stoppin-Mellet et al. 2013) did not allow to directly distinguish parallel versus antiparallel orientations as their MT bundles generally comprised more than 2 MTs with mixed polarities. While not ruling out a small effect size, our results do not confirm their modeling-based prediction that parallel bundles display more rescues than single MTs (our results do however confirm their prediction that antiparallel bundling induces more rescues than parallel bundling). Another point of misalignment with (Stoppin-Mellet et al. 2013) is our observation that Ase1 binding in our assay did affect MT shrinkage velocities. Notably, we observed the shrinkage velocity of both single and crosslinked MTs to be affected (Figure 1A). Additionally, plus ends in antiparallel overlaps shrunk decidedly slower than in parallel overlaps, or outside of overlaps. This suggests that Ase1 molecules within antiparallel overlaps have greater impact on MT shrinkage than Ase1 molecules in parallel overlaps, or outside of overlaps. It bears noting that the rescue frequency displayed by antiparallel overlaps did not differ between 42nM and 420nM, which can be explained by the apparent near-saturation of antiparallel overlap binding at already 42nM: Taking the median density of 0.19 molecules per nm and discounting for Ase1 binding to protofilaments not involved in the overlap by subtracting two times the median density for single MTs (0.03 molecules per nm, for density values see also Figure 1F), one arrives at a saturation of the available binding sites within the antiparallel overlap (assuming one protofilament per MT to be involved in overlap binding, i.e. one Ase1 binding site every 8nm). Further, as pointed out previously, at 420nM, bundles featured twice the density of single MTs, indicating that most Ase1 molecules which contributed to the bundle densities we measured at 420nM stemmed from Ase1 molecules not partaking in MT crosslinking. Thus, it seems likely that at 420nM, antiparallel and parallel overlaps featured a similar amount of Ase1 in their overlaps, rendering improbable that the differences we see between parallel and antiparallel MT dynamics are exclusively due to a lower amount of Ase1 molecules binding to parallel overlaps. Instead, the Ase1 inter-MT bond is likely to be stronger for antiparallel MTs, as indicated by the higher binding affinity for antiparallel overlaps and the heightened tendency of overlaps to form. We further confirmed this with a FRAP assay (Figure 2E).

## Figure 3

How does Ase1 interact with depolymerizing MT ends? A closer look at depolymerizing MTs suggests that there is an increased density of Ase1 at depolymerizing microtubule tips (Figure 3A-B, Figure S3A), reminiscent of what has previously been observed for Ndc80 complexes (Powers et al. 2009). Where we observed the occassional Ase1 cluster, the clusters were dragged with depolymerizing MT ends (Figure 3I, S3X). It thus suggests itself that the increase in Ase1 density we observed was due to sweeping of Ase1 molecules with the depolymerizing MT end, analogous to what has been proposed for the Dam1 complex and the Ndc80 complex. We investigated Ase1 sweeping by comparing the Ase1 density around a depolymerizing MT tip to the Ase1 density at the same spot on the MT before depolymerization had begun (Figures 3D-E, Methods). At 42 nM Ase1 in solution, we estimate that we observed a median number of 5 ± 13 Ase1 molecules being swept by depolymerizing single MTs, and 30 ± 35 molecules at 420 nM Ase1 (Figure 3E). During the course of depolymerization, the number of swept Ase1 molecules did not grow indefinetly, and at 420 nM saturated faster than we could resolve with our framerate (Figure S3A). In similar experiments with slightly different experimental conditions we could observe a gradual accumulation of Ase1 before saturation (Figure 3B) (for a quantification of the MT dynamics of these experiments, see Figure S2).

How does Ase1 engaged in MT crosslinking interact with depolymerizing MT ends? For MTs crosslinked in an antiparallel fashion, we observed a median of 3 ± 14 (42 nM Ase1) respectively 13 ± 19 (420 nM Ase1) molecules being swept (Figure 3E-F). Single MTs and antiparallely crosslinked MTs at 42 nM Ase1 thus swept similar amounts of Ase1 (Figure 3), despite the great difference in steady-state Ase1 density (Figure 1F). This suggests that few Ase1 molecules engaged in antiparallel crosslinking are being swept. For MTs crosslinked in a parallel fashion, we observed an amount of 0.1 ±4 molecules at 42 nM Ase1 respectively 14 ±31 molecules at 420 nM Ase1 being swept (Figure 3G-H).

## Mathematical modelling

What causes Ase1 sweeping? A model which has been proposed for Ram1 and Ndc80 is biased diffusion (Grishchuk 2017). In such biased diffusion, a diffusing molecule, upon encountering the depolymerizing microtubule end, is assumed to halt microtubule depolymerization, until the molecule diffuses away from the microtubule tip. We were interested in recapitulating the phenomena we observe in a mathematical model. FURTHER TEXT

Why do we have less sweeping for antiparallel overlaps? Could it be because of less depolymerization velocity? And/or less sweeping of bonding Ase1 molecules?

In order to understand the mechanism driving Ase1 accumulation at shrinking ends and its effect on microtubule depolymerization speed, we used a simple one-dimensional mathematical model (Fig. 4A, top). In this model, the microtubule is represented as a one-dimensional lattice. Assuming a constant concentration of Ase1 in solution, Ase1 molecules are represented as particles that can bind and unbind to a lattice site with constant rates (,). Binding is only allowed if the lattice site is empty. Bound Ase1 molecules undergo unbiased diffusion on the lattice with constant hopping rates (). Diffusion is only allowed if the target site is empty, and diffusion out of the lattice at either of the microtubule ends is forbidden (Red arrow in Fig. 4A). Systems containing microtubules and Ase1 have been previously modeled like this (Lansky et al. 2015, Johann et al. 2015, Lera-Ramirez et al. 2019). To introduce an assumption that would produce sweeping and slowing down of depolymerization velocity, we considered two mechanisms proposed previously for tip-tracking of a single diffusive particle on shrinking microtubules (Grishchuk 2017). (1) A power stroke model, in which the terminal subunit of the microtubule lattice, prior to its detachment, exerts a force on the attached diffusive molecule, effectively displacing it in the polymerization direction. (2) A biased diffusion model, in which the presence of the diffusive molecule at the terminal subunit of the microtubule reduces the rate of dissociation of the terminal subunit, and hence the depolymerization rate. We opted for the biased diffusion model, since it both reduces the depolymerization velocity and produces tip-tracking. We introduced the assumption that the detachment rate of the terminal subunit at the microtubule plus end is when Ase1 is not bound to it, and when Ase1 is bound to it. is simply a parameter that goes from 0 to 1. When it is 1, the microtubule never depolymerizes if Ase1 is at the tip. When it is 0, the presence or absence of Ase1 does not affect the depolymerisation rate (Fig. 4A, bottom). Importantly, if is 0 Ase1 does not accumulate, as it detaches with unbinding subunits. Therefore, the fact that the plus end acts as a barrier for diffusion of Ase1 molecules (red arrow on Fig. 4A) is not sufficient to produce accumulation of Ase1 at shrinking ends, as in principle tubulin subunits may unbind when Ase1 is attached to them. Instead, the ability to prevent tubulin subunit detachment is required for either tip-tracking of a single molecule, or for accumulation of multiple molecules at the shrinking plus end.

## Discussion

* We show that the presence of diffusible microtubule crosslinkers can suffice to establish enduring microtubule midzones, which may be important for organisms which do not possess strong microtuble rescue factors such as CLASP (see e.g. (Bratman et al. 2007)).
* Our results show that Ase1 can suffice to selectively yield enduring antiparallel midzones, while not substantially increasing the stability of parallel overlaps or single microtubules.
* For filament overlaps, it has been ovserved that F-actin crosslinkers slow down actin depolymerization (Schmoller et al. 2011), and that MAP-65-1 promotes rescues. Our results confirm that inhibition of depolymerization may be a widespread feature of cytoskeletal crosslinkers. This may also have significance for understanding how PRC1 and kinesin-4 are sufficient to generate stable bipolar overlaps (Hannabuss et al. 2019).
* The impact of Ase1 on microtubule dynamics may be tunable by posttranslational modifications of either Ase1 or the microtubule surface. Such a tunability has recently been proposed for a seemingly related capacity of Ase1, namely the braking of microtubule sliding caused by molecular motors (Thomas et al. 2020).
* The cause for Ase1 sweeping during microtubule depolymerization could also be powerstrokes by protofilaments (Grishchuk 2017), or possibly a combination of biased diffusion and powerstrokes.
* We observed sweeping of Ase1 to be low in antiparallel overlaps. If, as we estimate, we observed a saturation of binding sites within antiparallel overlaps at our experimental conditions, this is not surprising, as sweeping in such case likely cannot substantially raise the Ase1 density. The low mobility of Ase1 in antiparallel overlaps may also contribute to reduced sweeping in these instances. Lastly, it is possible that Ase1 sweeping on single microtubules is primarily caused by power strokes of bending protofilaments, which may not occur for crosslinked protofilaments due to protofilament straightening.
* The same hypotheses could explain why antiparallel microtubule crosslinking has such a pronounced effect on microtubule depolymerization, while its effect on single microtubules is weaker.
* Optical-trapping studies with Dam1 have shown that applying load on Dam1 complexes at depolymerizing microtubule ends reduces depolymerization velocity (Franck et al. 2007). We show that load-bearing is not necessary for diffusible MAPs to slow down microtubule depolymerization, which may contribute to the (SIGNIFICANCE?)
* Since such sweeping has not been previously been reported in a similar assay with the plant Ase1 homologue MAP65 (Stoppin-Mellet et al. 2013), we enquired the first author of that paper about similar observations. Indeed, in that assay, sweeping of MAP65 could indeed sometimes be observed (Figure S1), albeit to a lesser degree than in our assay.