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Microtubule nucleation during central spindle assembly requires NEDD1 phosphorylation on Serine 405 by Aurora A

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& - This manuscript is dedicated to Jean Yves Cremet who passed away April 7th, 2014

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ABSTRACT

During mitosis, the cell sequentially constructs two microtubule-based spindles to ensure faithful segregation of chromosomes. A bipolar spindle first pulls apart the sister chromatids, then a central spindle further separates them away. Although the assembly of the first spindle is well described, the assembly of the second remains poorly understood. We report here that the inhibition of Aurora A leads to an absence of the central spindle due to a lack of nucleation of microtubules in the midzone. In the absence of Aurora A, the HURP and NEDD1 proteins that are involved in nucleation of microtubules fail to concentrate in the midzone. HURP is an effector of RanGTP and NEDD1 serves as an anchor for the γ TURC. Interestingly, Aurora A already phosphorylates them during assembly of the bipolar spindle. We show here that the expression of a NEDD1 isoform mimicking Aurora A phosphorylation is sufficient to restore microtubule nucleation in the midzone in a context of Aurora A inhibition. These results reveal a new control mechanism of nucleation of microtubules by Aurora A during assembly of the central spindle.

INTRODUCTION

Achievement of faithful segregation of chromosomes relies on the coordination of a complex set of events: it begins with anaphase A and B, then continues with telophase and ends with the final abscission step which allows the physical separation of daughter cells. From anaphase onset to abscission the events controlling chromosome segregation and physical separation of the two daughter cells must be tightly coordinated (Mendoza and Barral, 2008) (Fededa et al., 2012). Any mistakes during the coordination of these events can lead to severe aneuploidy and chromosomal instability. These two defects are now largely recognized as responsible for tumorigenesis (Ganem et al., 2007), but are also believed to participate in cancer cell resistance to chemotherapeutic treatments (Puig et al., 2008). During anaphase A, the movement of chromosomes towards the spindle poles relies mainly on the depolymerization of microtubules (MTs) in the bipolar spindle. In anaphase B, on the other hand, the separation of chromosomes continues thanks to the polymerization of MTs in a central spindle assembled between the two sets of chromosomes already separated (Scholey et al., 2016). In addition, this anaphase central spindle is also involved in preventing mis-segregation of mis-attached chromosomes (Puig et al., 2008) (Scholey et al. 2016) and serves as a scaffold to recruit structural and regulatory proteins required for the establishment of the acto-myosin ring (Akhshi et al., 2014). The central spindle arises from the rapid growth of MTs that mainly emerge from the inter-chromosomal region during early anaphase (Uehara and Goshima, 2010) and are then stabilized and bundled to form an array of antiparallel MTs. The establishment of the central spindle requires MT associated proteins that participate in nucleation, stabilization and bundling, as well as signalling molecules that regulate the

different processes required for the assembly. Only a few molecular effectors have yet been identified such as the RanGTP effector HURP, TACC3 and CLASP proteins (Lioutas and Vernos, 2013) (Inoue et al., 2004) ((Maton et al., 2015). After this phase of polymerization, MTs are then amplified using pre-existing MTs as templates in an augmin-dependent manner (Uehara and Goshima, 2010) and concomitantly stabilized and bundled to form the robust central spindle through the recruitment of the centralspindlin complex (Uehara and Goshima, 2010) (Mishima et al., 2002) (Mishima et al., 2004). The size of the central spindle is regulated by Aurora B-dependent phosphorylation of Kif2A (Uehara et al., 2013) and Kif4A (Nunes Bastos et al., 2013) (Hu et al., 2011). All these proteins are also involved in the process of MT polymerization and/or stabilization during prometaphase (Koffa et al., 2006) (Wong and Fang, 2006) (Maton et al., 2015) (Zhang et al., 2018) (Ganem et al., 2004) (Wandke et al., 2004). The structural similarities between bipolar and central spindle assembly organization have led to the assumption that these two spindles are assembled and regulated according to similar principles. However, recent work has shed light on differences between the mechanisms controlling bipolar spindle and central spindle assemblies (Hu et al., 2011).

Aurora A kinase is one of the major regulators of mitotic spindle assembly. While the prometaphase functions of Aurora A are now quite well understood, its post-metaphase role has only recently been clearly demonstrated by Hegarat and colleagues (Hégarat et al., 2011). In their study they used a conditional knock-out of Aurora A and a pharmacological inhibition of Aurora B to show that these two kinases likely cooperate to control MT depolymerization at the end of anaphase. Additionally, Lioutas and Vernos have used the Aurora A kinase

inhibitor MLN8237 to show that Aurora A participates in nucleation and stabilization of central spindle MTs at least in part by phosphorylating TACC3 (Lioutas and Vernos, 2013). Moreover, we have previously used a chemical genetics approach to demonstrate that Aurora A is involved in central spindle stabilization through P150Glued phosphorylation (Reboutier et al., 2013) and in Spindle Assembly Checkpoint maintenance (Courthéoux et al., 2018). Whether Aurora A regulates MT nucleation at the midzone through phosphorylation of targets other than TACC3 during central spindle assembly remained an open question that we address in the present work. We show that inhibition of Aurora A during MT regrowth in anaphase leads to an absence of MT in the midzone demonstrating that the kinase is indeed required for central spindle MTs nucleation. We also show that upon Aurora A inhibition, one of its prometaphase substrate, the RanGTP effector HURP, failed to relocate from KT-MTs in prometaphase to central spindle MTs during anaphase. Finally, we show that phosphorylation by Aurora A, of the gamma-Tubulin Ring Complex (γ TURC) adaptor protein NEDD1 on Ser405, another prometaphase substrate of Aurora A, is required for MT nucleation in the midzone. These findings shed new light on the molecular mechanisms underlying the initial steps of central spindle MT nucleation, and reveal a role of Aurora A in the process of central spindle assembly.

RESULTS

Aurora A activity is required for midzone MT nucleation during central spindle assembly.

Aurora A has been implicated in MT nucleation from both centrosomes and chromosomes during prometaphase (Pinyol et al., 2013) (Sardon et al., 2008) (Reboutier et al. 2012) (Terada et al., 2003) (Scrofani et al., 2015). Here, we analysed its function in regulating MT nucleation during central spindle assembly in anaphase. Specific and rapid inhibition of Aurora A activity in early anaphase was achieved using two methods: a previously validated chemical genetics system as well as the Aurora A inhibitor MLN8237 (Reboutier et al., 2013) (Courthéoux et al., 2018). Briefly, in the first approach, we used two U2OS cell lines: WT-AurA cells (negative control) that stably express a GFP-tagged wild type version of Aurora A, and AS-AurA cells that stably express a GFP-tagged mutated version of Aurora A that can be specifically inhibited by the ATP analogue 1-Naphthyl-PP1 (1-Na-PP1). Both transgenic Aurora A coding sequences contain silent mutations that make them resistant to RNA interference (siRNA). As a consequence, following endogenous Aurora A downregulation by siRNA treatment, these two cell lines solely express exogenous wt-AurA or as-AurA proteins but at levels similar to the endogenous one (Figure 1A). In parallel, we also used U2OS cells that do not express any exogenous Aurora A (Ctl-AurA) in which we inhibited the kinase with its inhibitor MLN8237 (250 nM) (MLN-AurA) for a short time window (5 min) (Lioutas and Vernos, 2013) (Cheeseman et al., 2011) (Zheng et al., 2010) (Asteriti et al., 2014).

To address whether Aurora A is involved in the nucleation of MTs during anaphase, we carried out a depolymerization and regrowth assay and followed the growth of MTs using live-cell microscopy in cell lines expressing GFP-Tubulin. Briefly, WT-AurA and AS-AurA cells were first depleted of endogenous Aurora A with siRNA treatment (Figure 1B). All cell lines were then synchronized in anaphase by first arresting them in early G2 phase with the CDK1 inhibitor RO3306 (Vassilev, 2006) (Coldwell et al., 2013) (Ma and Poon, 2006) (Tsai and Zheng, 2005) and then by releasing the cells for 35 min. As soon as they entered anaphase (defined by the beginning of chromosomes separation), WT-AurA and AS-AurA cells were treated with 10 μ M 1-Na-PP1 and normal U2OS cells with 250 nM MLN (MLN-AurA) for five minutes. At these concentrations 1-Na-PP1 and MLN show high specificity for Aurora A (Reboutier et al., 2013) (Lioutas and Vernos, 2013). Cells were then maintained for 20 min in cold medium containing 1-Na-PP1 or MLN with 10 ng/mL nocodazole to depolymerize MTs. Nocodazole was then removed by washing cells with warm medium containing 1-Na-PP1 or MLN, and the cells were imaged using a spinning-disc confocal microscope. WT-AurA cells showed progressive nucleation of MTs both at the spindle poles and in the midzone (100%, n = 13, Figure 1B, first row) (like Ctl-AurA, not shown). In comparison, 90% of AS-AurA and MLN-AurA cells displayed a stronger rate of MTs growth at the spindle poles and a dramatic decrease in MT polymerization in the midzone (Figure 1B, second and third rows) with even a total absence of midzone MTs in the case of 33% of AS-AurA cells (n = 30).

To quantify the number of MTs in the midzone, we repeated the experiments, fixed the cells at different time points after the removal of nocodazole (t = 0, t = 30 sec, 2 min, or 5 min),

visualized MTs by immunofluorescence and manually counted them (Figure 1C and 1D). At $t = 0$, we observe that while there were no polar MTs in the WT-AurA, the Aurora A inhibited cells AS-AurA still presented MTs emerging from spindle poles (Figure 1C). In the midzone, however, the average number of MTs was significantly lower after inhibition of Aurora A (AS-AurA and MLN-AurA) than in cells containing active Aurora A (WT-AurA) (Figures 1D). We decided to inhibit Aurora A with MLN8732 in the rest of the study because we observed similar inhibition of central spindle assembly in AS-AurA or MLN-AurA, as already reported (Reboutier et al., 2013) (Lioutas and Vernos, 2013). Interestingly, when Aurora A was left active for the first five minutes of anaphase and inhibited only during the depolymerization and regrowth phases, we did not observe any defect in MT regrowth (Figure 2A and B). This likely means that Aurora A kinase activity is required for MT nucleation during the very beginning of anaphase. Inhibition of Aurora A kinase activity during anaphase is accompanied by decreased nucleation of MT in the midzone, indicating that Aurora A is critical for MT nucleation during central spindle assembly. But inhibition of Aurora A kinase activity in anaphase induces simultaneously an increased MT nucleation from centrosomes, such as if the factors necessary for the assembly of the central spindle remained localized to the centrosomes instead of migrating in the midzone. Aurora A might thus be required to relocalize MT nucleation factors from the bipolar spindle to the midzone. We decided to follow two of these factors HURP and NEDD1.

HURP localization is affected by Aurora A inhibition in anaphase.

HURP is involved in bipolar spindle assembly, KT-MT stabilization and interkinetochore tension prior to anaphase (Koffa et al., 2006) (Silljé et al., 2006) (Wong et al., 2008). HURP is phosphorylated by Aurora A, which increases its affinity for MTs and at the same time its stability. (Yu et al., 2005) (Wong et al., 2008). This prompted us to investigate the behaviour of HURP under Aurora A inhibition. We first decided to analyse Aurora A and HURP colocalization during mitosis. Using Aurora A-GFP expressing U2OS cells, we observed a partial colocalization between Aurora A and HURP in prometaphase (Figure 3A). As already reported, HURP and Aurora A localize along KT-MTs and accumulate at MT ends in the vicinity of kinetochore (Figure 3A, enlarge middle panel and graphic) (Courthéoux et al., 2018) (Wu et al., 2013). During anaphase A, HURP and Aurora A remain at KT-MTs but also appear at the new MTs in the central spindle (Figure 3B, first row enlarge zone, blue arrows). During anaphase B, KT-MTs are shorter, the HURP signal appears to decrease at KT-MTs but accumulates with Aurora A to freshly nucleated MTs at the central spindle (Figure 3B, second row, enlarged zone, blue arrows). We then asked whether inhibition of Aurora A with MLN8237 would affect HURP localization. In anaphase A, HURP still localizes to central spindle MTs but interestingly enough, no longer colocalizes with Aurora A (Figure 3B third row, enlarged view). In anaphase B however, HURP was not found any more at the midzone (Figure 3B, bottom row, 3C and 3D), only rare central spindle MTs remained decorated with HURP (Figure 3B, bottom row, blue arrow). Our data indicate that Aurora A kinase activity is required for the presence of HURP in the midzone, although this

may be indirectly due to the absence of microtubules. We then decided to focus on a MT nucleation factor.

NEDD1 is required for microtubule nucleation at the central spindle during Anaphase and its localization is affected by Aurora A inhibition.

NEDD1 is the anchoring protein of the γ TURC that targets this complex to centrosomes, chromosomes, and pre-existing MTs to promote MT polymerization and growth during bipolar spindle assembly in prometaphase (Haren et al., 2006) (Lüders et al., 2006) (Zhang et al., 2009). Although NEDD1 appears to be a key component of several MT assembly pathways, a role for NEDD1 during central spindle assembly has not been described. Therefore, we decided to check if NEDD1 was involved in the midzone MT nucleation process. As previously described for Aurora A (Marumoto et al., 2005) (Reboutier et al., 2013), NEDD1 was also present as dots on the central spindle during anaphase A (Figure 4B, first row). A reduction in the number of NEDD1 dots at the central spindle was observed after NEDD1 siRNA treatment (Figure 4A) indicating that the dots we followed corresponded to NEDD1 protein (Figure 4B, second row). The use of super resolution revealed that many NEDD1 dots on the central spindle also contained Aurora A suggesting that the proteins could organize into clusters (Figure 4C bottom row, blue arrows).

We examined the effect of NEDD1 depletion on central spindle assembly (Figure 4D). When significant depletion of NEDD1 was achieved, about two third of the cells presented serious mitotic spindle defects and did not progress through prophase. Yet, one third of the cells, likely less depleted of NEDD1, entered anaphase. Among these cells, 95% (n=20 in 3

independent experiments) displayed a strong central spindle assembly defect never observed in control conditions. In particular, NEDD1 depleted cells had an overall weaker α -Tubulin signal in the midzone compared to control cells (Figure 4D compare the first with the second row), meaning that NEDD1 depleted cells had nucleated fewer MTs in the midzone. Furthermore, the fluorescence intensity peaks in the midzone, that are characteristic of bundled MTs in control conditions, were absent when NEDD1 was depleted (Figure 4D).

NEDD1 phosphorylation on Serine 405 by Aurora A is involved in microtubule nucleation at the central spindle during Anaphase

We tested whether Aurora A is involved in NEDD1 phosphorylation during anaphase and whether this event is required for the nucleation of MTs in the midzone. In order to obtain a large majority of cells synchronized in late mitotic phases, particularly in anaphase, we used a pharmacological approach developed by Hu and colleagues (Hu et al., 2008). This approach involves blocking cells in monopolar prometaphase with S-trityl-L-cysteine (STLC, a Kinesin-5 inhibitor), and then forcing them to progress through and exit mitosis using Purvalanol A (a CDK1 inhibitor) (Hu et al., 2011) (Hu et al., 2008) (Ozlu et al., 2010). To evaluate the phosphorylation state of NEDD1 during anaphase, we blocked WT or ASAurA cells depleted for endogenous Aurora A in monopolar mitosis with STLC. We then treated cells with Purvalanol A, and after five minutes added 1-Na-PP1 to ensure that the cells had received the inhibitor while exiting mitosis (Figure 5A). Interestingly, while WTAurA cells treated with 1-Na-PP1 exhibited a robust spindle constituted of parallel MTs (Figure 5B, upper row), ASAurA cells treated with 1-Na-PP1 presented a diffused and disorganized MT

network (Figure 5B, lower row). Phosphorylation of NEDD1 was analysed by Western Blot in extract prepared from control cells or from Aurora A inhibited cells synchronized in monopolar mitotic exit (cells were lysed at $t = 15$ min) (Figure 5C). In control conditions (WTAurA + 1-Na-PP1), NEDD1 appears as two bands, with the upper band being the most abundant form (Figure 5C, first lane). Treatment of this extract with λ -Phosphatase (λ PP) resulted in the disappearance of the upper band and an increase in the intensity of the lower band, indicating that the upper band corresponds to the phosphorylated form of NEDD1 and the lower band to the unphosphorylated form (Figure 5C, third lane). In comparison, when Aurora A was inhibited (AS AurA + 1-Na-PP1), two bands with similar intensities were detected (Figure 5C, second lane), suggesting that Aurora A participates to NEDD1 phosphorylation during anaphase. To further investigate the role of Aurora A at the central spindle, we asked whether NEDD1 and Aurora A could colocalize at MT nucleation sites after recovery from cold shock. 15 seconds after recovery, cells stably expressing Aurora A-GFP were immunostained for NEDD1 and tubulin. And indeed, the Super-resolution images reveal a colocalization of NEDD1, Aurora A and tubulin at the midzone when MTs start to repolymerize (Figure 6A, blue arrows). Because NEDD1 is directly phosphorylated on Serine 405 by Aurora A in prometaphase to regulate MT nucleation from chromosome during bipolar spindle assembly (Pinyol et al., 2013), we decided to focus on this phosphorylation event in anaphase. We expressed various Flag-tagged phosphorylation mutants of NEDD1 in cells depleted for endogenous NEDD1 (Figure 6B). The level of Flag-NEDD1 protein expressed was controlled by western blot, the WT version of NEDD1 runs faster than the flag-tagged variants on SDS-PAGE, both isoforms can be seen on lane 3 of the figure 6B.

We then counted the number of microtubule fibers present in the midzone. Interestingly, depletion of NEDD1 or expression of an isoform that cannot be phosphorylated on Serine 405 by Aurora A (SA-NEDD1) leads to a major decrease in central MT nucleation after cold shock recovery (Figure 6C, compare #1 with #2 and #5). This defect is rescued either by the WT-NEDD1 or the phosphomimetic mutant SD-NEDD1 (Figure 6C, #4 and #6, and Sup table1). To confirm that Aurora A is responsible for NEDD1 phosphorylation on Serine 405 required for MT nucleation at the midzone, we inhibited Aurora A using the analogue sensitive allele and expressed NEDD1 mutants in a cold shock and recovery experiment. Endogenous Aurora A was depleted by siRNA and different Flag-tagged NEDD1 variants were expressed (WT, SA and SD) (Figure 7A, #3, #4, and #5). Cells were immunostained with anti-tubulin and anti-Flag antibodies, and MTs were counted in the midzone at different time points after recovery (Figure 7B, blue arrows). Only expression of the NEDD1 phosphomimetic mutant (SD) partially rescued Aurora A inhibition suggesting that NEDD1 phosphorylation on Serine 405 by Aurora A is necessary for MT nucleation at the central spindle (Figure 7C, series #5 and Sup table 2). We have analysed the localization of WT-NEDD1 and phospho-mutants S405A and S405D by expressing FLAG-tagged versions of the protein and quantifying their level in the midzone in the presence of active Aurora A or in the presence of MLN. In the presence of active Aurora A, the same level of WT-NEDD1 and S405D-NEDD1 was present in the mid-zone while the amount of S405A-NEDD1 was much smaller. More importantly, in the presence of MLN, the level of WT-NEDD1 decreased in the middle zone to reach a level equivalent to that of S405A-NEDD1. Also, the presence of S405D-NEDD1 in the middle zone was insensitive to MLN (Figures 8A and B). This

clearly demonstrates that Aurora A-dependent NEDD1 phosphorylation on serine S405 is necessary to localize NEDD1 in the mid-zone in order to nucleate MTs for central spindle assembly.

DISCUSSION

We have previously shown that Aurora A is involved in central spindle assembly (Reboutier et al., 2013). Here, we aimed to better understand the function of Aurora A during this event and identify the molecular mechanism(s) that contribute to this function. To this end, we used two complementary approaches: (i) a chemical genetics strategy we developed previously (Reboutier et al., 2013) (Courthéoux et al., 2018) and (ii) a pharmacological inhibition of Aurora A with the MLN8237 inhibitor. Using this drug, we confirmed results previously obtained with the chemical genetics strategy showing that inhibition of Aurora A during early anaphase caused the disappearance of the central spindle (Reboutier et al., 2013). We also confirmed results from Lioutas and Vernos showing that inhibition of Aurora A in early anaphase triggers a strong decrease in the nucleation rate of MTs within the midzone (Lioutas and Vernos, 2013). Furthermore, our data indicate that inhibition of Aurora A triggers the stabilization of polar MTs and increases their growth. Similar results have been previously obtained by Floyd and colleagues, who showed that stabilization of Aurora A after Cdh1 depletion, results in brighter tubulin signal at the spindle poles during mitotic exit (Floyd et al., 2008). An increase in the stability and/or growth of polar MTs may per se explain central spindle perturbation. However, these results suggest that factors normally contributing to midzone MT nucleation are likely retained at the centrosomes when Aurora A is perturbed.

Interestingly, our results indicate that Aurora A activity is required within a short window of time at the entry to anaphase. Inhibition of Aurora A more than five minutes after anaphase onset results in no observable effect on midzone MT nucleation or central spindle assembly. Furthermore, our results show that the earlier Aurora A is inhibited, the stronger the effect on the central spindle assembly is. This likely means that Aurora A is not required for central spindle assembly when midzone MT nucleation is already at an advanced stage. Overall, these results indicate that the central spindle defect related to inhibition of Aurora A is likely explained by the contribution of the kinase to the initial nucleation of midzone MTs.

Our goal was then to identify Aurora A targets implicated in the nucleation of midzone MTs. Since the Ran-GTP pathway has been shown to be involved in central spindle assembly (Uehara and Goshima, 2010), we chose to focus our attention on two prometaphase substrates of Aurora A, and components of the Ran-GTP pathway, HURP and NEDD1. HURP's interaction with MTs is regulated by Aurora A through phosphorylation of its C-terminal domain (Wong et al., 2008). Here we observed an absence of HURP in the midzone during anaphase under Aurora A inhibition, suggesting that the kinase regulates HURP also in anaphase. However, multiple reasons can explain the absence of HURP in the midzone. It could be a consequence of the absence of MT in this area, since the location of HURP depends on the presence of MTs (Koffa et al., 2006). It might also be due to an absence of HURP phosphorylation by Aurora A leading to its degradation (Wong et al., 2008).

But how does MT nucleation starts in the midzone? Could Aurora A participate in early central spindle assembly events? We decided to ask whether the γ TURC anchoring protein NEDD1 required for MT nucleation during prometaphase would play a similar function in

anaphase (Haren et al., 2006). To investigate the implication of NEDD1 in the early steps of central spindle assembly, we first examined NEDD1 localization and the consequences of its depletion. NEDD1 localized to the central spindle MTs with Aurora A, and in addition, Aurora A localized to NEDD1-tubulin dots after recovery from cold shock. Finally, NEDD1 depletion triggered strong defects in the central spindle assembly, with less MTs bundled. NEDD1 functions are highly regulated by mitotic kinases such as CDK1, PLK1, Aurora A, and Nek9. Its phosphorylation regulates the various functions of NEDD1 and is required, for example, for the binding of NEDD1 to the γ TURC and for its targeting to the centrosomes, the spindle, and the chromosomes (Haren et al., 2006) (Lüders et al., 2006) (Zhang et al., 2009) (Manning et al., 2010) (Johmura et al., 2011) (Gomez-Ferreria et al., 2012) (Sdelci et al., 2012) (Pinyol et al., 2013). NEDD1 is also phosphorylated by Aurora A on Ser405 during pre-anaphase stages. This event is critical for RanGTP aster formation and chromatin-driven MT assembly in *Xenopus* egg extracts (Scrofani et al., 2015). Despite this complex phosphorylation profile during the cell cycle, NEDD1 appears predominantly as two bands in western blots, the upper being the phosphorylated form (Lüders et al., 2006) (Zhang et al., 2009) (Johmura et al., 2011) (Sdelci et al., 2012) (Gomez-Ferreria et al., 2012) (Pinyol et al., 2013). In anaphase, we detected those two bands of NEDD1, the upper one being the strongest. Inhibition of Aurora A resulted in a decrease in intensity of the upper band concomitant with an increase in the lower one, indicating that Aurora A also participates to NEDD1 phosphorylation after metaphase. Interesting enough, the S405A NEDD1 mutant protein does not localise to the midzone while the S405D NEDD1 (that mimics constitutive phosphorylation) localises therein in an Aurora A independent manner. Furthermore, in

depolymerization and regrowth assays during anaphase, the S405D NEDD1 mutant partially rescued the midzone MT nucleation defect induced by Aurora A inhibition. The fact that this rescue is only partial suggests that the kinase phosphorylates multiple substrates to control anaphase, and that NEDD1 is one of them. And indeed Aurora A phosphorylates p150Glued and TACC3 during anaphase, a lack of phosphorylation of these substrates also affect central spindle (Reboutier et al., 2013) (Lioutas and Vernos, 2013). How those Aurora A substrates cooperate to control central spindle assembly remains to be found. For instance, does TACC3 activates Aurora A in anaphase like already reported during meiosis (Pascreau et al., 2005)? Does p150Glued help to transport NEDD1 with dynein? We are currently working on these aspects.

As a whole, the data presented here indicate that NEDD1 phosphorylation on Ser405 by Aurora A is crucial for midzone MT nucleation during the initial steps of the central spindle assembly. Our work provides new insight into the regulatory mechanisms of MT nucleation during the initial steps of central spindle assembly. Specifically, we show for the first time that, once the mitotic spindle is correctly built and the checkpoint satisfied, central spindle assembly requires the prompt redistribution of NEDD1, and likely other MT nucleation factors, from centrosomes to the midzone. Moreover, we show that this event requires Aurora A-dependent phosphorylation of NEDD1 on Ser405. This initial nucleation step is then followed by the processes of MT amplification and stabilization to ultimately build a robust and functional central spindle (Uehara and Goshima, 2010).

MATERIALS AND METHODS

DNA constructs, siRNA sequences

The DNA constructs allowing WT- or ASaurA stable expression in U2OS cells have previously been described in Reboutier and colleagues (Reboutier et al., 2013). The DNA constructs allowing the expression of the WT, S405A or S405D flag-tagged versions of NEDD1 have previously been described in Pinyol and colleagues (Pinyol et al., 2006). Aurora A and NEDD1 were depleted from cells using siRNA oligonucleotides with the sequences 5'-AUGCCCUGUCUUACUGUCA-3' and 5'-GGGCAAAAGCAGACAUGUG-3' respectively. For siRNA negative control experiments, cells were transfected with a siRNA oligonucleotide targeting Luciferase with the sequence 5'-CGUACGCGGAAUACUUCGA-3'.

Cell culture, synchronization and transfection

Human U2OS and HeLa cells were respectively grown in McCoy's and DMEM media with penicillin and streptomycin (Invitrogen) and 10% fetal calf serum (PAA). For siRNA transfection of co-transfection experiments, cells were transfected in culture medium using Jetprime (Polyplus Transfection) according to the manufacturer's instructions. The medium was changed after 24 h. For anaphase enrichment, cells were arrested in G2 with 10 μ M RO3306 (Calbiochem) for 16 h and then washed three times for 30 sec with the fresh pre-warmed medium. The first cells enter anaphase approximately 35 minutes after the first wash. For monopolar mitotic exit synchronization, we followed the protocol published by Hu and

colleagues (Hu et al., 2008). Briefly, cells were treated with 2 μ M STLC (Tocris) for 24 h and then released into mitotic exit by treatment with 30 μ M Purvalanol A (Tocris). Cells reached monopolar mitotic exit 15 minutes after Purvalanol A addition. 1-Na-PP1 was purchased from Tocris.

Microtubule regrowth quantification

After anaphase enrichment, the first anaphase was visualized using DIC and cells were then maintained in cold medium containing 1-Na-PP1 or MLN plus 10 ng/mL nocodazole for 20 min to depolymerize MTs. Nocodazole was then removed by washing cells with warm medium containing 1-Na-PP1 or MLN, and the cells were immunostained as described below. We quantified the number of MTs in the midzone by repeating the experiments in which we fixed the cells at different time points after the removal of nocodazole ($t = 0$, $t = 30$ sec, 2 min, or 5 min) visualized MTs by immunofluorescence (IF) and manually counted them.

Immunofluorescence

For experiments presented in figure 1B, 3E, 4B and S1C, cells were plated onto glass coverslips, fixed with methanol at -20°C for 10 min, and then washed three times in PBS and saturated with PBS-BSA 1% for 1 h at room temperature. For the other experiments, cells were plated onto glass coverslips, permeabilized in PBS Triton X100 0.1% for 30 sec and then fixed with 4% PFA in 10 mM MES, 138 mM KCl, 3 mM MgCl_2 , 0.1 g/mL sucrose and 2 mM EGTA, pH 6.1. Cells were then washed three times in TGS (Tris Glycin-SDS) buffer

and saturated with PBS-BSA 1% for 1 h at room temperature. Antibodies in PBS-BSA 1% were added onto the cells: Rat anti- α -Tubulin 1:1000 (clone YL1/2, Millipore), rabbit anti-HURP polyclonal 1:500 (HPA005546, Sigma), mouse anti-NEDD1 1:200 (clone MO5, Abnova), and mouse anti-Flag 1:1000 (clone M2, Sigma). The cells were incubated with antibodies overnight at 4°C, and then washed three times with PBS and incubated in the dark with secondary antibodies (Alexa 488-anti-Mouse or Alexa 555-anti-rabbit 1:1000, Invitrogen) for 1 h at room temperature. After three final washes with PBS, the coverslips were mounted with ProLong Gold (Invitrogen) with 1 μ g/mL DAPI (Sigma). Imaging was performed using Olympus IX-71 microscope, with a 60X oil immersion objective lens, controlled by Delta Vision SoftWorx (Applied Precision). Image stacks were deconvolved and quick-projected, except when specified in the text. For midzone MT quantifications, we manually counted each α -Tubulin labeled MT located in the midzone. Three independent experiments with 20 fixed cells per replicate were counted. For midzone tubulin fluorescence measurement, the mean fluorescence in a cropped region corresponding to the midzone was measured after background subtraction using ImageJ. The ImageJ software was used for NEDD1 dots quantification. The midzones were cropped, then we used the “Threshold function” to automatically create a mask showing NEDD1 dot as clusters of black pixels (using the Otsu algorithm), and the numbers of clusters were counted using the “Analyze particles” function.

Super resolution microscopy

Images from Figures 2A and B, 3A and B were acquired using Airyscan Zeiss microscope, using full Airyscan function for the four channels. Cells stably expressing Aurora A-GFP were immunostained using the PFA modified protocol described in the previous section. GFP Nanobooster was used as a secondary antibody (Chromotek, 1:500) to enhance GFP signal.

Live-cell imaging

Cells were grown in LabTek I chambered coverglass (Nunc). Before microscopy, the medium was changed to CO₂-independent medium supplemented with 10% FBS and 200 mM L-glutamine (Invitrogen). Time-lapse images were acquired using a Plan Apo 60x/1.4 NA objective on an Eclipse Ti-E microscope (Nikon) equipped with a spinning disk (CSU-X1, Yokogawa), a thermostatic chamber (Life Imaging Service), a Z Piezo stage (Marzhauser), and a charge-coupled device camera (CoolSNAP HQ2, Roper Scientific). Metamorph Software (Universal imaging) was used to collect the data. Frames were recorded every 30 seconds. Images are maximum projections of 20 Z planes acquired at 0.6 μ m steps. Time-lapse data were processed using Metamorph. Anaphase B onset corresponding to the beginning of central spindle assembly was determined as the moment when the first MTs of the central spindle was visible.

Statistical analysis

T-test of the mean was performed using IGOR © (Wavemetrics) and p-values are presented in table 1 and 2.

Western blot analysis

Cells were directly lysed in Laemmli buffer and proteins were resolved by SDS-PAGE (7% / 13% discontinuous gradient for Aurora A and Flag-tagged NEDD1, and 10% Anderson gels for NEDD1 phosphorylation (Anderson et al., 1973)) and transferred onto nitrocellulose membranes. The membranes were blocked for 1 h at room temperature with TBST 4% milk, and incubated overnight with the following antibodies: mouse anti-Aurora A 1:100 (clone 35C1) (Cremet et al., 2003), mouse anti-Flag 1:5000 (clone M2, Sigma), and mouse anti-NEDD1 1:2000 (clone MO5, Abnova). Membranes were incubated with secondary antibodies coupled to HRP (Jackson) for 1 h and antibody binding was detected by enhanced chemiluminescence (Pico or Dura, Pierce). Proteins extracts were treated with λ -Phosphatase according to the supplier's protocol (New England Biolabs).

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AUTHOR CONTRIBUTIONS

T.C. and D.R. contributed equally to this manuscript. Conceptualization, methodology, software, formal analysis, investigation and data curation: T.C. and D.R. Validation: T.C, D.R. and C.P. Resources: T.C., D.R., J-Y.C., I.V., C.B, Writing, review and editing: T.C, D.R and C.P. Supervision and project administration: C.P. Funding acquisition: T.C and C.P.,

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Figures

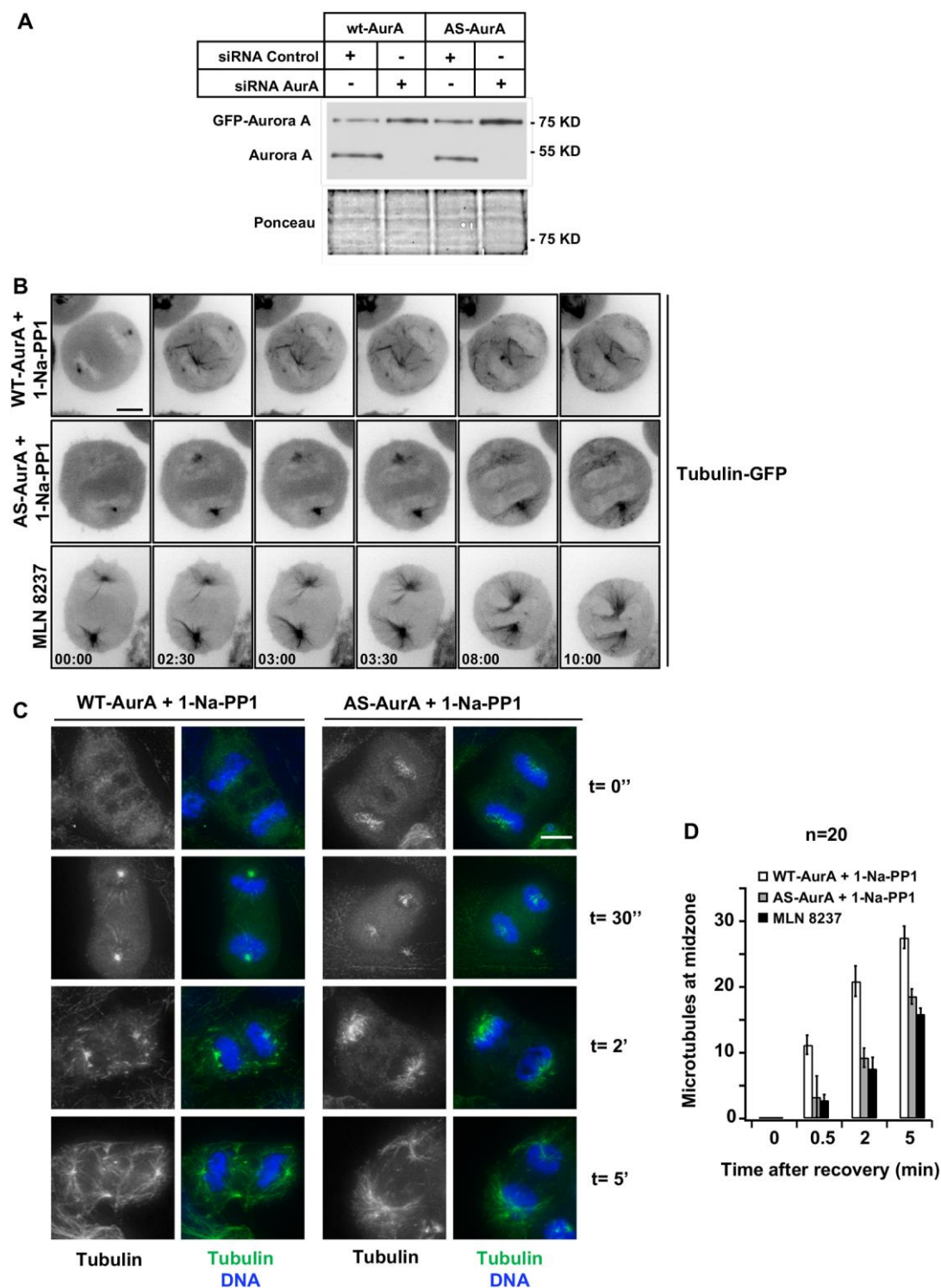


Figure 1 - Aurora A activity is required for midzone MT nucleation during central spindle assembly.

(A) Western blot analysis showing the abundance of endogenous Aurora A (bottom bands) and GFP-tagged Aurora A (upper bands) in WT- or AS-Aurora A cells depleted, or not depleted, of endogenous Aurora A.

(B) Fluorescence time-lapse microscopy images showing MT nucleation in WTAurA, ASaurA, and untransfected cells expressing GFP-Tubulin, which were depleted of endogenous Aurora A and treated with 10 μ M 1-Na-PP1 (for WTAurA and ASaurA) or U2OS GFP-Tubulin cells treated with 250 nM of MLN8237. Cells were treated at the metaphase to anaphase transition. MT regrowth begins at $t = 0$ (time in minutes). Scale bar, 5 μ m.

(C) Immunofluorescence microscopy images showing MT nucleation in WTAurA or ASaurA cells counted in **(D)**. Scale bar, 10 μ m. **(D)** Histogram showing the number of MTs in the midzone of WTAurA, ASaurA or MLN treated cells. Values are the mean of three independent experiments. MTs were counted in 20 cells for each experiment. Error bars show standard deviations.

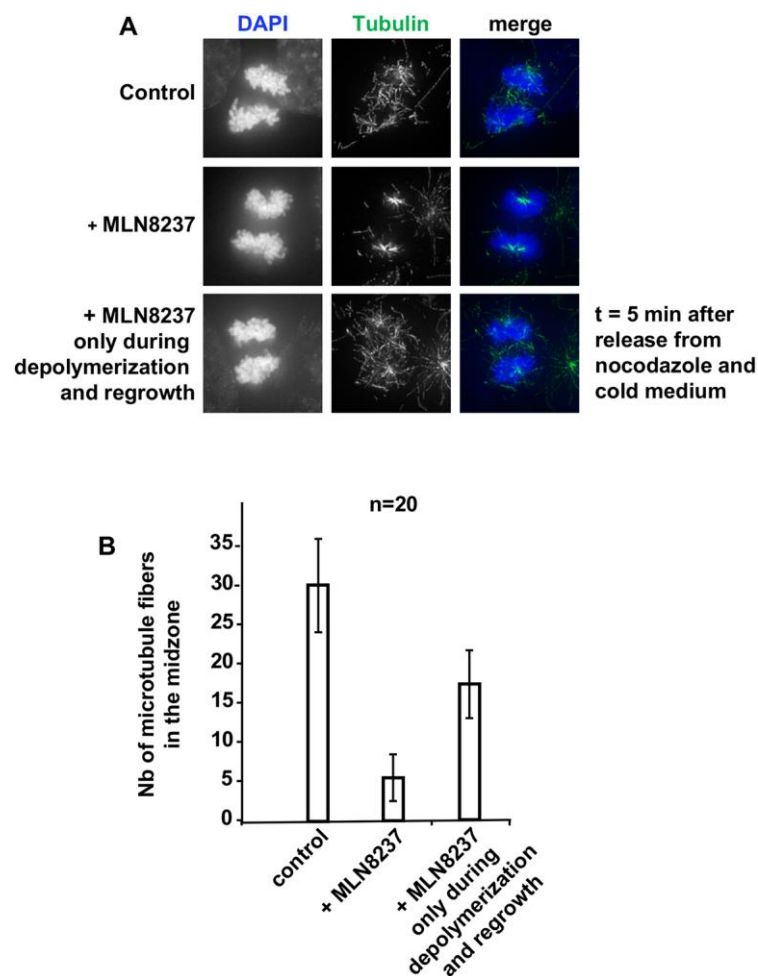


Figure 2 - Aurora A activity is required for midzone MT nucleation at the beginning of central spindle assembly.

(A) Fluorescence microscopy images showing MT nucleation in control or MLN8237 treated cells. Cells were treated at the metaphase to anaphase transition or only during depolymerization and regrowth. Scale bar, 10 μ m.

(B) Bar graph representing the number of MTs in the midzone of control cells or cells treated with MLN8237 only during depolymerization and regrowth. Values are the mean of three independent experiments. MTs were counted in 20 cells for each experiment. Error bars show standard deviations.

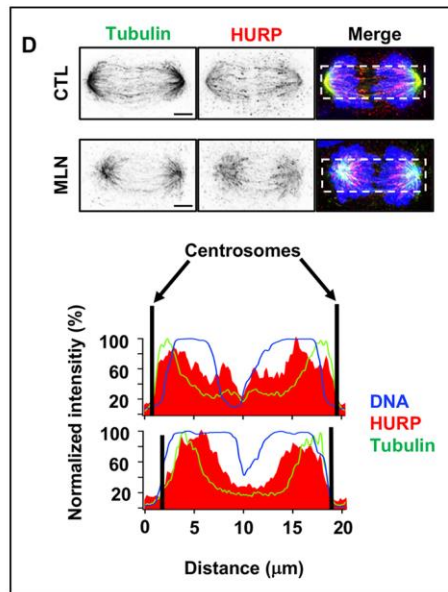
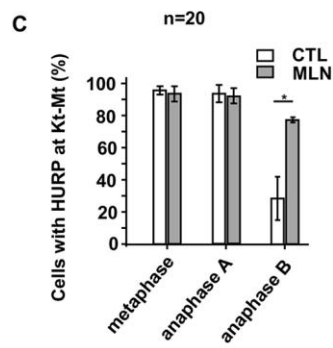
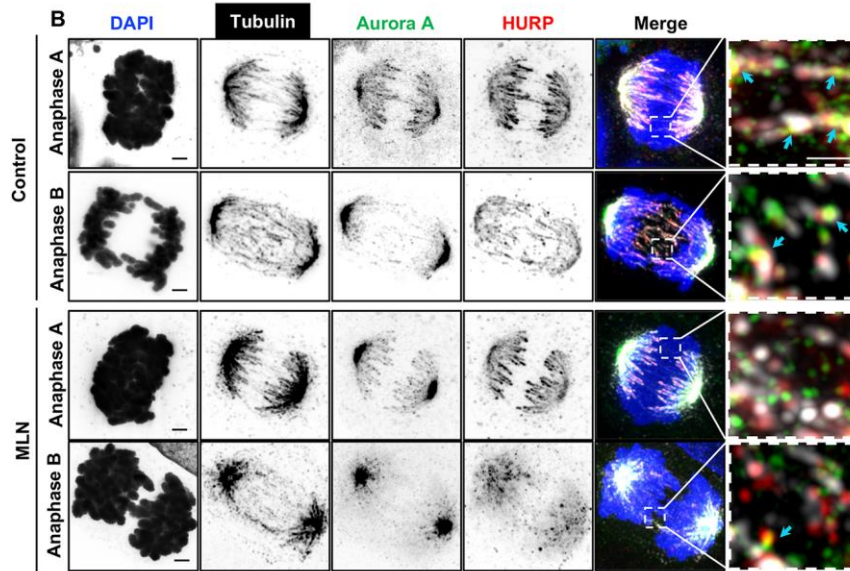
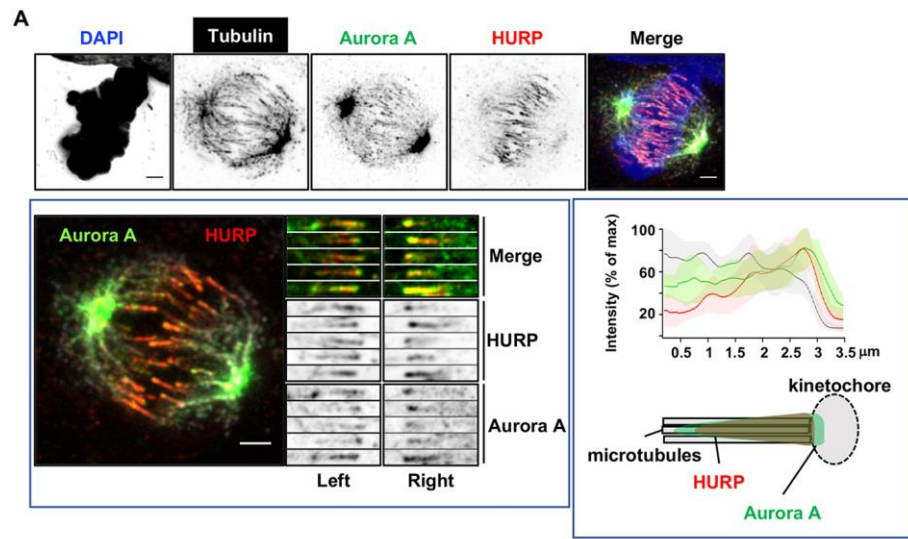


Figure 3 - HURP localization is affected by Aurora A inhibition during mitosis.

(A and B) Super resolution images of U2OS cell expressing Aurora A-GFP under an endogenous promoter. Cells were immuno-stained in prometaphase **(A)** or in anaphase **(B)** and projected in z. Scale bar, 3 μ m.

(A) top row prometaphase cells stained for DNA, tubulin, Aurora A and HURP. Bottom-left panel, enlarged picture of prometaphase cell showing only HURP and Aurora A colocalization at kinetochore-microtubule bundles. Bottom-middle small panels, crop sections of kinetochore-microtubule bundles at the left and right side of the spindle shown in left. Bottom-right panel, Line scans from black middle crop sections. Aurora A (green) colocalizes with HURP (red) at the minus end of KT-MT bundles (black). HURP, Tubulin, and Aurora A intensities were collected along 20 KT-MTs. Mean intensities with standard deviation were plotted over distance. A scheme representing the position of both HURP and Aurora A relative to MTs and kinetochore is shown.

(B) HURP and Aurora A localization during anaphase A and B. Right panel, an enlarged section of the central spindle showing HURP, Aurora A, and Tubulin colocalization (blue arrows) in control and MLN treated cells. Scale bar, 3 μ m.

(C) Bar graph showing the number of cells with HURP enriched toward KT-MTs in control or MLN8237 treated cells. Values are the mean of three independent experiments. At least 20 were counted for each experiment. Error bars show standard deviations. * $p < 0.001$.

(D) Fluorescence microscopy images showing the localization of HURP in control or MLN8237 treated HeLa cells that do not express any exogenous Aurora A. Scale bar, 5 μ m. Right panel, a diagram showing the intensity of the HURP and DNA labelling along the

spindle axis in control or MLN8237 treated HeLa cells. Insets show the area of measurement in both cells.

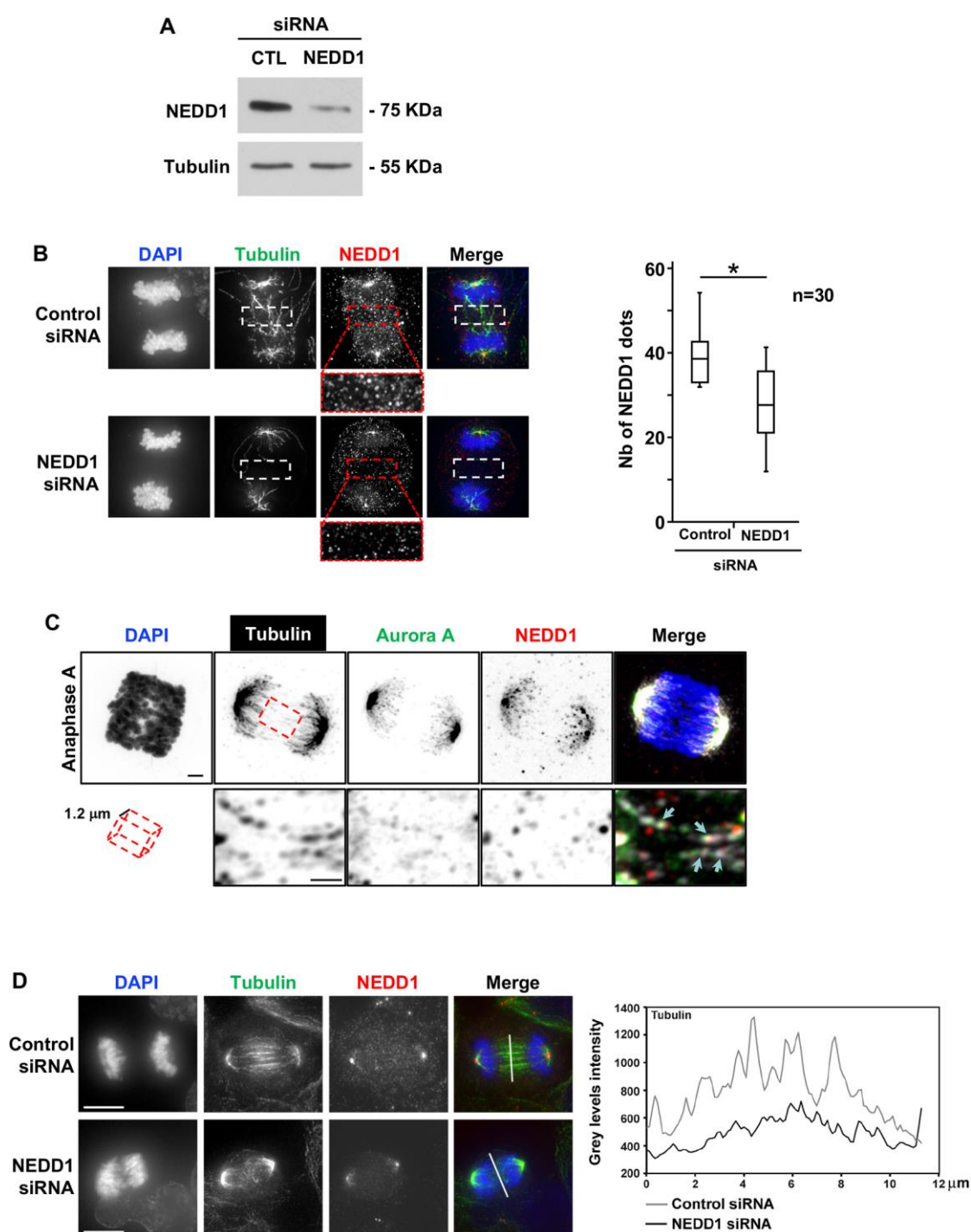


Figure 4 - NEDD1 localizes at the midzone and its depletion affects MT nucleation at the central spindle.

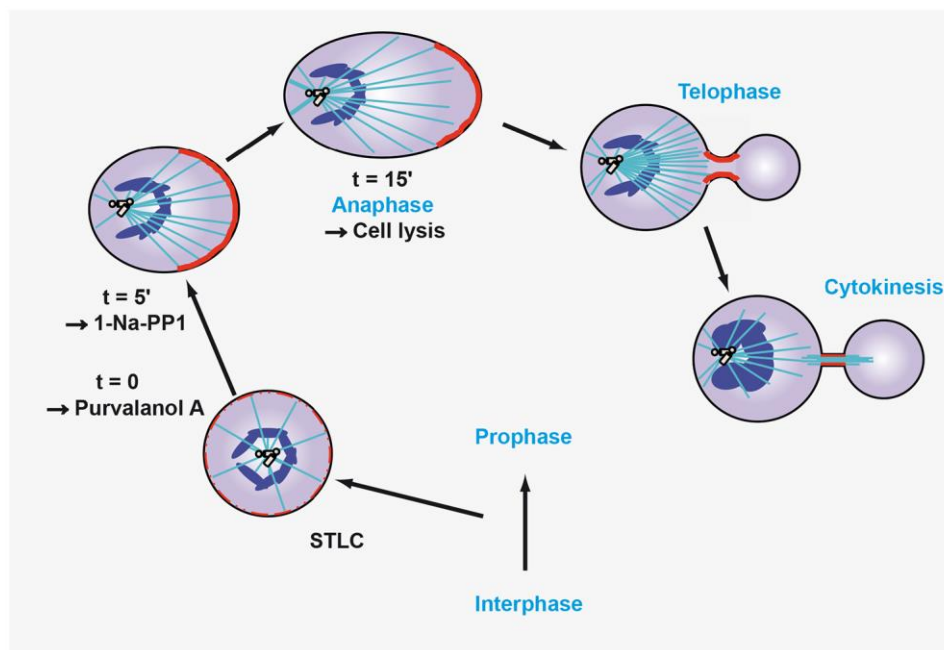
(A) Western blot showing the efficiency of NEDD1 protein depletion analysis by siRNA in the extracts prepared from cells representative of those imaged in (B and D).

(B) Fluorescence microscopy images showing the MTs (tubulin) and the localization of NEDD1 during MTs depolymerization and regrowth assay in control or NEDD1 depleted cells. Insets show enlargements of the midzone area. Scale bar, 6 μm . Right, the number of NEDD1 dots in the midzone of control or NEDD1 depleted cells. * $P \leq 0.004$. Values originate from three independent experiments. Dot numbers were measured in 30 cells for each experiment.

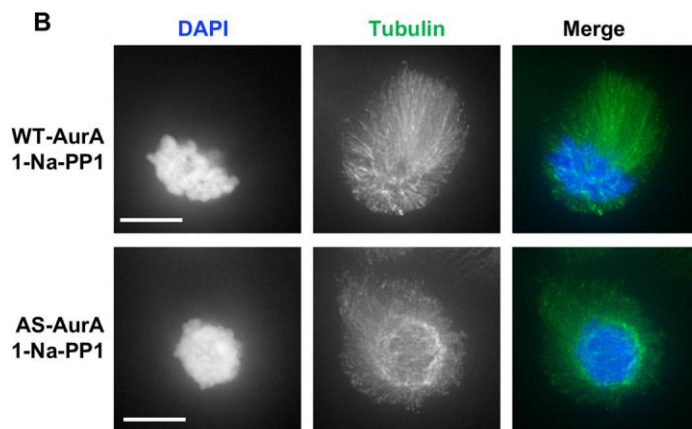
(C) Super resolution images of U2OS cell expressing Aurora A-GFP under an endogenous promoter. Anaphase A spindle showing Aurora A and NEDD1 localization. Lower row, enlarged region of the spindle midzone composed of the Z-projection of a 1.2 μm section. Colocalization dots of Aurora A, NEDD1 and tubulin are indicated by blue arrows. Scale bar, 2 μm

(D) Fluorescence microscopy images showing the effect of NEDD1 depletion on the central spindle in fixed cells. Cells were stained for DNA, MTs, and NEDD1. The fluorescence intensity signal of tubulin was quantified along the line across the central spindle (white line). Left, fluorescence intensities were plotted as a function of the line length drawn on the merge images. Scale bar, 10 μm .

A



B



C

WT-AurA	+		+
AS-AurA		+	
1-Na-PP1	+	+	+
λ PP			+

NEDD1	
Ponceau	

Figure 5 - Aurora A activity is required NEDD1 phosphorylation during for monopolar anaphase

(A) Diagram showing the different steps required for the synchronization of cells in monopolar anaphase (adapted from Hu *et al.*, 2008).

(B) Fluorescence microscopy images showing the effect of the inhibition of Aurora A on the spindle of cells synchronized in monopolar anaphase. Scale bars, 10 μm .

(C) Western blot analysis (10%, Anderson gel) (Anderson et al., 1973) of cell extracts from WT- or ASaurA cells depleted of endogenous Aurora A, synchronized in monopolar mitotic exit, and treated with 1-Na-PP1. The extract in the third lane corresponds to that in the first lane, but was treated with λ -Phosphatase. Ponceau staining is shown as a loading control.

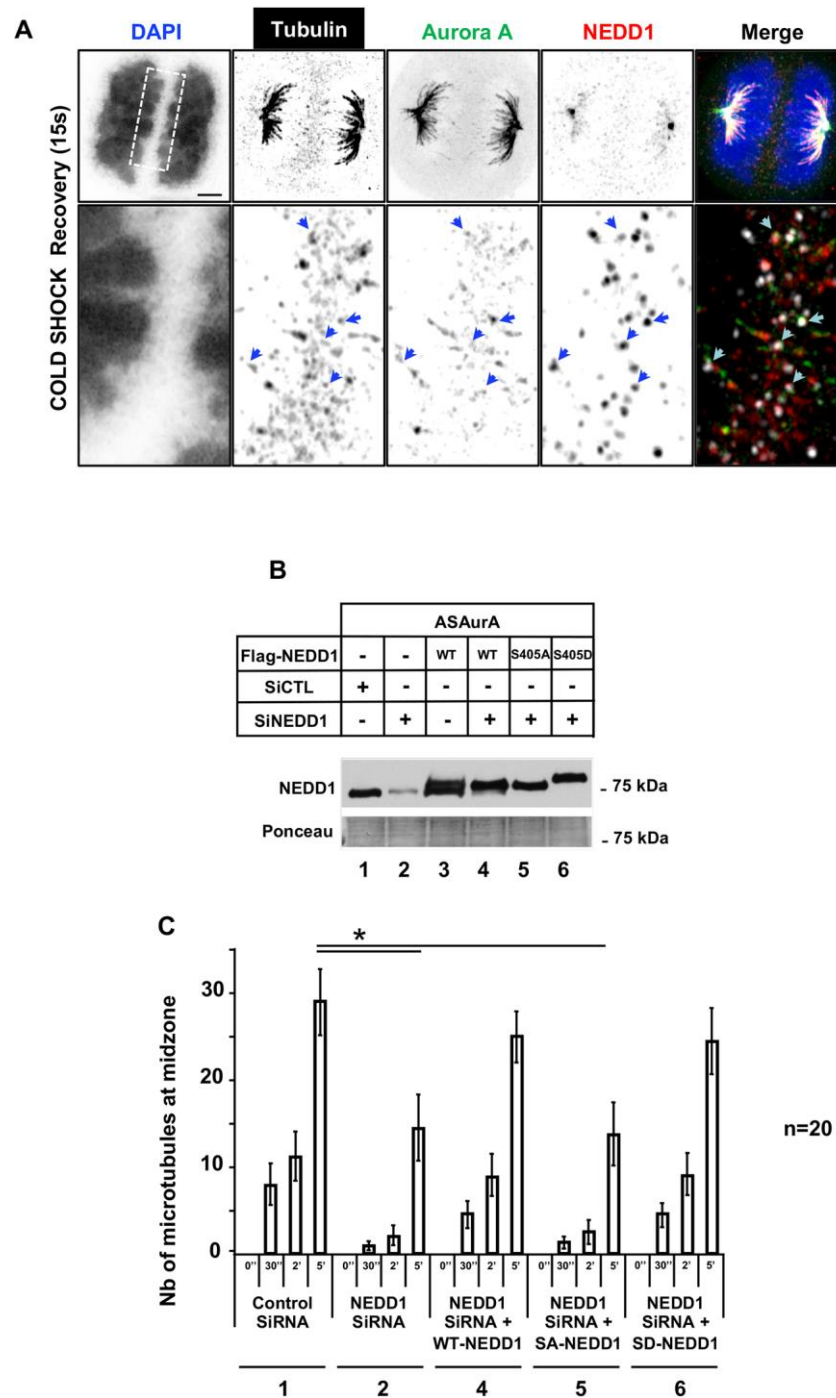


Figure 6 - NEDD1 phosphorylation on Serine 405 is required for microtubule nucleation at the spindle midzone during anaphase.

(A) Super resolution images of U2OS cell expressing Aurora A-GFP under an endogenous promoter. Anaphase A spindle 15 sec after recovery from cold shock. Lower row, an enlarged region of central spindle presenting microtubule nucleation spots where Aurora A, NEDD1, and tubulin colocalized (blue arrows). Scale bar, 2 μ m.

(B) Western blot analysis of extracts from cells treated as indicated and counted in **(C)** excepted lane 3 that shows endogenous WT and exogenous Flag tagged WT NEDD1. Ponceau staining is shown as loading control.

(C) Bar graph reporting the number of MT fibers in the midzone of cells treated as described in **(B)**, the numbers correspond to those of the western blot in **(B)**. Values are the mean of three independent experiments. MTs were counted in 20 cells for each experiment. Error bars show standard deviations. *=P value < 1e-6.

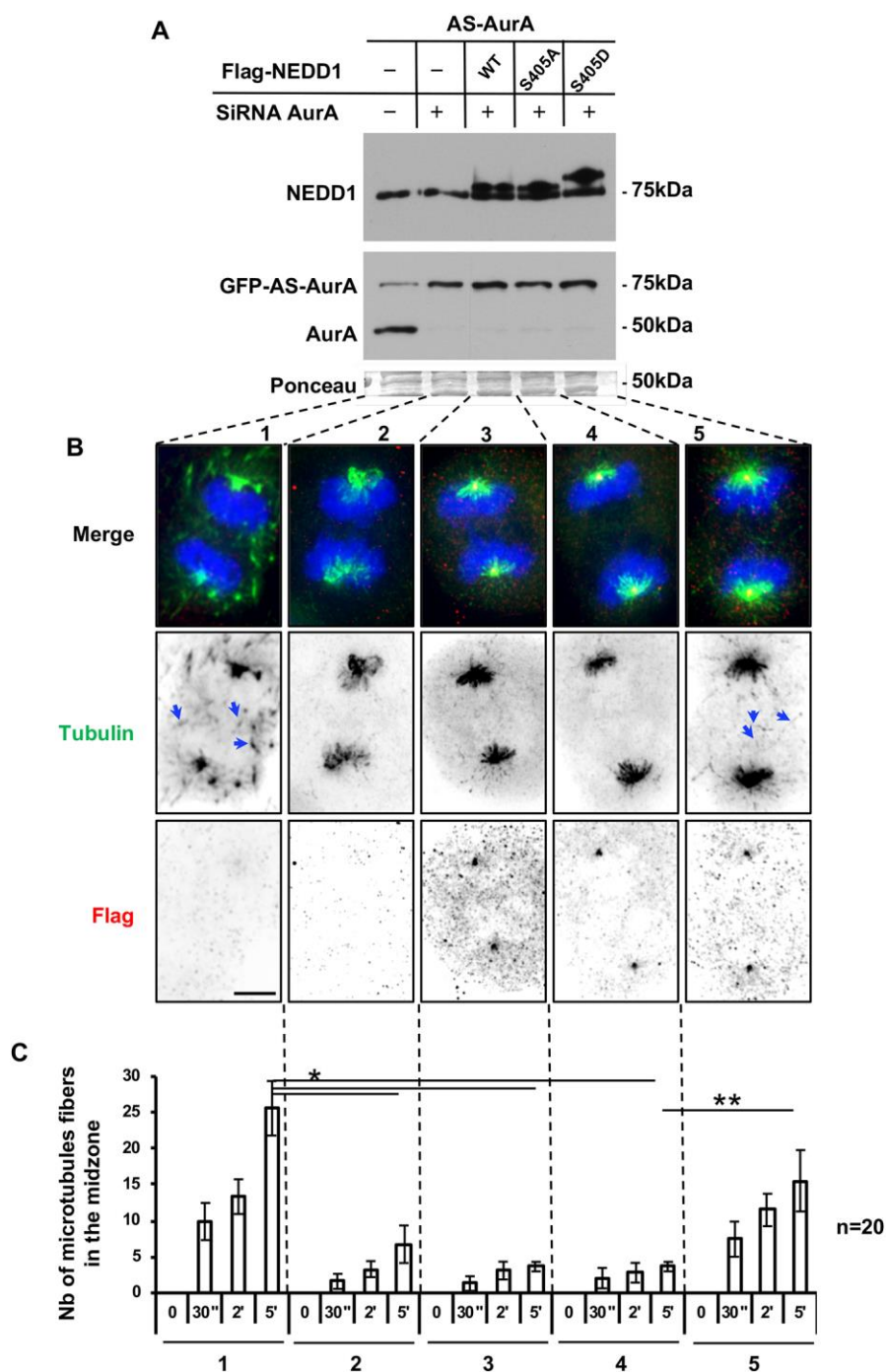


Figure 7 - Aurora A is responsible for NEDD1 phosphorylation on Serine 405 during anaphase.

(A) Western blot analysis of the extracts prepared from cells representative of those used in **(B)** before 1-Na-PP1 treatment. Ponceau staining is shown as a loading control.

(B) Fluorescence microscopy images showing MT nucleation, 2 min after release from Nocodazole supplemented cold medium. Scale bar, 5 μm .

(C) Bar graph reporting the number of MT fibers in the midzone of various cell populations treated as described in A same numbering. Values are the mean of three independent experiments. MTs were counted in 20 cells for each experiment. Error bars show standard deviations. *:P value < 1×10^{-10} . **:P value < 1×10^{-4}

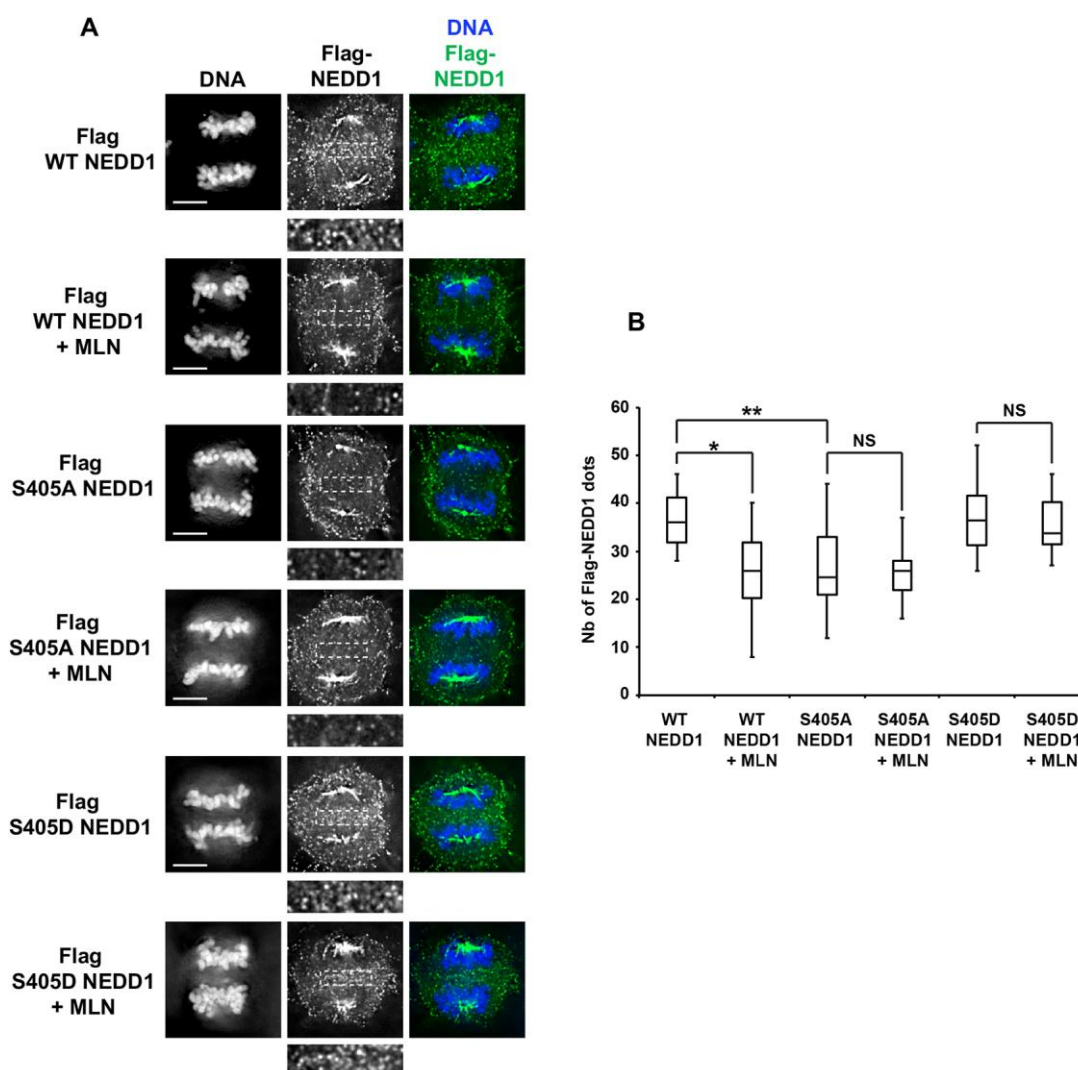


Figure 8 - Aurora A dependent phosphorylation of S405 in NEDD1 is required for its the midzone localization in anaphase.

(A) Fluorescence microscopy images showing the localization of Flag-tagged NEDD1 in control or 250 nM MLN8237 treated HeLa cells expressing the indicated versions of Flag-tagged NEDD1. Inserts show enlargements of the midzone area. Bars, 5 μ m.

(B) Boxplot diagram showing the number of NEDD1 dots into the midzone in control or 250 nM MLN8237 treated HeLa cells expressing the indicated versions of Flag-tagged NEDD1.

Values are representative of three independent experiments. Intensities were measured in 30 cells for each experiment. * $p < 3.02 \times 10^{-6}$; ** $p < 3.8 \times 10^{-6}$; NS = Not significant.

TABLES

Table S1- P Values of t-test on the mean from Figure 6 C.

Test performed on values from the 5 minute time point for each condition. In green, P values for significantly different populations. In red, P values for t-test with no differences.

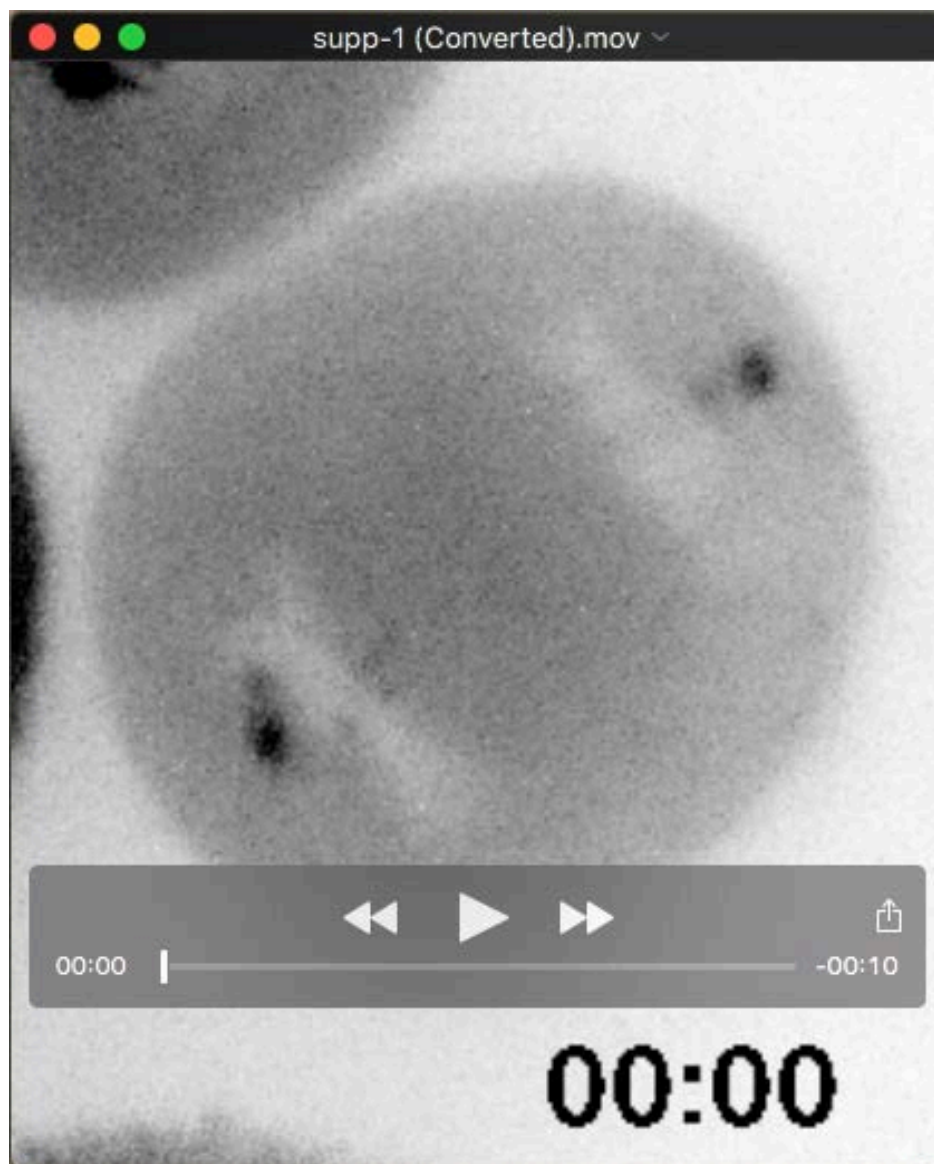
[Click here to Download Table S1](#)

Table S2- P Values of t-test on the mean from Figure 7 B.

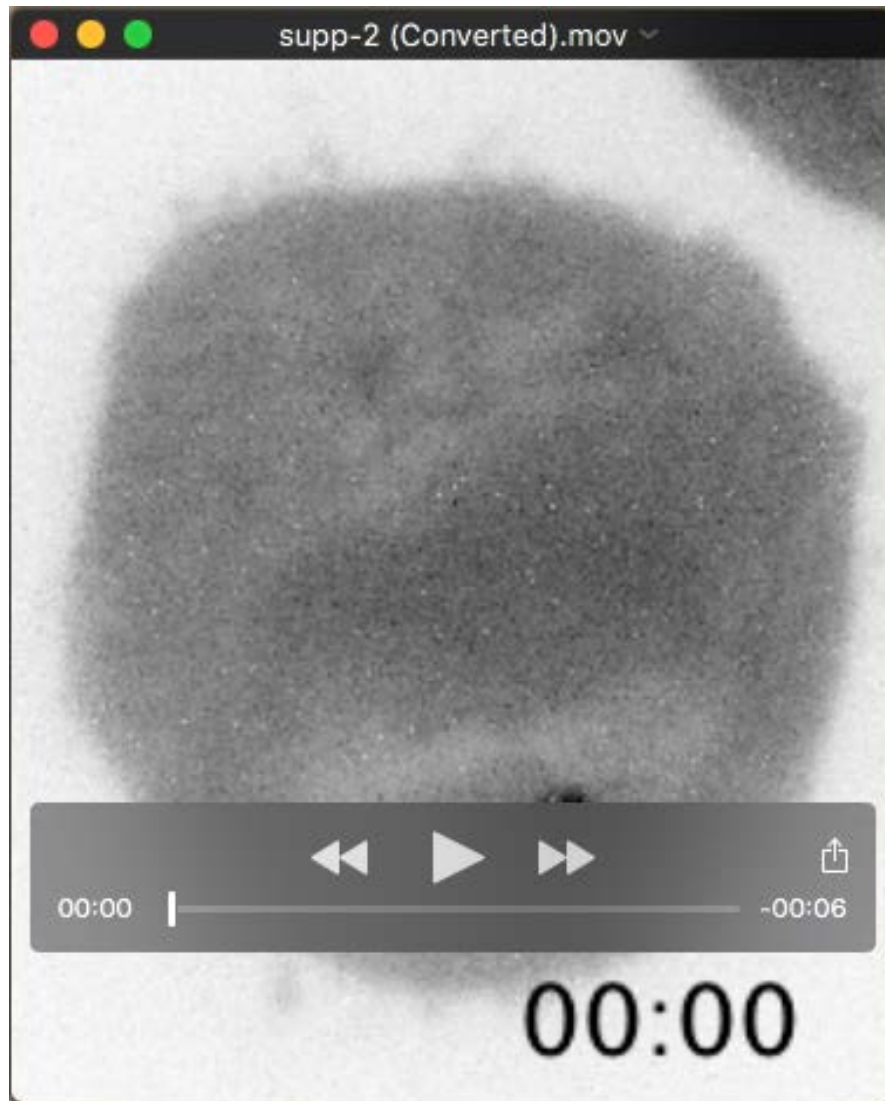
Test performed on values from the 5 minute time point for each condition. In green, P values for significantly different populations. In red, P values for t-test with no differences.

[Click here to Download Table S2](#)

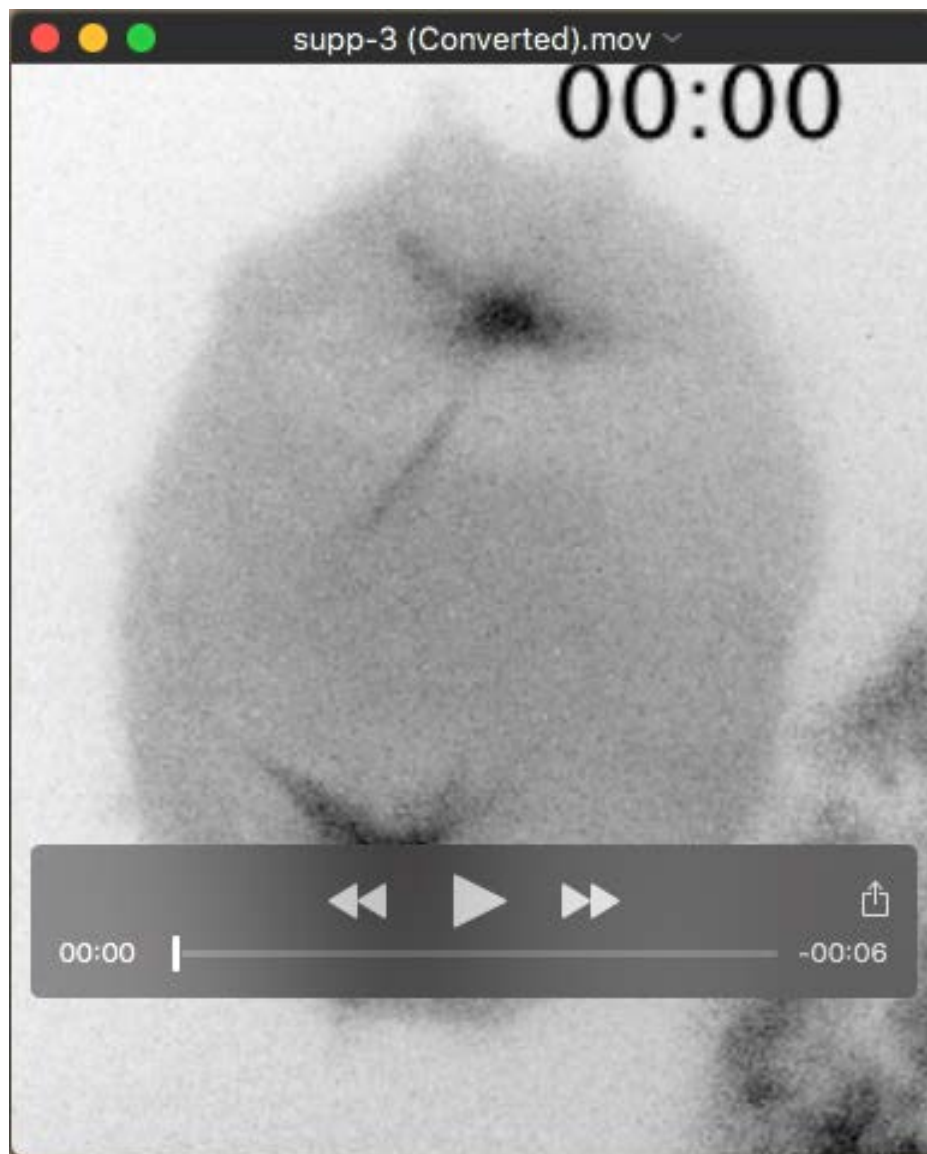
MOVIES



Movie 1- Fluorescence time-lapse microscopy showing MT nucleation in WTAurA cells expressing GFP-Tubulin, which were depleted of endogenous Aurora A and treated with 10 μ M 1-Na-PP1. MT regrowth begins at $t = 0$ (time in minutes)



Movie 2- Fluorescence time-lapse microscopy showing MT nucleation in ASaurA cells expressing GFP-Tubulin, which were depleted of endogenous Aurora A and treated with 10 μ M 1-Na-PP1. MT regrowth begins at $t = 0$ (time in minutes)



Movie 3- U2OS GFP-Tubulin cells treated with MLN8237. Cells were treated at the metaphase to anaphase transition. MT regrowth begins at $t = 0$ (time in minutes).