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Aurora A kinase and its substrate TACC3 are required for central spindle assembly

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Cell division entails a marked reorganization of the microtubule network to form the spindle, a molecular machine that ensures accurate chromosome segregation to the daughter cells. Spindle organization is highly dynamic throughout mitosis and requires the activity of several kinases and complex regulatory mechanisms. Aurora A (AurA) kinase is essential for the assembly of the metaphase bipolar spindle and, thus, it has been difficult to address its function during the last phases of mitosis. Here, we examine the consequences of inhibiting AurA in cells undergoing anaphase, and show that AurA kinase activity is necessary for the assembly of a robust central spindle during anaphase. We also identify TACC3 as an AurA substrate essential in central spindle formation.

Keywords: Aurora A; TACC3; central spindle; anaphase; MLN8237

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INTRODUCTION

During mitosis, microtubules (MTs) form the spindle, an extremely dynamic but robust macromolecular apparatus that faithfully distributes the duplicated genetic material to the two forthcoming daughter cells. Spindle organization and dynamics change throughout mitosis [1]. During the initial phases of mitosis, MTs organize into a bipolar spindle consisting of two antiparallel interdigitating arrays that connect the chromosomes to the two spindle poles and align them on the metaphase plate. Anaphase involves a switch in MT dynamics: kinetochore MTs (K-fibres) shorten and pull the chromatids apart while a new MT-based structure called the central spindle forms in between the separating chromosomes. The central spindle consists of two arrays of parallel MTs that interdigitate at the equator of the separating chromosomes and slide apart contributing to their net separation. The central spindle also acts as a platform for the

recruitment and accumulation of several proteins involved in furrow ingression and cytokinesis [1,2].

Spindle assembly and dynamics are under tight temporal and spatial control achieved in large part by reversible phosphorylation of spindle assembly factors that altogether regulate MT dynamics and organization. Aurora A (AurA) is a member of the Aurora Serine/Threonine kinase family that localizes to the centrosome and spindle MTs in dividing cells [3]. AurA is required for mitotic entry, centrosome maturation and separation and for bipolar spindle formation [3]. Further insights into AurA function have been obtained through the identification of some of its substrates [3–5]. One of its best characterized substrate is TACC3, a protein that promotes MT stabilization through its interaction with chTOG/XMAP215 [6]. AurA-dependent phosphorylation of TACC3 on S558 was shown to be required for TACC3 function in promoting MT stability [7,8].

Although some data indicate that AurA also has a role in post-metaphase events, no clear picture has emerged, [9–11] as it is experimentally challenging to address this question because of its early mitotic functions. Here we have examined the role of AurA kinase activity during anaphase using a specific inhibitor in cells starting anaphase. We show that AurA kinase activity and one of its substrates TACC3 are required for the assembly of the central spindle during anaphase.

RESULTS AND DISCUSSION

To determine whether AurA kinase is active after metaphase we performed immunofluorescence analysis in HeLa cells using the anti-pT288 AurA antibody that specifically recognizes the active form of the kinase [12]. Active AurA was strongly enriched at the centrosome and spindle poles during prometaphase and metaphase (as previously described). Interestingly, it was still detectable at the spindle poles during anaphase (supplementary Fig S1A online). We therefore decided to investigate its function during the final phases of mitosis.

To address the role of AurA kinase activity after metaphase without interfering with the previous events, we decided to use the AurA inhibitor MLN8237 (MLN) [13]. We first determined the optimal concentration of this compound to efficiently inhibit AurA by monitoring the active kinase with the anti-pT288 AurA antibody. Western blots and immunofluorescence of HeLa cells

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Fig 1 | AurA kinase activity is required for bipolar spindle maintenance. (A) Images of anaphase cells incubated with DMSO (CTRL) or MLN. pT288 Aurora A, green; MTs, red; DNA, blue. Scale bar, 5 μ m. On the right, the graph shows the fluorescence intensity quantification for the pT288 Aurora A signal at the spindle poles (CTRL, $n = 14$; MLN, $n = 14$). The data were obtained from three independent experiments. Bars, s.e.m. (B) Images from time-lapse recordings of cells arrested in metaphase with MG132 and incubated with DMSO (CTRL) or MLN. Scale bar, 5 μ m. The graph shows the pole-to-pole distance over time for CTRL ($n = 13$)- and MLN ($n = 10$)-treated cells. Red, tubulin-mRFP; green, H2B-eGFP. Bars, s.d. AurA, Aurora A; CTRL, control; DMSO, dimethyl sulphoxide; eGFP, enhanced green fluorescent protein; mRFP, monomer red fluorescent protein.

incubated with a range of MLN concentrations revealed that incubation of cells with 250 nM MLN was sufficient to abolish the signal for active pT288 AurA (Fig 1A, supplementary Fig S1B online). Under these conditions, the signal for the active form of AurB at the spindle midzone as detected with an anti-pT232 AurB antibody was not compromised (supplementary Fig S1C online). As expected, active AurB was not detectable anymore in cells incubated with the AurB inhibitor AZD1152 (AZD) [14] (supplementary Fig S1C online). We conclude that MLN inhibits AurA at 250 nM without compromising AurB activity and we performed all the subsequent experiments using this inhibitor concentration.

Metaphase spindle maintenance requires AurA activity

We first checked whether AurA activity is required for the maintenance of the bipolar metaphase spindle. HeLa cells stably expressing H2B-eGFP and α -tubulin-mRFP were arrested in metaphase by incubation with the proteasome inhibitor MG132 and placed under the microscope for time-lapse analysis. Dimethyl sulphoxide (DMSO) or MLN were then added to the medium.

Control cells maintained a metaphase spindle with aligned chromosomes for more than two hours (Fig 1B, supplementary Movie 1 online). By contrast, addition of MLN induced the rapid collapse of the bipolar spindle and by 30 min the two spindle poles were closely juxtaposed to the chromatin (Fig 1B, supplementary Movie 2 online). These results indicate that AurA kinase activity is essential for the maintenance of the bipolar spindle in metaphase. Therefore, the only way to address the function of AurA in the last phases of mitosis is to interfere with its kinase activity just after the metaphase-anaphase transition. This can be achieved by applying the AurA inhibitor in a timely fashion.

AurA inhibition promotes anaphase defects

We first examined the consequences of inhibiting AurA in early anaphase by time-lapse experiments using HeLa cells stably expressing H2B-eGFP and α -tubulin-mRFP. Cells were partially synchronized in metaphase with a low concentration of the proteasome inhibitor MG132 for 30 min. After MG132 washout the cells were placed under the microscope for time-lapse

imaging. As cells entered anaphase (30 min after MG132 washout) MLN or DMSO were added to the media. Both control and MLN-treated cells progressed into anaphase (Fig 2A, supplementary Movies 3,4,5 online). However, MLN-treated cells required 23.7% longer than control cells to reach cytokinesis (16.7 ± 0.5 min versus 13.5 ± 0.3 min for control cells) (Fig 2B). These results indicate that AurA activity is necessary for the timely progression of the late mitotic phases.

AurA inhibition did not prevent chromosome segregation altogether. However, it induced several segregation defects including chromatin bridges and lagging pieces of chromatin (Fig 2A) in 88% of the cells whereas this type of defects were observed in 33% of control cells and under the same conditions in 30.43% of nonsynchronized control cells. The increase of chromatin segregation defects observed in MLN-treated cells indicated that AurA kinase activity is important for accurate chromosome segregation during anaphase.

As one function of AurA is to promote MT stability, we then examined whether MT function was compromised by AurA inhibition. We first quantified spindle elongation by measuring the separation of the spindle poles over time and found that in the presence of MLN, pole-to-pole separation was reduced compared to controls (supplementary Fig S2A online). We then looked at the potential relative contribution of K-fiber shortening and/or central spindle elongation in the overall reduction of pole-to-pole separation. K-fibres of cells incubated in MLN shortened slightly faster than in control cells suggesting that AurA kinase activity might have a role in the control of K-fiber dynamics during anaphase (supplementary Fig S2A online). However, the main contribution to the reduction in pole-to-pole separation induced by MLN was at the level of the central spindle (Fig 2C) as detected by measuring central spindle elongation over time. The central spindles of MLN cells elongated at a slower rate and were shorter than controls (on average 27.8% shorter at 8 min). Moreover, these central spindles often appeared weak and disorganized (Fig 2A).

These results indicated that AurA kinase activity is important during anaphase and suggested that it has a role in central spindle assembly.

AurA activity is required for central spindle assembly

To examine the central spindle more closely, we performed immunofluorescence studies in HeLa cells incubated with DMSO or MLN. Most control cells (92%) showed the typical central spindle organization characterized by two dense arrays of tightly packed MTs extending from the chromosomes towards the cell centre where they were separated by a thin dark line (Fig 2D). Although 26% of MLN-treated cells did not appear to present major defects, quantification of the MT fluorescence intensity along the pole-to-pole axis showed that MT density was specifically reduced at the central spindle (Fig 2D,E). The other MLN-treated cells (74%) showed highly disorganized central spindles characterized by a range of defects including reduced and/or poorly aligned MTs (Fig 2D,E). To further support these observations, we quantified the central spindle and astral MT densities in anaphase cells incubated in DMSO or MLN. Inhibition of AurA by MLN resulted in a significant and specific reduction of MT density at the central spindle (Fig 2F, supplementary Fig S2C online).

Altogether our results strongly indicate that AurA activity is important for the control of MT assembly and organization during anaphase, in particular at the level of the central spindle.

TACC3 is required for central spindle assembly

Our results suggested so far that AurA kinase activity is required for MT stabilization during anaphase. TACC3 is a well-characterized substrate of AurA that promotes MT stabilization in early mitosis in partnership with XMAP215/chTOG [6–8,15]. As the role of TACC3 in anaphase has not been addressed, we decided to explore whether it might have a function in central spindle formation.

We performed time-lapse imaging on control and TACC3-silenced HeLa cells stably expressing H2B-eGFP and α -tubulin-mRFP. TACC3-silenced cells progressed into anaphase and telophase although they required 11% longer time than control cells. (siCTRL: 12.78 ± 1.21 min; siTACC3: 14.17 ± 2.20 min) (Fig 3A,B, supplementary Movie 6,7 online). We then measured the central spindle elongation over time in control- and TACC3-silenced cells (Fig 3C). TACC3-silenced cells showed reduced rates of central spindle elongation. Moreover, the central spindles of TACC3-silenced cells were shorter than controls (on average 31.5% shorter at 8–10 min). These data suggested strongly that TACC3 is required for central spindle assembly and dynamics. As these data were reminiscent of the phenotypes observed in the AurA-inhibited cells, they suggested that TACC3 is one of the targets of AurA for the regulation of central spindle formation in anaphase.

To address this question, we first examined whether TACC3 phosphorylation at the AurA site S558 was altered upon incubation of cells with MLN. Immunofluorescence analysis on anaphase cells with an anti-pS558-TACC3 antibody showed that phospho-TACC3 localized to the anaphase spindle poles and the central spindle MTs in control cells (supplementary Fig S3A online). Moreover, the signal for phospho-TACC3 was greatly reduced in cells incubated with MLN (supplementary Fig S3A online) suggesting that AurA phosphorylates TACC3 on S558 during anaphase.

To determine whether TACC3 phosphorylation by AurA is required for central spindle formation, we performed rescue experiments by expressing either Flag-TACC3 WT or Flag-TACC3 S558A, a phosphorylation mutant on the AurA site, [16] in TACC3-silenced cells (supplementary Fig S3C online) and analysed the cells by immunofluorescence quantifying the central spindle MT fluorescence intensities (Fig 3D,E). TACC3 silencing promoted a substantial reduction of central spindle MT density. Expression of Flag-TACC3 WT in the silenced cells partially restored the density of MT density at the central spindle, however, the expression of the phosphorylation-null mutant Flag-TACC3 S558A did not (Fig 3D).

Altogether our data suggest that TACC3 and its phosphorylation by AurA at S558 are important in central spindle assembly during anaphase.

AurA regulates TACC3 function in central spindle assembly

To further characterize the role of AurA activity and TACC3 on central spindle assembly and dynamics, we used a depolymerization/regrowth assay [17]. Control and TACC3-silenced cells incubated in DMSO or MLN were placed on ice and fixed at different time

Fig 2 | Aurora A kinase activity is required for central spindle assembly and accurate chromosome segregation. (A) Images from time-lapse recordings of HeLa cells released from a metaphase arrest in the presence of DMSO (CTRL) or MLN. White arrowheads point to chromatin pieces in between the segregating chromosomes. MTs, red; DNA, green. Numbers on top left corner, time in minutes. Scale bar, 5 μm . (B) Quantification of the time from anaphase onset to the complete contraction of the cytokinesis furrow for CTRL ($n = 15$) and MLN ($n = 7$) treated cells ($*P = 0.0377$). Bars, s.e.m. (C) Central spindle length over time during anaphase in DMSO (CTRL) ($n = 15$, blue line)- and MLN-treated cells ($n = 7$, green line). The difference in central spindle length between the two conditions is statistically significant ($P = 0.0004$). Bars, s.e.m. (D) Confocal maximum intensity projections of anaphase HeLa cells incubated in DMSO (CTRL) or MLN. 92% of CTRL ($n = 633$) and 26% of MLN-treated cells ($n = 666$) scored as having organized central spindles. 74% of MLN cells had disorganized central spindles. Data from three independent experiments. Representative images for these different categories are shown. Scale bar, 5 μm . (E) MT fluorescence intensity profiles measured along the pole-to-pole axis (red box in the CTRL cell in D) for each of the representative cells shown in (D). CTRL, blue line; MLN, yellow, red and light blue lines as indicated in (D). (F) Total MT intensity of the central spindles in CTRL ($n = 75$)- and MLN ($n = 86$)-treated cells. ($***P < 0.0001$). Bars, s.e.m. Aurora, Aurora A; CTRL, control; DMSO, dimethyl sulphoxide; MT, microtubules.

points. Cells were processed for immunofluorescence and the density of MTs in the central spindle region was quantified (Fig 4A). MT depolymerization occurred faster in cells incubated with MLN than in control cells with only a few MT remnants left in the central

spindle area after 2 min of incubation on ice (Fig 4A,B). Similar levels were only achieved after more than 10 min on ice for the control cells. These data indicate that Aurora A activity is essential for the stability of the central spindle MTs.

Fig 3 | TACC3 and its regulation by Aurora A are necessary for central spindle formation. (A) Images from time-lapse recordings of control (siCTRL) and TACC3 (siTACC3) silenced HeLa cells during the last phases of mitosis. Numbers on top left corner, time in minutes. Red, tubulin-mRFP; green, H2B-eGFP. Scale bar, 5 μm . (B) Quantification of the time in anaphase and telophase for CTRL ($n = 18$) and siTACC3 ($n = 24$) cells ($*P = 0.0206$). Bars, s.e.m. (C) Central spindle length over time during anaphase for siCTRL (blue line, $n = 40$) and siTACC3 (green line, $n = 37$) cells. The difference in central spindle length between the two conditions is statistically significant ($P = 0.0045$). Bars, s.e.m. (D) Representative immunofluorescence images of a CTRL (siCTRL) silenced cell expressing Flag and TACC3-silenced cells expressing, Flag alone, Flag-TACC3 WT or the AurA phosphorylation-null mutant Flag-TACC3 S558A (Flag-TACC3 SA). On the right, the graph shows the quantification of the total MT intensity in the central spindle for siCTRL ($n = 72$) and siTACC3 cells expressing Flag alone ($n = 64$), Flag-TACC3 WT ($n = 69$) and Flag-TACC3 S558A ($n = 77$). ($***P < 0.0001$; NS, $P = 0.4929$). Bars, s.e.m. CTRL, control; eGFP, enhanced green fluorescent protein; siCTRL, silenced control; siTACC3, silenced TACC3; WT, wild type.

We had already observed that TACC3 silencing had a marked effect on MT density at the central spindle (see Fig 3D and 0-min time point in Fig 4B). Ice-induced depolymerization of the remaining central spindle MTs in these cells occurred in the first 5 min of incubation on ice and with a similar kinetics

whether cells were incubated in DMSO or MLN (Fig 4B). Altogether our data show that inhibiting AurA or silencing TACC3 or both results in the destabilization of MTs in the central spindle. As AurA inhibition and TACC3 silencing did not generate additive destabilization phenotypes on central

Fig 4 | AurA kinase activity and TACC3 are required for central spindle assembly and stability. (A) Maximum projections of confocal images of representative anaphase cells showing different MT intensities of DMSO (CTRL) treated cells after 0 (a), 2 (b), 5 (c) and 10 min (d) on ice. Scale bar, 5 μ m. (B) Quantification of total MT intensities of the central spindles of control (siCTRL) and TACC3-silenced (siTACC3) cells incubated with DMSO (CTRL) or MLN after different times on ice. For every condition 69–78 cells were quantified in three independent experiments. Bars, s.e.m. (C) Quantification of total MT intensities of the central spindles of control (siCTRL) and TACC3-silenced (siTACC3) cells incubated with DMSO (CTRL) or MLN placed at 37 °C after a 15 min pre-incubation on ice. For every condition 66–85 cells were quantified in three independent experiments. Bars, s.e.m. AurA, Aurora A; CTRL, control; DMSO, dimethyl sulphoxide; MT, microtubules; siCTRL, silenced control; siTACC3, silenced TACC3.

spindle MTs, it is possible that these two components act in the same pathway.

We then examined MT regrowth in the different experimental conditions by placing cells pre-incubated on ice for 15 min back at 37 °C. Cells were fixed at different time points, processed for immunofluorescence and the MT density in the central spindle area quantified as before (Fig 4C). In control cells, MT regrowth was very efficient showing a substantial recovery of the MT density as early as 2 min after placing the cells at 37 °C reaching an almost full recovery after 10 min. By contrast, MT assembly was strongly delayed for cells incubated in MLN as well as for TACC3-silenced cells whether incubated with DMSO or MLN (Fig 4C). This suggests that both AurA and TACC3 are essential for the assembly of MTs in the central area of the anaphase cell and for central spindle assembly.

Previous data have shown that centrosomal MTs contribute very little if any to the assembly of the central spindle that relies mostly on the RanGTP-dependent pathway and the augmin MT–MT amplification pathway [17]. Interestingly, AurA has been shown to be required for MT nucleation/stabilization through the RanGTP-dependent pathway in the early phases of mitosis [4,8,18]. We have shown here that AurA kinase activity is required during anaphase for central spindle assembly. Our data suggest that during anaphase AurA kinase might promote non-centrosomal MT nucleation as well as MT stabilization at least in part through TACC3 phosphorylation. This also suggests that AurA

might have a role in MT regulation throughout mitosis, maybe acting on similar substrates and/or processes in the different phases of mitosis.

Interestingly, while we were preparing this paper, Reboutier *et al* [19] also found that AurA is required for central spindle assembly using a different experimental approach. Although here we found that TACC3 is one of the important substrates of AurA for central spindle assembly, they described that another important substrate is P150Glued [19]. Altogether, this highlights the existence of multiple targets through which this mitotic kinase exerts key regulatory functions throughout mitosis for the assembly of the various complex MT architectures required for accurate chromosome segregation and cell division.

METHODS

Immunofluorescence and Western Blotting. Cells fixed in methanol at –20 °C for 10 min were incubated with the primary antibodies diluted in phosphate-buffered saline (PBS); 2% bovine serum albumin (Sigma); 0.1% Triton X-100 (Sigma) for 45 min at room temperature. Secondary antibodies and Hoechst33342 (Invitrogen) were incubated for 45 min. Washes were performed with PBS/0.1% Triton X-100. Coverslips were mounted in Mowiol (Sigma). For pS558 TACC3 immunofluorescence, cells were fixed with 3% formaldehyde in PBS at 37 °C for 10 min. Cells were then permeabilized with PBS/0.5% triton for 10 min at room temperature and the anti-pS558–TACC3 incubated

on the cells for 48 h at 4 °C. For Western blots, nitrocellulose membranes were scanned with Li-cor Odyssey. A list of antibodies is provided in the supplementary Information online.

TACC3 silencing and rescue experiments. An small interfering RNA against the 83–103 nucleotide region of TACC3 [8,9] and short RNA-mediated interference oligonucleotides scrambled sequence (as control) (Dharmacon) were transfected with Lipofectamine 2000 (Invitrogen). Sixteen hours after transfection, cells were synchronized with thymidine (2 mM) for 24 h, released for 10 h and blocked in prometaphase with 5 μ M S-Trityl-L-Cysteine (STLC) for 16 h. For the TACC3 rescue experiments, a pool of small interfering RNAs targeting the 5'-CGGCCAUAAGGGCUAGAAUU-3' and 5'-GCUUUGAAAACAUGACUCAUU-3' UTR of TACC3 were used. Twenty-four hours after small interfering RNA transfection, cells were transfected with pCMV constructs for expression of Flag-tagged TACC3 WT and TACC3 S558A (gift from F. Gergely) with XtremeGene9 according to manufacturer instructions (Roche).

Cell synchronization and inhibitors. MLN8237 and AZD1152 were purchased from Selleck chemicals. HeLa cells were grown at 37 °C in Dulbecco's Modified Eagle Medium (Cambrex) containing 10% fetal bovine serum and 2 mM L-glutamine (Invitrogen) with 5% CO₂ in a humid atmosphere. For live cell imaging, cells were either incubated in 10 μ M MG132 (Sigma) for metaphase arrest or in 2.5 μ M MG132 for 30 min for synchronization and release into anaphase. For immunofluorescence analysis, HeLa cells were incubated in Thymidine 2 mM for 24 h, released for 8 h and incubated with 5 μ M STLC (Sigma) for 16 h. Mitotic cells were harvested with a mitotic shake off and after STLC washout plated on 0.1% Poly-D-Lysin coated glass coverslips. DMSO or MLN were added to the medium 1 h 25 min after STLC release when most of the cells were progressing to anaphase.

Microscopy. Fixed cells were imaged on an inverted widefield Leica fluorescent microscope (DMI-6000) or a TCS SPE Leica confocal microscope with $\times 63$ objectives using the Leica Application Suite acquisition software. Three-dimensional optical section images were taken at 0.55 μ m intervals and projected to a maximum intensity image. For live imaging, cells were maintained in standard culture condition in an incubation chamber built on Olympus Andor Revolution XD spinning disk microscope. Four optical sections (0.8 μ m z-stack interval) were acquired every 2 min for ~ 2 h using a $\times 63$ oil immersion objective (1.42 NA). Images were processed using Andor IQ software and ImageJ.

MT depolymerization/regrowth assay. MT depolymerization and regrowth assay were performed as previously described [17]. In short, cells were incubated in ice-cold L-15 medium containing 20 mM HEPES and DMSO (CTRL) or MLN and fixed at different time points over a 15-min incubation period on ice. For MT regrowth, the medium was exchanged with pre-warmed Dulbecco's Modified Eagle Medium containing DMSO or MLN and the cells fixed at different time points over a 10-min incubation period at 37 °C.

Image and statistical analysis. For quantifications of the central spindles, the total integrated fluorescence intensity of the MTs in the central spindle of anaphase cells was obtained and the background was subtracted for each cell in different conditions using ImageJ. For the quantification of the astral MTs, the total

integrated fluorescence intensity of the MTs in the central spindle (minus background) was subtracted from the total integrated fluorescence intensity of the MTs in the whole cell (minus background). Prism (GraphPad) was used to perform statistical analysis (Student's *t*-test) and to create graphs.

Supplementary information is available at EMBO reports online (<http://www.emboreports.org>).

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CONFLICT OF INTEREST

The authors declare that they have no conflict of interest.

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