A non-canonical function for Centromere-associated protein-E (CENP-E) controls centrosome integrity and orientation of cell division

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1 **Summary**

2 Centromere-associated protein-E (CENP-E) is a kinesin motor localizing at kinetochores.
 3 Although its mitotic functions have been well studied, it has been challenging to investigate
 4 direct consequences of CENP-E removal using conventional methods because CENP-E
 5 depletion results in mitotic arrest. In this study, we harnessed an auxin-inducible degron
 6 system to achieve acute degradation of CENP-E. We revealed a kinetochore-independent role
 7 for CENP-E that removes pericentriolar material 1 (PCM1) from centrosomes in G₂ phase.
 8 After acute loss of CENP-E, centrosomal Polo-like kinase 1 (Plk1) is sequestered by
 9 accumulated PCM1, resulting in aberrant phosphorylation and destabilization of centrosomes,
 10 which triggers loss of astral microtubules and oblique cell divisions. Furthermore, we also
 11 observed centrosome and cell division defects in cells from a microcephaly patient with
 12 mutations in *CENPE*. Orientation of cell division is deregulated in some microcephalic
 13 patients, and our unanticipated findings provide a unifying principle that explains how
 14 microcephaly can result from centrosomal defects.

15

16 Introduction

17   The centrosome is an organizing center for microtubules (MTs) in metazoan
18 cells. This organelle functions as a hub for MT-based protein transport during interphase, and
19 it organizes spindle poles in mitosis. Within the centrosome, the two centrioles are embedded
20 in pericentriolar material (PCM), which consists of γ -tubulin ring complexes, a nucleator of
21 MTs, together with a cohort of proteins that regulate centrosome integrity¹. Centrosomes and
22 the environment surrounding this organelle are dynamically remodeled throughout the cell
23 cycle. In interphase, centriolar satellites, dense granules that consist of multiple proteins
24 essential for controlling centriole duplication, are scattered around centrosomes^{2,3}. These
25 granules are anchored on microtubules (MTs) through a scaffold protein, pericentriolar
26 material 1 (PCM1; despite its name, this is not a component of the PCM). Beyond its ability
27 to organize centriolar satellites on the MTs, PCM1 also prevents its interacting partners from
28 being inappropriately relocated to centrosomes, as suggested by the observation that satellite
29 proteins are constitutively localized at centrosomes after PCM1 depletion⁴⁻⁸. Centriolar
30 satellites accumulate around centrosomes through dynein-driven transport during and after
31 centriole duplication⁹⁻¹¹. Subsequently, they are re-distributed within the cytoplasm as
32 prophase commences^{4,5}. On the other hand, Plk1 accumulates on the centrosome in prophase,
33 where it phosphorylates PCM components, including pericentrin (PCNT) and Wdr62^{12,13}.
34 These modifications accelerate PCM expansion and formation of robust astral MTs, which
35 anchor the cell cortex to maintain spindle orientation. Despite our knowledge of these
36 dynamic events during prophase, the mechanisms underlying the dispersal of centriolar
37 satellites—and its functional implications—remain obscure, and how PCM expansion is
38 spatio-temporally regulated likewise remains unclear.

Recent proteomic studies have identified motor proteins that potentially interact with centriolar satellites^{14, 15}, and centromere-associated protein-E (CENP-E) is one such candidate. CENP-E is a kinesin motor localizing to kinetochores, where the protein reinforces MT-kinetochore interactions¹⁶⁻²⁰, and contributes to the spindle assembly checkpoint²¹⁻²³. CENP-E depletion in mammals leads to mitotic arrest^{21, 22}, and deletion of *CENPE* in mice results in early embryonic lethality²⁴. However, all known functions for CENP-E pertain to mitosis, during which time centriolar satellites are dispersed in the cytoplasm. Therefore, the functional consequences for interactions between CENP-E and centriolar satellite proteins remain elusive. In this study, we show that CENP-E has a non-canonical role around centrosomes in interphase. CENP-E removes PCM1 from the peri-centrosomal region in G₂ phase, and this transport is critical for structural stability of centrosomes and maintenance of spindle orientation in mitosis. Moreover, our findings can explain phenotypes associated with microcephaly, a developmentally-related brain disorder, using patient-derived cells mutated in *CENPE*²⁵. Thus, we have unveiled an unanticipated role for CENP-E in centrosome dynamics and have linked it to mechanisms that result in microcephaly.

Results

CENP-E is recruited around centrosomes in G₂ phase

It was previously reported that CENP-E protein is initially synthesized prior to mitosis¹⁶. Western blotting of lysates from synchronized wild-type RPE-1 cells confirmed that cytoplasmic CENP-E levels were elevated in late S/G₂ phase, and they peaked in mitosis

(Fig. 1a). However, in late S/G2 phase, MTs are not attached to kinetochores, and therefore, whether cytoplasmic CENP-E had a function in interphase was unclear. To understand its interphase role, we first explored CENP-E localization from interphase to mitotic onset using immunofluorescence. In early S phase, CENP-E signal was not detectable in the cytoplasm, in accordance with its low expression levels (Fig. 1b,c, 0 and 6h). On the other hand, CENP-E was specifically enriched around centrosomes from late S to G2 phase (Fig. 1b,c, 10 and 12h), and was dispersed throughout the cytoplasm by prophase (Fig. 1b,c, prophase). This pericentrosomal localization of CENP-E is consistent with recently identified interactions between centriolar satellites, typified by the PCM1 protein, and CENP-E¹⁵. Given that centriolar satellites are not localized at kinetochores^{4, 5}, these data suggest that CENP-E has a previously uncharacterized role around the centrosome in interphase.

Loss of CENP-E leads to cell cycle exit after one cell division cycle

CENP-E depletion using siRNA or antibody injection leads to mitotic arrest^{17, 20, 22}. However, these methods require extended periods of time for complete depletion or inactivation and depend on the specificity of siRNAs or antibodies. Therefore, it has been challenging to study the immediate consequences of CENP-E removal. To overcome these problems and investigate the peri-centrosomal function of CENP-E, we established a conditional knockout (KO) cell line using gene-editing to biallelically introduce Auxin-inducible degrons at the endogenous locus (CENP-E-AID)²⁶⁻²⁹. One hour after auxin (IAA) addition, CENP-E was degraded and undetectable by western blotting (Fig. 2a). Further, CENP-E signal was not detectable around centrosomes in interphase or at kinetochores in mitosis upon auxin treatment (Fig. 2b). Most mitotic CENP-E KO cells had misaligned chromosomes near spindle poles, a phenotype consistent with other CENP-E depletion studies^{17, 20, 22, 23, 30} (Fig. 2b arrowheads; Extended Data Fig. 2a). In agreement with

a previous report in mice²³, the complete loss of CENP-E did not lead to mitotic arrest but instead prompted a delay in metaphase (Fig. 2c). Importantly, nearly all CENP-E KO cells stopped growing and exited from the cell cycle after one cell division cycle (Fig. 2d,e; Extended Data Fig. 1b). Loss of CENP-E led to chromosome mis-segregation in mice²³, suggesting that our CENP-E KO cells might exit from the cell cycle due to aneuploidy in daughter cells.

Loss of CENP-E promotes PCM fragmentation and oblique cell divisions

A previous study reported that spindle MTs were not properly focused at spindle poles in a small fraction of cells treated with CENP-E siRNA, despite exclusive localization of CENP-E at kinetochores in wild-type metaphase cells²². Focusing of spindle MTs at poles is dependent on dynein-driven transport along astral MTs, nucleated at the PCM³¹. Therefore, the possibility remained that unfocused spindle MTs in CENP-E-depleted cells arose from defects in PCM structure. Interestingly, by immuno-staining for PCM markers, we showed that loss of CENP-E also provoked severe defects in PCM morphology. In controls without auxin treatment, γ -tubulin and PCNT were sharply focused at each spindle pole (Fig. 3a, -IAA). On the other hand, γ -tubulin and PCNT foci were fragmented in 55% of CENP-E KO cells upon auxin addition (Fig. 3a, +IAA). Furthermore, cells treated with GSK923295 (GSK)³², an inhibitor of CENP-E motor activity, also showed PCM fragmentation (77%; Fig. 3b), as well as misaligned chromosomes (Extended Data Fig. 2b), suggesting that fragmentation in CENP-E KO cells results from loss of CENP-E-dependent protein transport. Although a recent study suggested that prolonged prometaphase induced by drug treatments led to precocious centriole disengagement and PCM fragmentation in a separase-dependent manner³³, we did not observe a significant increase in separated centrioles after GSK treatment (Extended Data Fig. 3a). Instead, centrioles were misaligned with respect to the

equator in ~16% of metaphase CENP-E KO cells (Extended Data Fig. 3b). These results suggest that PCM fragmentation in the CENP-E KO was not driven by enhanced separase activity, but rather that PCM was detached from centrioles.

Strikingly, we also found that ~44% of mitotic CENP-E KO cells lost astral MTs, consistent with PCM fragmentation in those cells (Fig. 3c). To further investigate this phenotype, we performed live-cell imaging of cells with and without auxin treatment. The appearance of abnormal astral MTs in the CENP-E KO resulted in a high proportion of cells undergoing rotation of their metaphase plates (~50%; Fig. 3d,e; Supplementary Movie 1) and an oblique cell division angle (~41%; Fig. 3 d,f). Intriguingly, these phenotypes are reminiscent of knock-down of *ASPM* and *Wdr62*, two genes implicated in microcephaly that encode mitotic spindle and centrosome proteins^{13, 34, 35}. *CENPE* mutations have also been implicated in microcephaly²⁵, and our results imply that analogous mechanisms could link the disease phenotypes arising from these three mutations.

PCM fragmentation in CENP-E KO is linked to aberrant Plk1 activity

Since centrosomal Plk1 phosphorylates PCM components in prophase, and this phosphorylation is essential for robust PCM expansion^{1, 12}, it was possible that PCM fragmentation in CENP-E KO reflected an aberrant modification of its constituents. To test this possibility, we first explored centrosomal Plk1 levels in prophase CENP-E-AID cells. By immunofluorescence, we revealed that centrosomal Plk1 levels in CENP-E KO cells were significantly diminished as compared to controls (Fig. 4a,b). We next investigated the impact of this Plk1 reduction on PCNT, as it is a key substrate and an essential factor for PCM expansion¹². Previous studies have identified sites on PCNT phosphorylated by Plk1, including S1241¹², which is essential for PCM expansion, and multiple carboxy-terminal sites around R2331³⁶ that are essential for PCNT cleavage during mitotic exit. After

immuno-staining with a phospho-specific antibody (PCNT-pS1241)¹², we found that the fluorescence intensity of pS1241 was significantly lower on centrosomes in prophase CENP-E KO cells as compared to controls, similar to our findings with Plk1 (Fig. 4c,d). We further tested PCNT cleavage to examine the C-terminal phosphorylation of PCNT. Treatment with ZM447439 (ZM, an inhibitor of Aurora kinases) results in forced exit of mitotically arrested cells from M phase, and PCNT is cleaved in a Plk1-phosphorylation dependent manner³⁶. Although we did not detect the cleaved form of PCNT, most likely due to rapid degradation after cleavage, full-length PCNT was nearly undetectable in ZM-treated controls replete with CENP-E, suggesting near-complete cleavage during mitotic exit (Fig. 4e, -IAA). As a control, and as described previously³⁶, we showed that this cleavage was substantially blocked by inhibition of Plk1 through BI2536 (BI) treatment, attesting to its dependence on Plk1 (Fig. 4e, compare lanes 2 and 6). Importantly, similar to Plk1-inhibited cells, ZM-treated CENP-E KO cells retained full-length PCNT, at levels approximating those in untreated cells (Fig. 4e, +IAA; compare lanes 3 and 4). Thus, we conclude that Plk1 fails to efficiently phosphorylate PCNT in CENP-E KO cells.

Finally, to determine whether the reduction in Plk1 levels could explain the PCM defects, we ectopically expressed Plk1 in CENP-E KO cells. Notably, over-expression of constitutively active Plk1 (Plk1-T210D) in CENP-E KO rescued PCM fragmentation, whereas expression of the catalytically inactive protein (Plk1-K82R) did not (Fig. 4f,g). In contrast, chromosome misalignment was not rescued by over-expression of Plk1-T210D (Extended Data Fig. 2c). These data strongly suggest that (1) the reduction of Plk1 levels on centrosomes in CENP-E KO cells results in PCM fragmentation and (2) PCM defects in CENP-E KO cells are independent of kinetochore dysfunction associated with chromosome misalignment.

CENP-E prevents PCM1 accumulation around centrosomes prior to mitosis

As described above, CENP-E is recruited around centrosomes in G₂ phase and dispersed by prophase, during which time centriolar satellites are re-distributed⁴. Since CENP-E is a processive kinesin that moves toward the plus end of MTs¹⁷, we hypothesized that CENP-E transports PCM1 from the vicinity of centrosomes, as it is redistributed throughout the cytoplasm by the initiation of prophase. We therefore investigated the distribution of PCM1 around centrosomes in prophase cells. In control cells, PCM1 exhibited the expected centriolar satellite-like localization, with PCM1 foci scattered around the centrosome (Fig. 5a, -IAA). In contrast, PCM1 was tightly concentrated within the immediate vicinity of centrosomes in CENP-E KO cells (Fig. 5a, +IAA; Fig. 5b). These data suggest that CENP-E removes PCM1 from the peri-centrosomal region by prophase, during which time Plk1 phosphorylates PCM proteins.

As described above, a recent proteome-wide study identified interactions between CENP-E and PCM1, although this interaction was not explored in detail¹⁵. To determine whether these interactions reflected the observed CENP-E-dependent PCM1 redistribution and to clarify the cell cycle stage in which CENP-E interacts with PCM1, we performed immunoprecipitations with lysates from CENP-E-AID cells synchronized by treatment with mimosine (late G₁ phase) or monastrol (M phase). PCM1 co-precipitated with CENP-E in samples released from mimosine for 6 and 10 hours (Fig. 5c, 6h and 10h), and the amount of co-precipitated PCM1 increased in accordance with CENP-E levels. On the other hand, PCM1 was not detected in precipitates from lysates of cells released for 12 hours (Fig. 5c, 12h) or from monastrol-synchronized cells (Fig. 5c, M). Given that most cells reach G₂ phase by 10 h after release from mimosine (Extended Data Fig. 4), these data demonstrate that CENP-E interacts with PCM1 most likely in late S/G₂ phase, consistent with the idea that CENP-E transports PCM1 and centriolar satellites during this period.

PCM1 depletion rescues PCM-related defects in CENP-E KO

In CENP-E KO cells, peri-centrosomal PCM1 accumulation coincided with Plk1 reduction at centrosomes in prophase (Fig. 4a and Fig. 5a). Since PCM1 is thought to restrict centriolar satellite proteins from being recruited to centrosomes⁴⁻⁸, it was conceivable that PCM1 aberrantly sequestered Plk1 from centrosomes in KO cells. Therefore, we performed PCM1 ablation experiments in CENP-E-AID cells to examine whether PCM1 that concentrated in CENP-E KO cells (Fig. 5a,b) physically sequesters Plk1 from centrosomes. With control siRNA treatment, centrosomal intensity of Plk1 in prophase CENP-E KO cells was reduced as expected (Fig. 6a,b, Cntrl; compare with Fig. 4a,b). In striking contrast, loss of CENP-E did not result in a reduction in centrosomal Plk1 in PCM1-depleted cells (Fig. 6a,b, siPCM1), suggesting that the accumulation of PCM1 in CENP-E KO cells is directly responsible for perturbing Plk1 recruitment to centrosomes.

Importantly, and consistent with the recovery of centrosomal Plk1 levels, siPCM1 treatment reversed the reduction in PCNT phosphorylation at S1241 observed in CENP-E KO cells (Fig. 6c,d). Consequently, PCM1 depletion also rescued both PCM fragmentation (Fig. 6e) and the appearance of aberrant astral MTs caused by the loss of CENP-E (Fig. 6f). These data strongly suggest that the PCM-related phenotypes in CENP-E KO cells stem from the accumulation of PCM1 around centrosomes triggered by the absence of a motor protein that normally moves it *away* from the organelle. Importantly, chromosome misalignment in CENP-E KO was not rescued by PCM1 depletion (Extended Data Fig. 2d), indicating that the PCM1-Plk1 pathway is not involved in kinetochore defects caused by the loss of CENP-E.

Patient LCLs mutated in *CENPE* exhibit PCM and cell division defects

Heterozygous mutations in *CENPE* have been identified in two siblings with microcephalic primordial dwarfism²⁵. These two mutations (D933N and K1355E) are located within the coiled-coil region in the middle of the protein but do not affect *CENPE* expression levels. Moreover, as the mutations did not result in embryonic lethality, lymphoblastoid cell lines (LCLs) derived from these patients have been successfully established, providing further evidence that disease mutations in *CENPE* do not lead to cell cycle exit, in contrast with our CENP-E KO cells. To examine whether the mutations drive PCM-related defects observed in the CENP-E KO, we explored PCM structures by immunofluorescence using LCLs from one of the patients and an apparently healthy donor (wild-type). Although PCM fragmentation was rarely observed in the patient LCLs, centrosomal PCNT levels in metaphase were significantly decreased compared to wild-type cells (Fig. 7a,b), suggesting that the mutations in *CENPE* affect PCM expansion and/or maintenance of PCM structures. Consistently, centrosomal p1241 levels also significantly decreased in prophase patient cells (Fig. 7c,d). In agreement with the morphological defects in patient PCM, live-cell imaging revealed that chromosomes were obliquely segregated into daughter cells in 41% of patient LCLs, whereas the percentage of oblique cell divisions in wild-type cells was 13% (Fig. 7e,f). These data indicate that the heterozygous mutations in *CENPE* in microcephaly patients lead to PCM defects resulting in oblique cell divisions, which is a typical phenotype associated with the disease.

Discussion

CENP-E is a kinesin motor that transports chromosomes to the metaphase plate along spindle MTs. Persistent, complete loss of CENP-E causes delays in metaphase, most likely due to incomplete chromosome alignment, followed by cell cycle exit (Fig. 2c-e; Extended Data Fig. 1b)^{22, 23}. Therefore, it has been challenging to investigate direct consequences of CENP-E removal using siRNA or conventional KO cell lines. Here, acute degradation of CENP-E in our AID cell line enabled us to identify and characterize a second, distinct function for CENP-E apart from its role at kinetochores (Fig. 7g). Namely, we found that in G₂ phase, CENP-E transports PCM1 from the vicinity of centrosomes to a location occupied by centriolar satellites. This redistribution of PCM1 is important to maintain the level of centrosomal Plk1 in prophase, when it phosphorylates PCNT to facilitate PCM expansion^{12, 36}. Loss of CENP-E leads to reductions in phosphorylation levels of PCM, resulting in PCM fragmentation, loss of astral MTs, and oblique cell divisions. We propose that CENP-E represents the first kinesin identified as a transporter of centriolar satellites, moving them away from the centrosome. However, we note that ~50% of CENP-E KO cells retained a normal PCM appearance (Fig. 3a), implying that another kinesin or MT remodeling in mitosis could also contribute to the redistribution of PCM1 cooperatively or redundantly with CENP-E.

A recent study reported that centromere dysfunction causes dispersion of PCM during mitosis³⁷. Since CENP-E is a component of the kinetochore, it was possible that this effect may partially overlap phenotypes caused by the loss of CENP-E. However, the dispersion under kinetochore dysfunction was limited to the vicinity of spindle poles, in contrast to PCM fragmentation caused by loss of CENP-E or its inhibition (Fig. 3a,b). Furthermore, the PCM-related phenotypes observed in CENP-E KO cells were rescued by over-expression of active-Plk1 or PCM1 depletion (Fig. 4f,g; Fig. 6), whereas chromosome misalignment, a

phenotype induced by kinetochore dysfunction, was not rescued through these manipulations (Extended Data Fig. 2c.d). These findings indicate that the second, interphase function for CENP-E is functionally separable from its role at kinetochores, and the interphase pathway we propose in our model is most critical for maintenance of PCM integrity during mitosis.

Despite PCM fragmentation and loss of astral MTs after CENP-E removal, most of our KO cells formed bipolar spindles. Since astral MTs are essential for bipolar spindle formation³¹, our results suggest that PCM in CENP-E KO cells is fragmented by MT-induced forces between the cortex or chromosomes and centrosomes *after* bipolar spindle formation. Indeed, live-cell imaging revealed that all of the CENP-E KO cells formed a metaphase plate after NEBD (Fig. 3d; Supplementary Movie 1). Furthermore, the fragmented PCM in CENP-E KO cells did not capture chromosomes, suggesting that the PCM foci did not ectopically accumulate but that they represent PCM that has dissociated from centrosomes after bipolar spindle formation due to aberrant phosphorylation of its components. Spindle MTs attached to kinetochores are cross-bridged to each other by a clathrin-mediated complex^{38,39}. Thus, we speculate that once bipolar spindles are formed, KO cells are able to maintain bipolarity even if the focused ends of the fibers are detached from spindle poles. On the other hand, astral MTs are the only identified structures that connect spindle poles with the cell cortex. Loss of astral MTs in CENP-E KO cells therefore leads to release of the bipolar spindles from the cortex, resulting in rotation of metaphase plates and oblique cell divisions.

To date, 25 genes have been implicated in autosomal recessive primary microcephaly (MCPH; OMIM phenotypic series: PS251200)^{35,40}, and *CENPE* (MCPH13) is one of these genes. Although *PCNT* is not included among the MCPH genes, mutations in the gene cause microcephalic osteodysplastic primordial dwarfism type II (MOPDII)⁴¹⁻⁴³, suggesting strong

correlations between PCM defects and microcephaly. Since CENP-E was first identified as a component of the kinetochore involved in chromosome alignment, it was anticipated that the mutations in *CENPE* provoke microcephaly through defects in chromosome segregation. However, CENP-E patient cells do not exhibit cell cycle exit seen in our CENP-E KO, suggesting that CENP-E mutant proteins in patients retain partial activity essential for cell cycle progression. These data therefore suggest a new mechanism for diseases associated with CENP-E mutations. Using patient LCLs, we revealed that the mutations lead to reductions in the mass of PCM and oblique cell divisions. ASPM (MCPH5) and Wdr62 (MCPH2) also enforce proper spindle orientation through astral MT organization^{13, 34}, and our findings suggest a generalized mechanism for control of cell division orientation, which is de-regulated during brain development in some microcephalic patients.

Thus, our findings have clarified an unexpected and non-canonical function for interphase CENP-E--separable from its role at kinetochores--that is critical for maintenance of PCM structure, astral MTs, and spindle orientation, akin to the function of other genes mutated in microcephaly.

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424 **Acknowledgements**

425 We are grateful to Drs. M. Berkseth and G. Mirzaa (Seattle Children's Institute) for providing
 426 wild-type and *CENPE* mutant patient LCLs and K. Rhee (Seoul National University) for
 427 providing antibodies against PCNT. We thank Masahide Kikkawa (The University of Tokyo),
 428 Masafumi Hirono (Hosei University), and Ken-ichi Wakabayashi (Tokyo Institute of
 429 Technology) for critical reading of the manuscript. B.D.D. was supported by NIH grant
 430 5R01GM120776-09. M.O. was supported by a JSPS Overseas Research Fellowship.

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432 **Author contributions**

433 M.O. and B.D.D. designed experiments, and M.O. performed all the experiments.
 434 M.O. and B.D.D. wrote the manuscript.

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436 **Competing interests:** The authors declare no competing interests.

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Methods

Cell culture

hTERT-RPE1 cells were cultured in Dulbecco's Modified Eagle's Medium: Nutrient Mixture F12 (DMEM/F12; Corning, 10-092-CV) supplemented with 10% fetal bovine serum (FBS) and 50 U/ml penicillin/streptomycin (P/S; Corning, 30-001-CI). HeLa cells were cultured in DMEM (Corning, 10-013-CV) supplemented with 10% FBS, 1% L-Glutamine and P/S (50U/ml). Control and patient LCLs were cultured in Roswell Park Memorial Institute Medium (RPMI 1640; Corning, 10-040-CV) supplemented with 15% FBS and P/S (50U/ml). RPE-1 and HeLa cells were obtained from the American Type Culture Collection (ATCC). Wild-type (LR09-293m) and patient (LR05-054a2) LCLs were gifts from Dr. Mirzaa (Seattle Children's Hospital). All cells were cultured at 37°C with 5% CO₂ and routinely tested with the Universal Mycoplasma Detection Kit (ATCC, 30-1012K).

Production of CENP-E-AID cell line with CRISPR/Cas9

For the CENP-E-AID cell line, an sgRNA (5'-TTGGCACGCCTCCTCAGGCA-3') was sub-cloned into PX458 (Addgene, 48138). The C-terminal region of the *CENP-E* gene was amplified with the following primers; 5'-TGCCCAAACCTGGCCTTGAAC-3', 5'-CCAAAGAGCCGAGAATGACTTGA-3'. The amplicon was integrated into pBluescript SK(-) digested with EcoRV. A template vector which carries full-length AID-3 × FLAG-P2A-BSD was produced with the backbone of pMK392 (Addgene, 121193). Full-length AID-3×FLAG-P2A-BSD was integrated into the site just before the terminal codon of the sub-cloned *CENP-E* gene. 250 ng of each of the donor and Cas9-sgRNA vectors were transfected to RPE-1 cells cultured in 6 wells with 1.5 µl of FuGENE® HD (Promega, E2311) overnight. Transfected cells were then selected with blasticidin S (5 µg/ml) for 10

days, and biallelic mutations in the clones were validated by PCR, followed by DNA sequencing. OsTIR1 and the puromycin-resistant cassette in pBABE TIR1-9myc (Addgene, 64945) were replaced with ARF16-PB1-HA-P2A-OsTIR1 from pMGS46 (Addgene, 126580) and the neomycin-resistant cassette, respectively. Retrovirus was produced with the vector and infected to the CENP-E-AID clones, followed by selection with G418 (500 µg/ml).

Cell cycle synchronization

To increase the number of mitotic cells, RPE1 cells and LCLs were treated with 3 mM or 2 mM thymidine (Sigma, T9250) for 22 hours, respectively. The RPE1 cells were released for 8 hours and the LCLs for 5 hours before fixation. For synchronization at late G1 phase, RPE-1 cells were treated with 0.4 mM mimosine (Sigma, M0253) for 22 hours. Mimosine was washed out twice with fresh media. For synchronization in mitosis, RPE-1 cells were first treated with 3 mM thymidine for 22 hours. After washing out thymidine, the cells were incubated with 5 µM paclitaxel (Selleck, S1150) for 12 hours or 100 µM monastrol for 9 hours. For inhibition of Plk1 activity, cells synchronized with paclitaxel were treated with 100 nM BI2536 (Selleck, S1109) for 3 hours. For forced cell cycle exit, mitotic cells were treated with 2 µM ZM447439 (Selleck, S1103) for 1 hour. 0.5 mM IAA (Sigma, I5148) for CENP-E KO cells or 200 nM GSK923295 (APExBIO, A3450) was added right after release from mimosine or thymidine.

Immunofluorescence microscopy

RPE-1 cells or LCLs on coverslips were fixed with cold methanol at -20°C for 5 min, washed twice with PBS, and blocked with 3% donkey serum in 0.1% Triton X-100/PBS for 15 min. The coverslips were then incubated with primary antibodies for 120 min at room temperature. Cells were washed with 0.1% Triton X-100/PBS three times, and the coverslips

were incubated with secondary antibodies for 90 min at room temperature, nuclei were stained with DAPI, and coverslips were mounted with ProLong^{TM} Diamond Antifade Mountant (Invitrogen, P36961). Cells were observed using an Axiovert 200M (63×, NA 1.4, Zeiss) or an LSM800 (63×, NA 1.4, Zeiss). Maximum intensities in Z-stack images were projected to a single image for figures. Signal intensities were measured after subtracting background intensities using ImageJ. For LCLs, coverslips were coated with 0.1% polyethylenimine (PEI), followed by fibronectin (50 µg/ml; Sigma, F2006). Antibodies used for IF are listed in Supplementary Table 1.

Live-cell imaging

H2B-GFP was introduced into CENP-E-AID cells using retroviral delivery as described above, followed by selection with 5 µg/ml of puromycin. Cells expressing H2B-GFP were seeded onto glass bottom dishes (Greiner, 627870) and treated with 3 mM thymidine for 22 hours. 7 hours after thymidine wash-out, cells were observed using an LSM800 confocal microscope (20×, NA 0.8, Zeiss) operated by Zen software (Zeiss). LCLs were synchronized with 2 mM thymidine for 22 hours. 1 hour after release from thymidine, cells were seeded onto glass bottom dishes coated with PEI, and incubated with 2 µM Hoechst33342 for 15 min. After changing media, cells were observed using the same equipment and conditions described above. Z-stack (thickness of a slice was 4 µm) images were acquired every 4 min, and multiple slices were projected to one image for figures using ImageJ. We defined cells as obliquely dividing if the center of segregated chromosomes were in different Z-stacks in the first frame of anaphase.

Immunoprecipitation and western blotting

Cells were harvested and resuspended in lysis buffer (30 mM Tris-HCl pH 7.5, 150 mM NaCl, 0.5% NP-40, 2mM EDTA, 2 mM EGTA, 1 mM DTT, 10 mM NaF, 50 mM β -glycerophosphate, 5% glycerol and protease inhibitors) on ice for 15 min, followed by centrifugation at 16,000 $\times g$ for 15 min. For immunoprecipitation, supernatants were mixed with ANTI-FLAG® M2 Affinity Gel (Sigma, A2220) and rotated at 4°C for 3 hours. The beads were washed with lysis buffer four times, and proteins were eluted with 0.1 M Glycine-HCl pH 2.8. In SDS-PAGE, 50 μ g of lysates were loaded into each lane, and proteins were transferred to nitrocellulose membranes (GE Healthcare, 10600007). Antibodies used for western blots are listed in Supplementary Table 1, and uncropped blots are shown in Extended Data Fig. 5.

FACS analysis and cell proliferation assays

For FACS analysis, harvested cells were fixed with 70% ethanol for 5 min at -20°C, washed twice with PBS containing 1% BSA, and then stained with Propidium iodide (PI). All data were acquired with an LSRII UV cell analyzer (BD Bioscience) and analyzed by FlowJo software. For cell proliferation assays, 10,000 cells were seeded in 3.5 cm dishes and harvested at indicated time-points. Cells were counted with a Vi-Cell Cell Viability Analyzer (Beckman Coulter).

Statistical analysis

Statistical tests, *n* values, and the number of times that the measurements were repeated were described in figure legends.

Data and materials availability

536 Data supporting the findings of this manuscript are available from the corresponding authors
537 upon reasonable request. Source data for all graphs are available. For LCLs, please contact
538 Dr. G. Mirzaa (Seattle Children's Institute).

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542 **Supplementary Table 1: antibodies used in this study**

Antibody	Host	Dilution	Reference
γ -tubulin	Mouse	1:2000 (IF)	Sigma #T5326
Centrin (20H5)	Mouse	1:1000 (IF)	Merc Millipore #04-1624
Centrin1	Rabbit	1:400 (IF)	Proteintech #12794-1-AP
Plk1 (F-8)	Mouse	1:200 (IF)	SantaCruz #sc-376685
CENP-E (C-5)	Mouse	1:100 (IF), 1:500 (WB)	SantaCruz #sc-17783
PCNT	Rabbit	1:1000 (IF)	Covance #PRB-432C
PCNT	Rabbit	1:20000 (IF), 1:5000 (WB)	Lee and Rhee ,2011
PCNT-p1241	Rabbit	1:400 (IF)	Lee and Rhee ,2011
α -tubulin (B5-1-2)	Mouse	1:3000 (IF)	Sigma #T5168
V5	Mouse	1:1000 (IF)	Invitrogen #R960-25
PCM1	Rabbit	1:1000 (IF), 1:500 (WB)	SantaCruz #sc-67204
CyclinB1 (GNS1)	Mouse	1:1000 (WB)	SantaCruz #sc-245
β -actin	Mouse	1:10000 (WB)	Abcam #ab6276

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Owa and Dynlacht. Fig. 1

Fig. 1 Cytoplasmic CENP-E is recruited around centrosomes in G₂ phase.

a, Mimosine synchronized RPE-1 cells were released into fresh media and harvested 0, 4, 6, 10 and 12 hours after mimosine removal. M phase cells were synchronized with monastrol. Total cell lysates were immuno-blotted with indicated antibodies. **b**, RPE-1 cells released from mimosine were immuno-stained with antibodies against CENP-E (magenta) and centrin1 (green). Representative pictures 0, 6, and 12 hours after release or in prophase are shown (scale bar=10 μm). Inset in each panel is a magnified image around centrosomes. **c**, Signal intensities of CENP-E around centrosomes in **(b)** were measured and plotted (N=148, two independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median).

Owa and Dynlacht. Fig. 2

Fig. 2 Loss of CENP-E induces cell cycle exit.

a, Cell lysates from wild-type RPE-1 and CENP-E-AID (-IAA: not treated; +IAA: treated with IAA for 1 hour) were immuno-blotted with indicated antibodies. The CENP-E band was shifted up in CENP-E-AID due to addition of AID and 3×FLAG tags. **b**, Representative images of CENP-E-AID cells immuno-stained with antibodies against CENP-E (magenta) and centrin1 (green). Kinetochores-specific (top panels) and pericentriolar localization (bottom) of CENP-E diminished after IAA treatment. Arrowheads indicate misaligned chromosomes. Scale bar=10 μm. **c**, Time from NEBD to anaphase onset in CENP-E-AID cells with or without IAA was measured by live cell imaging and plotted (N=60, three independent experiments). **d**, Cell growth assays in CENP-E-AID cells with or without IAA.

569 Each experiment commenced with ~10,000 cells (day1). Percent increases of cells were
 570 plotted (three independent experiments). **e**, Proportions of cells that exited from the cell cycle
 571 were analyzed by FACS with Ki67-FITC staining. Asynchronous or serum starved
 572 CENP-E-AID cells without IAA were used for positive or negative controls, respectively.
 573 Time on the histograms indicates duration of IAA treatment.

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Owa and Dynlacht Fig. 3

Fig. 3 Loss of CENP-E leads to PCM fragmentation and oblique cell divisions

a,b, Synchronized CENP-E-AID (a) or wild-type RPE-1 (b) cells with a single thymidine block were released into fresh media for 8 hours with or without indicated drugs. The cells were then fixed and co-immunostained with antibodies against γ -tubulin (magenta) and PCNT (green). Representative images of metaphase cells in each sample are shown (scale bar=10 μ m). Percentages of metaphase cells with fragmented PCM are compared in the bar graph (>100 cells in total from three independent experiments; error bars: SD). **c**, CENP-E-AID cells treated as in panel (a) were co-immunostained with antibodies against α -tubulin (magenta) and centrin1 (green). Representative images of metaphase cells in each sample are shown (scale bar=10 μ m). Percentages of metaphase cells with absent astral MTs are compared in the bar graph (>100 cells in total from three independent experiments; error bar: SD). **d**, Live-cell imaging of CENP-E-AID cells stably expressing H2B-GFP.

589 Representative time-course images of control (-IAA) or CENP-E KO cells (+IAA) are shown
 590 (scale bar=10 μ m). **e**, Percentages of cells in which the metaphase plate was rotated within 1
 591 hour after NEBD are shown in the bar graph (>100 cells in total from three independent
 592 experiments; error bars: SD). **f**, Percentages of oblique cell divisions (see methods for
 593 definition) are shown in the bar graph (>100 cells in total from three independent
 594 experiments; error bars: SD). p-values were calculated by unpaired *t*-tests.

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Owa and Dynlacht. Fig. 4

Fig. 4 PCM1 fragmentation in CENP-E KO results from reductions in centrosomal Plk1 levels and aberrant phosphorylation of PCNT. **a,b**, Representative images for IAA-treated or un-treated CENP-E-AID cells in prophase co-immunostained with antibodies against Plk1 (magenta) and centrin1 (green) (**a**, Scale bar=10 μ m). The area enclosed by the square in each image is magnified and shown under the panel. Relative Plk1 intensities on the centrosome in prophase cells were plotted in (**b**) (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). **c,d**, Representative images for IAA-treated or untreated CENP-E-AID cells in prophase co-immunostained with antibodies against γ -tubulin (magenta) and PCNT-pS1241 (green) (**c**, Scale bar=10 μ m). The area enclosed by the square in each image is magnified and shown under the panel. Relative PCNT-pS1241 intensities on the centrosome in prophase cells were plotted in (**d**) (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). **e**, Control (-IAA), CENP-E KO (+IAA), and Plk1 inhibited (-IAA+BI) cells synchronized with 100 nM paclitaxel were shaken off from plates

and forced to exit from mitosis with 2 μ M ZM44739 for 1 hour. Cell lysates were immuno-blotted with indicated antibodies. The arrow indicates full-length PCNT. **f,g**, CENP-E-AID cell lines stably expressing doxycycline (dox)-inducible V5-tagged Plk1 (T210D: constitutively active; K82R: catalytically inactive) were synchronized with thymidine for 22 hours, and released for 8 hours. The cells were stained with antibodies against V5-tag (magenta) and PCNT (green). In +dox samples, 1 μ g/ml dox was maintained throughout the experiments. Representative images for metaphase cells in each sample are shown (**f**, scale bar=10 μ m). Percentages of metaphase cells with fragmented PCM are compared in (**g**) (>100 cells in total from three independent experiments; error bars: SD). p-values were calculated by Mann-Whitney *U* tests (b, d) or unpaired *t*-tests (g).

Owa and Dynlacht. Fig. 5

Fig. 5 Loss of CENP-E induces accumulation of PCM1 around centrosomes in prophase.

a,b, Representative images for IAA treated or untreated CENP-E-AID cells in prophase co-immunostained with antibodies against centrin (magenta) and PCM1 (green) (**a**, Scale bar=10 μ m). The area enclosed by the square in each image is magnified and shown under the panel. Relative PCM1 intensities around the centrosome in prophase cells were plotted in (**b**) (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). The p-value was calculated by a Mann-Whitney *U* test. **c,** Cell lysates from mimosine (6h, 10h, and 12h, indicating hours after release) or monastrol (M) synchronized CENP-E-AID cells were immunoprecipitated with FLAG-M2 beads. 10h and 12h (+IAA) samples were for negative controls. The precipitates were immuno-blotted with anti-CENP-E and anti-PCM1 antibodies (left). Inputs in each sample were also blotted with indicated antibodies (right).

Owa and Dynlacht Fig. 6

Fig. 6 Accumulation of PCM1 around centrosomes perturbs centrosomal Plk1 recruitment and PCNT phosphorylation in CENP-E KO. **a**, CENP-E-AID cells were synchronized with thymidine for 22 hours. In the first 8 hours of the synchronization, cells were treated with control siRNA or siPCM1. The cells were then released for 8 hours with or without IAA, fixed, and co-immunostained with antibodies against Plk1 (magenta) and centrin1 (green). Representative images for prophase cells in each sample are shown. The area enclosed by the square in each image is magnified and shown under the panel. Scale bar=10 μ m. **b**, Box plots of relative Plk1 intensities on centrosomes in prophase cells (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center

line: median). **c,d**, Samples prepared as **(a)** were co-immunostained with antibodies against γ -tubulin (magenta) and PCNT-pS1241 (green). Representative images for prophase cells in each sample are shown (**c**, Scale bar=10 μ m). The area enclosed by the square in each image is magnified and shown under the panel. Relative PCNT-pS1241 intensities on the centrosome in prophase cells were plotted in **(d)** (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). **e,f**, CENP-E-AID cells were synchronized with siRNA treatments as **(a)**. The cells were then released for 8 hours with IAA, and percentages of PCM fragmentation (**e**) or abnormal astral MTs (**f**) were calculated as in Fig. 3a and c (>100 cells in total from three independent experiments; error bars: SD). p-values were calculated by Mann-Whitney *U* tests (b, d) or unpaired *t*-tests (e, f).

Owa and Dynlacht. Fig. 7

Fig. 7 LCLs from a microcephalic patient with *CENP-E* mutations show PCM defects and oblique divisions. **a,b**, Wild-type and patient LCLs were synchronized with 2 mM thymidine for 22 hours, and released for 5 hours. Representative images for metaphase cells co-immunostained with antibodies against γ -tubulin (magenta) and PCNT (green) are shown (**a**, Scale bar=10 μ m). The area enclosed by the square in each image is magnified and shown under the panel. Relative PCNT intensities on the centrosome in metaphase cells were plotted in (**b**) (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). **c,d**, Representative images for prophase LCLs co-immunostained with antibodies against γ -tubulin (magenta) and PCNT-pS1241 (green) (**c**, Scale bar=10 μ m). Relative PCNT-pS1241 intensities on the centrosome in metaphase cells were plotted in (**d**) (N=90, three independent experiments; whisker: 95% confidence interval; box: interquartile; center line: median). **e**, Live-cell imaging of wild-type and patient LCLs. DNA was visualized with Hoechst 33342. Representative time-course images of wild-type

(top) or patient (bottom) LCLs are shown (scale bar=10 μ m). 3D reconstructions in the last time points (viewed from arrowheads) are also shown next to the images. **f**, Percentages of oblique cell divisions observed in the live-cell imaging (see methods for definition) were compared in the bar graph (>100 cells in total from three independent experiments; error bars: SD). p-values were calculated by Mann-Whitney *U* tests (b, d) or an unpaired *t*-test (f). **g**, A model summarizing results in this study. See text for details.

Owa and Dynlacht. Extended Data Fig. 1

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682 **Extended Data Fig. 1 Further characterization of the CENP-E-AID cell line.**

683 **a**, Cell cycle profiles of asynchronous wild-type cells and asynchronous CENP-E-AID cells
684 without IAA treatment. DNA content was analyzed by FACS using propidium iodide (PI)
685 staining. **b**, Cell cycle profiles of CENP-E-AID cells treated with IAA. Asynchronous
686 CENP-E-AID cells were treated with IAA for indicated hours and analyzed by FACS (PI).
687 Time on the histograms indicates duration of IAA treatment.

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Owa and Dynlacht Extended Data Fig. 2

Extended Data Fig. 2 Percentages of misaligned chromosomes.

Percentages of cells that possess one or more misaligned chromosomes around the spindle pole. Error bars: SD. **a**, CENP-E-AID cells with or without IAA; **b**, wild-type cells treated with DMSO or GSK; **c**, CENP-E-AID cells stably expressing doxycycline (dox)-inducible V5-tagged Plk1 (T210D or K82R), treated with IAA only or IAA and dox; **d**, CENP-E-AID cells treated with control siRNA and IAA or siPCM1 and IAA.

Owa and Dynlacht Extended Data Fig. 3

Extended Data Fig. 3 Centriole detachment from the spindle pole in CENP-E KO.

Wild-type RPE-1 cells were synchronized with thymidine and released for 8 hours with DMSO or GSK. The cells were co-immuno-stained with antibodies against γ -tubulin (magenta) and centrin1 (green). **a**, Percentages of precocious centriole disengagement (>100 cells in total from three independent experiments; error bars: SD). **b**, Representative images for normal (top, DMSO) or misaligned centrioles (bottom, GSK). The arrowhead indicates centrioles detached from the spindle pole. Percentages of centrioles detached from the spindle pole are shown in the bar graph (>100 cells in total from three independent experiments; error bars: SD). p-values were calculated by unpaired *t*-tests.

Owa and Dynlacht. Extended Data Fig. 5

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712 **Extended Data Fig. 4 Cell cycle profiles of mimosine-synchronized cells.**

713 Mimosine synchronized wild-type RPE-1 cells or CENP-E-AID cells treated with indicated

714 drugs were released for 4, 6, 10 and 12 hours and analyzed by flow cytometry. Histograms of

715 DNA content at each time point are shown (2N: G₁; 4N: G₂/M)

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Owa and Dynlacht Extended Data Fig. 5

Extended Data Fig. 5 Uncropped images of western blots.