Supplemental Materials
Molecular Biology of the Cell

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Supplemental Figure 1. Cetn3 inhibits trans-autophosphorylation of the Mps1 kinase domain.

GST-Mps1 (M), GST-tagged catalytically inactive Mps1 kinase domain (M^KDD; Mps1 residues 515-794 containing the D664A mutation) and His-Cetn3 (C3) were used for *in vitro* kinase assays that were incubated for a total of 60 min. As summarized in the chart at the top of the figure, various reaction components were added for either the first 30 min or the second 30 min, as follows: In lanes 1 and 2, Mps1 (M) and M^KDD were incubated on their own for the entire 60 min, verifying that M^KDD lacks kinase activity. In lane 3, C3 was incubated alone for the first 30 min, and Mps1 was added for the remaining 30 min, showing that C3 reduces Mps1
autophosphorylation. In lane 4, Mps1 was incubated alone for the first 30 min and $M^{KDD}$ was added for the remaining 30 min. In lanes 5-7, $M^{KDD}$ was pre-incubated alone (lane 5) or with 1.0 (lane 6) or 0.1 µg (lane 7) C3 for 30 min, and Mps1 was added for the remaining 30 min, while in lane 8, Mps1 was pre-incubated with 1 µg C3 for 30 min, and $M^{KDD}$ was added for the remaining 30 min, showing that C3 reduces $M^{KDD}$ phosphorylation whether it is pre-incubated with $M^{KDD}$ (lane 6) or Mps1 (lane 8). In lane 9, Mps1 and $M^{KDD}$ were pre-incubated for the first 30 min and 1 µg C3 was added for the remaining 30 min, showing that C3 cannot reverse phosphorylation of $M^{KDD}$. After 60 min kinase assays were analyzed by SDS-PAGE and autoradiography. Coomassie staining (showing equal loading of $M^{KDD}$) and 30-min autoradiographic exposure are shown.

A.

B.

C.
Supplemental Figure 2. Cetn3 reduces Cetn2 phosphorylation over a wide concentration range and reduces the ability of Mps1 to phosphorylate a generic substrate.

(A) Determining the concentrations of recombinant proteins. GST-Mps1 (M) was treated with and without lambda phosphatase (λ) to revert auto-phosphorylated forms into a single full-length band. GST-Mps1 (M), GST-Mps1KDD (M117-794), His-Cetn2 (C2), His-Cetn3 (C3), and known amounts of BSA and Lysozyme were then run on an SDS-PAGE gel, stained with SYPRO-Ruby, and scanned on a Typhoon imager. A standard curve was generated from BSA and Lysozyme, and the concentration of GST-Mps1, MKDD, His-Cetn2 and His-Cetn3 was calculated using this standard curve. (B) Cetn3 inhibits Cetn2 phosphorylation over several a wide concentration range. Recombinant GST-Mps1 (M), His-Cetn2 (C2) and His-Cetn3 (C3) were used for in vitro kinase assays. Kinase assays were incubated for a total of 60 min. Mps1 was present for the entire 60 min in all assays. In lanes 1 and 2, either C2 or C3 (0.5 µg) was added after 30 min. In lanes 3-8, varying amounts of C3 (1, 0.5, 0.05, 0.005, 0.0005, or 0.00005 µg) were co-incubated with Mps1 for 30 min, after which 1 µg C2 was added for an additional 30 min. The concentrations of the proteins in lane 3 are as follows; GST-Mps1, 3.55 µM; Cetn2, 7.28 µM; Cetn3, 22 µM. Thus, the concentration of Cetn3 in lanes 4-8 varied from 11 µM to 1.1 nM. After 60 min assays were analyzed by SDS-PAGE. Shown are the Coomassie stained gel and corresponding 30 min autoradiograph exposure. Cetn3 reduced phosphorylation of Cetn2 after dilution by several orders of magnitude, and full phosphorylation of Cetn2 is only restored when Mps1 is present in 100-fold molar excess over Cetn3 (compare lanes 7 and 8 to lane 1). (C) Cetn3 also reduces phosphorylation of the generic substrate Myelin Basic protein (MBP) by Mps1. GST-Mps1 (M), His-Cetn2 (C2), His-Cetn3 (C3), and MBP were used for in vitro kinase assays that were incubated for a total of 60 min. As summarized in the chart at the top of the figure, various reaction components were added for either the first 30 min or the second 30 min, as follows: Mps1 was present for the entire 60 min in all reactions. In lanes 1-3, C3, C2, or MBP were co-incubated with Mps1 for the entire 60 min. In lane 4, C3 was pre-incubated with Mps1 for 30 min prior to addition of C2 for an additional 30 min. In lane 5, MBP was pre-incubated with Mps1 for 30 min prior to addition of C3 for an additional 30 min. In Lane 6, MBP and C3 were co-incubated with Mps1 for the entire 60 min. In lanes 7 and 8, 1 or 0.5 µg C3, respectively, was pre-incubated with Mps1 for 30 min prior to addition of MBP for an additional 30 min. In lane 9, Mps1 was incubated alone for the entire 60 min. After 60 min kinase assays were analyzed by SDS-PAGE. Shown are the Coomassie stained gel and corresponding 30 min autoradiograph exposure. The red arrow corresponds to the position of C3, blue arrow to C2, black arrow to MBP, and arrowhead to Mps1 autophosphorylation. Lane 4 recapitulates the inhibition of Cetn2 phosphorylation by pre-incubation of Mps1 and Cetn3, as shown in Figure 2. As with C2, MBP phosphorylation is not inhibited when Cetn3 and MBP are co-incubated with Mps1 for the entire 60 min (lane 6) or when Cetn3 is added to the reaction after 30 min (lane 5). However, as for Cetn2, pre-incubation of Cetn3 and Mps1 (lanes 7 and 8) reduces the phosphorylation of subsequently added MBP. The asterisk represents a non-specific phosphorylated species present in the MBP preparation.
Supplemental Figure 3. Interaction of endogenous Mps1 and Cetn3 in HEK293 cells.

(A) Validation of rabbit anti-Cetn3. Rabbit anti-Cetn3 was produced against His-Cetn3 as described previously for rabbit anti-Cetn2 (Yang et al., 2010). HeLa cells were transfected with sicontrol (siCon) or siCetn3 and arrested in S-phase for 48 hr. Cells were lysed and immunoblotting was performed with rabbit Cetn3 (1:1000). The antibody detects a band at approximately 20 kDa in the siCon, and this band was reduced by roughly 80% in the siCetn3 lane. The lower panel shows immunoblotting of the same samples with α-Tubulin to show equal loading. (B, C) Reciprocal co-IP is observed between endogenous Mps1 and Cetn3 in HEK 293 cells. Lysates from S-phase arrested HEK 293 cells were incubated with rabbit antibodies against (B) Mps1 (αMps1) or (C) Cetn3 (αC3) then analyzed by SDS-PAGE and immunoblotting with mouse antibodies against Mps1 or Cetn3. The bracket in (C) denotes the abundant species of Mps1 while the black arrow indicates the less abundant, lower mobility species of Mps1 that interacts with Cetn3. Input (In) is 1% of the lysate. Pc denotes the pre-clearing beads (e.g. binding to beads alone).
Supplemental Figure 4. Estimation of Cetn3 overexpression levels.

(A-C) In-Cell Western showing the amount of Cetn3 overexpression. HeLa cells were transfected with GFP (G), GFP-Cetn3 (G-Cetn3), m-Cherry (mCh) or m-Cherry Cetn3 (mCh-Cetn3) and arrested in S-phase for 48 hr. Cells were fixed and antibody against Cetn3 was used to estimate the amount of Cetn3 overexpression. γ-Tubulin (γ-Tub) was used as a control for cell number. (A) Representative images showing the relative amount of Cetn3 (red), (γ-Tub) (green) in each well. Higher exposure of Cetn3 is also presented to show the endogenous Cetn3 in control cells transfected with GFP or mCh. No primary antibody (No Ab) was used in adjacent wells to correct for background. (B) Graphical representation of fold increase in the Cetn3 signal in the wells transfected with GFP-Cetn3 (G-Cetn3) compared to the GFP alone (set at 1) control. Background corrected Cetn3 signal was normalized to background corrected γ-Tub signal and the fold change was calculated as the ratio of average Cetn3 signal in the GFP-Cetn3 wells (performed in triplicates) to the average Cetn3 signal in the GFP alone wells. (C) Graphical representation of fold increase in the Cetn3 signal in the wells transfected with mCh-Cetn3 compared to mCh alone (set at 1) control. The fold change was calculated as described in (B).
Supplemental Figure 5. Depletion of Cetn3 does not induce any obvious S-phase defects, and the effects of Cetn3 depletion are reversed by overexpression of siRNA-resistant Cetn3.

(A, B) Asynchronously growing HeLa cells transfected with control (siCon) or Cetn3-specific (siCetn3) siRNAs were incubated with BrdU for 4 hr. Cells were fixed and stained with an antibody against acetylated tubulin (green) to identify cell boundaries, and an antibody against BrdU (blue) to estimate the percentage of BrdU positive cells in siControl cells (siCon) compared to siCetn3 cells. (A) Image of representative fields of BrdU positive asynchronously growing HeLa cells transfected with either siCon or siCetn3. (B) Graphical representation of percent BrdU positive cells. Values represent the mean ± SD of triplicate samples where at least 100 cells were counted per replicate. (C-E) HeLa cells were sequentially transfected with control or Cetn3-specific siRNAs and either mCh alone, or siRNA-resistant mCh-Cetn3 (mCh-C3siR),
then arrested in S-phase for 48 hr with HU. (C) Immunoblot showing the expression of mCh and mCh-C3siR in cells transfected with siControl (Con) or siCetn3 (C3). (D) Graphical representation of the percentage of S-phase arrested cells transfected as indicated that had more than 4 Cetn2 foci. (E) Graphical representation of percentage of S-phase arrested cells transfected as indicated that had long linear Cetn2 structures. Values in D and E represent mean ± standard deviation of triplicate samples where at least 100 cells were counted per replicate.

Supplemental Figure 6. Overexpression of Cetn3 inhibits centrosome reduplication in U2OS cells.

U2OS cells were transfected with mCh-Cetn3, arrested in S-phase for 24 hr as described in materials and methods, and centrosome number was determined by γ-Tubulin staining in transfected cells and non-transfected cells. (A) Representative image of an mCh-Cetn3 (red) expressing cell adjacent to a non-transfected cell; γ-Tubulin (green), bar is 5 μm. (B) Graphical representation of the percentage of S-phase arrested U2OS cells with more than 2 γ-Tubulin foci in mCh-Cetn3 transfected cells compared to untransfected cells. Cetn3 overexpression leads to a decrease in the cells with extra γ-Tubulin foci. Values represent mean ± standard deviation of triplicate samples where at least 100 cells were counted per replicate.
Supplemental Figure 7. Mps1 pT676 staining at centrosomes, and Cetn3 depletion does not grossly affect centrosomal or cellular Mps1 levels.

(A) Representative images of asynchronously growing HeLa cells showing active Mps1 at centrosomes stained with pT676 (green) and γ-Tubulin (red) in various stages of the cell cycle. BrdU was incorporated during a 4 hr pulse, and images were taken after a 4 hr chase. Bar is 5 μm. Mps1 pT676 phosphorylation is seen at kinetochores and centrosomes in prometaphase, but not in anaphase when pT676 phosphorylated Mps1 is present at the midbody; white boxes indicate the positions of γ-Tubulin/centrosomes, and white arrows indicate pT676 staining at the midbody. (B-D) HeLa cells were transfected with either control (siCon) or Cetn3-specific siRNA (siCetn3) for 68 h, then labeled with BrdU for 4 h and analyzed by IIF with the M02 pan Mps1 antibody. (B) Representative micrographs showing weak, moderate and strong total centrosomal Mps1 staining (green) at centrosomes (γ-tub, red). (C) Box and whisker plot showing the normalized level of total centrosomal Mps1, determined as described in materials and methods, for 25 BrdU-positive cells each for siCon and siCetn3 imaged under identical optical condition. p value was determined by unpaired T-test. (D) Immunoblots show whole-cell level of Mps1 and Cetn3 in siCon and siCetn3 cells where α-Tubulin (α-tub) was used as loading control. siCetn3 effectively depleted Cetn3 but did not affect the whole cells levels of Mps1 and had minimal effect on total centrosomal Mps1.