Supplementary Information

CAMP (C13orf8, ZNF828) is a novel regulator of kinetochore-microtubule attachment

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Supplementary Methods

Cell culture and transfections

HeLa cells were used unless otherwise indicated. Cells were cultured in DME medium supplemented with 10% fetal bovine serum. For synchronization at the G1/S boundary, cells were cultured in the presence of 2 mM thymidine for 18 h. For synchronization at M phase, cells were cultured in the presence of 2 mM thymidine for 18 h, washed once with PBS, cultured in fresh medium for 3 h, and then cultured in the presence of 100 ng/ml nocodazole or 1 μM taxol for 12 h. Transfection of siRNA oligonucleotides was carried out by incubating 100 nM duplexed siRNA with RNAi MAX (Invitrogen) in antibiotic-free growth medium. For control transfections, the same annealing reaction was performed using H₂O instead of siRNA oligos. Transfection of expression plasmids was performed using FuGENE 6 Transfection Reagent (Roche Applied Science). Rescue experiments were performed by transfecting siRNA-treated cells 36 h later with the indicated expression plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s protocol. Cells were observed 24 h after transfection of the expression plasmids.

Plasmid construction

The cDNAs coding for human MAD2L2 and CAMP were PCR-amplified from a human brain cDNA library. The cDNAs were subcloned into pcDNA5/FRT (Invitrogen), pGEX-4T3, or pEGFP-C1 (Clontech) expression vectors. The expression plasmids for
CAMP deletion mutants were constructed using PCR amplification. The expression plasmids for non-phosphorylatable CAMP mutants and RNAi-resistant CAMP constructs were constructed using the QuikChange Multi Site-Directed Mutagenesis Kit (Stratagene).

**Generation of cell lines, immunoprecipitation (IP), and nanoflow liquid chromatography tandem mass spectrometry (nano-LC/MS/MS)**

Cell lines stably expressing Flag-MAD2L2 were established using the Flp-In system (Invitrogen) according to the manufacturer’s instructions. Flp-In-293 cells (Invitrogen) were transfected with the Flag-MAD2L2 expression vector using FuGENE 6 Transfection Reagent and selected for hygromycin resistance. IP was carried out with anti-Flag M2 affinity gel (Sigma) as previously described (Hong et al, 2008). The IP product was separated by SDS-PAGE and stained using a silver stain kit (Wako). Protein bands were excised from the gels, digested with trypsin and subjected to nano-LC/MS/MS analysis as described previously (Hong et al, 2008).

**Immunoprecipitation**

Cells were lysed in IP buffer (20 mM Tris (pH 7.5), 150 mM NaCl, 20 mM β–glycerophosphate, 5 mM MgCl₂, 0.1% NP-40, 5% glycerol) supplemented with protease inhibitor cocktails (Complete Mini EDTA-free, Roche Diagnostics) and phosphatase inhibitors (Wako), and cleared by centrifugation for 30 min at 4°C at 13,000 rpm. After preclearing with sheep anti-rabbit IgG conjugated to magnetic beads
(Dynabeads M-280, Invitrogen), the lysates were incubated with anti-CAMP antibody (3 µg) for 1 h in the presence of RNase A and DNase I at 4°C, followed by incubation with anti-rabbit IgG conjugated to magnetic beads for 2 h at 4°C. After washing twice with IP buffer and twice with TBS containing 0.1% Tween 20, the beads were suspended in SDS-PAGE sample buffer and subjected to western blotting.

**Western blotting**

Cells were lysed in IP buffer supplemented with protease and phosphatase inhibitors. Protein concentration was measured and adjusted by the Bradford method (Protein Assay system, Bio-Rad Laboratories), and the cell extracts were resolved by SDS-PAGE and transferred to a PVDF membrane (Immobilon-P, Millipore). Blocking and antibody incubations were performed in 5% non-fat dry milk. Horseradish peroxidase-labelled secondary antibodies (Santa Cruz) were visualized by chemiluminescence using luminal and couramic acid (Amersham).

**Far western assay**

GST-recombinant proteins were separated by SDS-PAGE and transferred to a PVDF membrane. After the transferred proteins were renatured with guanidine hydrochloride, the membrane was blocked in 5% non-fat dry milk and incubated with 1 µg/ml His-MAD2L2 overnight at 4°C. After extensive washing, the membrane was subjected to western blotting with an anti-His antibody.
Reference for Supplementary Methods

Supplementary Figure Legends

Supplementary Figure 1. CAMP interacts with MAD2L2 and localizes to kinetochores

(A) Identification of the proteins associated with MAD2L2. Lysates of asynchronous HEK293 cells (Control) or asynchronous HEK293 cells expressing Flag-MAD2L2 were immunoprecipitated with anti-Flag M2 antibody. The immunoprecipitations (IPs) were separated by SDS-PAGE and stained with silver.

(B) Coprecipitation of endogenous CAMP with Flag-MAD2L2. Total cell lysates prepared from HEK293 cells expressing Flag-MAD2L2 treated with thymidine (Thy) or nocodazole (Noc) were subjected to IP with an anti-Flag antibody. Lysates and IPs were separated by SDS-PAGE and probed by western blotting with anti-CAMP and anti-Flag antibodies, respectively.

(C) Schematic representation of the structure of CAMP. Upper panel: The structure of CAMP. The C2H2-type zinc-finger domains, SPE motifs, WK motifs, and FPE motifs are shown. Lower panel: Sequences of the SPE, WK, and FPE motifs. The coordinates of the first residue of each repeat in the protein sequence are given. The consensus motif of each domain is indicated at the top.

(D) Schematic representation of CAMP fragments expressed as GST-fusion proteins. The coordinates of the first and the last residues of each construct on the original protein sequence are indicated.

(E) CAMP interacts with MAD2L2 through the WK region. The GST-tagged domains
of CAMP shown in (D) were blotted on a membrane and incubated with a 
His-MAD2L2 probe, followed by detection of the probe with an anti-His antibody.

(F) Distribution of CAMP on chromosomes. HeLa cells were treated with 50 ng/ml 
nocodazole for 6 h to enrich mitotic cells. Mitotic cells were shaken off, cytospun, fixed, 
and stained with anti-CAMP antibody (green) and CREST serum (red). DNA was 
stained with DAPI (blue). Right panels are magnifications of the boxed area. Scale bar 
= 5 μm.

Supplementary Figure 2. Phenotype of CAMP-depleted cells

(A) Localization of MAD2L2 in CAMP-depleted cells during mitosis. HeLa cells 
expressing GFP-MAD2L2 (green) were treated with mock, MAD2L2, or CAMP siRNA 
for 48 h. DNA was stained with DAPI (blue). Note that GFP-MAD2L2 localizes to the 
spindle (arrows indicate spindle poles). Scale bar = 10 μm.

(B) Chromosome misalignment in U2OS cells depleted of CAMP. i. Depletion of 
CAMP with siRNA. Total cell lysates prepared from U2OS cells treated with mock or 
CAMP siRNA for the indicated periods were separated by SDS-PAGE and probed by 
western blotting with an anti-CAMP antibody. ii. U2OS cells were treated with mock or 
CAMP siRNA for 48 h and stained with anti-CAMP antibody (green). DNA was stained 
with DAPI (blue). Scale bar = 10 μm.

Supplementary Figure 3. Multipolar spindle and γ-tubulin localization in 
CAMP-depleted cells
(A) Multipolar spindles in CAMP-depleted cells. i. HeLa cells were treated with mock or CAMP siRNA for 48 h and with MG132 (10 μM) for the final 2 h. Cells were stained for tubulin (green), kinetochores (CREST, red), and DNA (blue). Scale bar = 10 μm. ii. Pie charts showing the percentage of mitotic cells with bi- and multipolar spindles. iii. Live cell imaging of a HeLa cell expressing histone H2B-GFP that exhibited multipolar spindle formation 48 h after CAMP siRNA transfection. Scale bar = 10 μm.

(B) Localization of γ-tubulin in CAMP-depleted cells. HeLa cells were treated as in (A)i. Cells were stained with an antibody against γ-tubulin (green) and for DNA (blue). Scale bar = 10 μm.

Supplementary Figure 4. Chromosome misalignment in cells expressing CAMP deletion mutants

(A) Expression of GFP-CAMP deletion mutants. Lysates were prepared from HeLa cells transfected with RNAi-resistant GFP-CAMP mutants for 24 h, preceded by transfection with CAMP siRNA for 36 h. Cell lysates were separated by SDS-PAGE and probed by western blotting with an anti-GFP or anti-Actin antibody. Arrowheads indicate the positions of corresponding mutants.

(B) The FPE region is responsible for the effect of CAMP on chromosome alignment. HeLa cells were transfected with RNAi-resistant Flag-CAMP mutants for 24 h, preceded by transfection with CAMP siRNA for 36 h. Cells were treated with MG132 (10 μM) for the final 2 h. The percentage of cells with misaligned chromosomes is shown for each mutant. Experiments were repeated three times and representative data
are shown. Oligo 1 was used for RNAi, and similar results were obtained for oligo 2 (data not shown).

(C) Expression of GFP-CAMP fragments was evaluated as in (A).

Supplementary Figure 5. Phosphorylation of CAMP.

(A) Schematic representation of the distribution of the SPE/D sequences in CAMP. The coordinate of the first residue of each sequence in the protein sequence is indicated.

(B) Expression of non-phosphorylatable GFP-CAMP mutants. Lysates were prepared from HeLa cells transfected with RNAi-resistant GFP-CAMP mutants for 24 h, preceded by transfection with CAMP siRNA for 36 h. The lysates were separated by SDS-PAGE and probed by western blotting with an anti-GFP or anti-Actin antibody. Arrowheads indicate the positions of corresponding mutants.

(C) Mitotic phosphorylation is dispensable for the spindle localization of CAMP. Localization of GFP-tagged full-length CAMP, CAMP-ΔC-ZNF, or CAMP-FPE, with or without the replacement of serines with alanines in the SPE/D sequences, when expressed in HeLa cells (green). DNA was stained with DAPI (blue). Scale bar = 10 μm.

Supplementary Figure 6. Effect of CAMP depletion on kinetochore/centromere-localizing proteins

(A) Localization of CENP-C, HEC1, and Aurora B in CAMP-depleted cells. HeLa cells were treated with mock or CAMP siRNA for 48 h and with MG132 (10 μM) for the
final 2 h. Cells were stained for antibodies against the indicated proteins (green), together with kinetochores (CREST, red) and DNA (blue). Insets are magnified images of the boxed areas. Scale bar = 10 μm.

(B) Kinetochore localization of Blinkin (KNL1), RanGAP1, RanBP2, Sgo1, and Ska1 in cells treated as in (A). Insets are magnified images of the boxed areas. Scale bar = 10 μm.

(C) Kinetochore localization of BUB1 and BUBR1 in cells treated as in (A). Scale bar = 10 μm.

**Supplementary Figure 7. Effect of CAMP depletion on spindle/chromosome-localizing proteins**

(A) Localization of EB1, CLIP-170, and ch-TOG in CAMP-depleted cells. HeLa cells were treated with mock or CAMP siRNA for 48 h and with MG132 (10 μM) for the final 2 h. Cells were stained for antibodies against the indicated proteins (green), together with microtubules (red) and DNA (blue). Scale bar = 10 μm.

(B) Localization of phospho-H3S10 and phospho-H3Th3 in cells treated as in (A). Cells were stained for an antibody against phosphorylated histone H3S10 (left panels, green) or H3Th3 (right panels, green), as well as DNA (blue). Scale bar = 10 μm.

(C) Localization of KID in cells treated as in (A). Scale bar = 10 μm.

(D) Localization of HP1α in cells treated as in (A). Scale bar = 10 μm.

(E) Localization of condensin I (CAP-G; left panels) and condensin II (CAP-G2; right panels) in cells treated as in (A). Scale bar = 10 μm.
Supplementary Figure 8. Chromosome misalignment in cells depleted of CAMP/CENP-E/CENP-F

(A) Depletion of CAMP/CENP-E/CENP-F with siRNAs. Total cell lysates prepared from HeLa cells treated with mock or indicated siRNAs for 48 h were separated by SDS-PAGE and probed by western blotting with indicated antibodies.

(B) Representative images of CAMP/CENP-E/CENP-F-depleted cells. HeLa cells were transfected with indicated siRNAs for 48 h, and treated with MG132 (10 μM) for the final 2 h. DNA was stained with DAPI. Scale bar = 10 μm.

(C) Quantitative analysis of chromosome misalignment in cells treated as in (B). The number of misaligned chromosomes per cell was scored. Error bars represent the s.d.

Supplementary Figure 9. Kinetochore localization of CENP-E and CENP-F in cells expressing non-phosphorylatable CAMP mutants.

HeLa cells were treated as in Figure 6D and stained for Hec1 (green), CENP-E or CENP-F (red), and DNA (blue). The GFP-CAMP signal was also detected in the green channel. Scale bar = 10 μm.
Supplementary Movies

Supplementary Movie 1. Live cell imaging of a mock-treated HeLa cell expressing H2B-GFP. Stills of this movie are displayed in Figure 3A. Images were collected every 3 min, and the display rate is 10 frames/s.

Supplementary Movie 2. Live cell imaging of a CAMP-depleted HeLa cell expressing H2B-GFP. Stills of this movie are displayed in Figure 3A. Images were collected every 3 min, and the display rate is 10 frames/s.

Supplementary Movie 3. Live cell imaging of a mock-treated HeLa cell expressing GFP-CENP-A and GFP-α-tubulin. Images were collected every 1 min, and the display rate is 10 frames/s.

Supplementary Movie 4. Live cell imaging of CAMP-depleted cells expressing GFP-CENP-A and GFP-α-tubulin. Stills of this movie are displayed in Figure 4A. Images were collected every 1 min, and the display rate is 10 frames/s.
**Supplementary Figure 2**

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**B**

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Supplementary Figure 3
Supplementary Figure 7
**A**

Supplementary Figure 8

[Supplementary Figure showing gel electrophoresis results with lanes labeled 1 to 7. Lane 1 is marked as mock, Lane 2 as CAMP, Lane 3 as CENP-E, Lane 4 as CENP-F, Lane 5 as CAMP/CENP-E, Lane 6 as CAMP/CENP-F, and Lane 7 as CAMP/CENP-E/F.]

**B**

[Supplementary Figure showing images of chromosome misalignment. The images are labeled mock, CAMP, CENP-E, CENP-F, CAMP/CENP-E, CAMP/CENP-F, and CAMP/CENP-E/F.]

**C**

[Supplementary Figure showing a bar graph representing chromosome misalignment (%). The graph shows RNAi treatments (mock, CAMP, CENP-E, CENP-F, CAMP/CENP-E, CAMP/CENP-F, CAMP/CENP-E/F) and misalignment categories (0-1 misaligned, 2-5 misaligned, >5 misaligned).]
Supplementary Figure 9