The microtubule-associated protein HURP recruits the centrosomal protein TACC3 to regulate K-fiber formation and support chromosome congression

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Kinetochoore fibers (K-fibers) are microtubule bundles attached to chromosomes. Efficient K-fiber formation is required for chromosome congression, crucial for faithful chromosome segregation in cells. However, the mechanisms underlying K-fiber formation before chromosome biorientation remain unclear. Depletion of hepatoma up-regulated protein (HURP), a RanGTP-dependent microtubule-associated protein localized on K-fibers, has been shown to result in low-efficiency K-fiber formation. Therefore, here we sought to identify critical interaction partners of HURP that may modulate this function. Using co-immunoprecipitation and bimolecular fluorescence complementation assays, we determined that HURP interacts directly with the centrosomal protein transforming acidic coiled coil–domain, and a guanylate kinase–associated protein; aa, amino acid(s); GKAP, guanylate kinase–associated protein; HEK, human embryonic kidney; EGFP, enhanced GFP; BIFC, bimolecular fluorescence complementation; VC, Venus-C; VN, Venus-N; MSD, mean square displacement; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; HA, hemagglutinin; IP, immunoprecipitation; shRNA, short hairpin RNA; ch-TAG, colonic and hepatic tumor-overexpressed gene; CHC, clathrin heavy chain; PA, photo-activated.

Efficient bipolar spindle formation is crucial for proper chromosome alignment and segregation. During mitosis, immediately after nuclear envelope breakdown, the interphase microtubules (MTs) quickly reorganize and nucleate to the mitotic spindles regulated by various MT nucleation pathways, including centrosome-dependent nucleation, RanGTP-driven chromatin-dependent MT nucleation, the chromosomal passenger complex (CPC) pathway, and MT-dependent MT nucleation through the Augmin complex (1–5). Mitotic spindles are characterized as three different populations: kinetochore microtubules (kMTs), astral microtubules, and interpolar microtubules (6–8); however, it is unclear how specific microtubule populations are formed.

K-fibers, bundles of 20–40 kMTs attached to the chromosomes, play an important role in chromosome congression and biorientation (9, 10). Successful attachment between MTs and kinetochores enables sufficient force to be delivered through K-fibers to allow segregation of a chromosome into two daughter cells (11). Thus, understanding K-fiber formation and dynamics is crucial for the study of mechanisms essential for chromosome alignment and segregation. The formation of K-fibers is tightly regulated by a group of proteins that specifically localize on kinetochore MTs or on bundles of spindle MTs that attach end-on to kinetochores, but the mechanism underlying K-fiber formation remains unclear (12).

The establishment of chromosome biorientation occurs in a stepwise manner. After nuclear envelope breakdown, the kinetochoore initially interacts with the lateral surface of the newly formed mitotic spindle. This unstable lateral attachment in a mono-oriented manner is a dominant form in early prometaphase (13). After the kinetochores are attached laterally, the chromosomes align in an equatorial ring on the surface of the nascent spindle. This distribution excludes the chromosome arms from the inside of the spindle so that the kinetochores are able to interact with MTs from both spindle poles (14). Later, the mono-oriented chromosomes are transported by the motors along the mitotic spindle to the MT plus end so that chromosome biorientation is established (15–17). However, the role of K-fiber dynamics in regulating the behavior of laterally attached chromosomes prior to chromosome biorientation is not fully understood.

Hepatoma up-regulated protein (HURP) is defined as a RanGTP-dependent microtubule-associated protein (MAP) that is known to stabilize kinetochore MTs and regulate chromosome congression (18–21). HURP consists of two microtubule-binding domains in its N-terminal (1–280 aa), a coiled-coil domain, and a guanylate kinase–associated protein (GKAP) domain with an unknown function (22–24). Intrigu-
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ingly, HURP is known to regulate the dynamics of K-fibers by contributing to chromatin-dependent MT nucleation pathways (21, 25, 26).

TACC3, a member of the transforming acidic coiled-coil (TACC) family, is a centrosomal protein that localizes along K-fibers with a number of known functions (27). TACC3 regulates centrosomal MT nucleation through interacting with ch-TOG (28, 29). TACC3 is involved in the chromatin-dependent MT nucleation pathway, which is responsible for MT sorting and kinetochore capture (30). TACC3 also exists in a TACC3–ch-TOG–clathrin complex to form inter-MT bridges cross-linking the kMTs (31–33). The transition function of the TACC3–ch-TOG–clathrin complex from MT polymerase activity to microtubule cross-linking activity is regulated by the activation of Aurora protein kinase A through TACC3 (34). However, it remains unclear how these kMT-localized proteins specifically regulate K-fiber formation.

Here we study the relationship between two important proteins, HURP and TACC3, present on K-fibers. We demonstrate a novel function of HURP in recruiting TACC3 to newly nucleated kMTs around the chromosome region via a direct protein–protein interaction. Successful sorting and bundling as a result of this interaction promotes the formation of K-fibers, which is important for stable lateral attachment in prometaphase to ensure efficient chromosome congression and promote mitotic progression. Thus, our study provides new insight into the coordinating mechanisms present between MAPs that promote successful K-fiber formation and chromosome congression in prometaphase.

Results

HURP modulates TACC3 spindle recruitment by interaction

The microtubule-associated protein HURP consists of a total of 846 amino acids and is localized to the kinetochore microtubules (23). To study its function, an RNAi of hurp was constructed into a pSUPER vector to knock down endogenous HURP (>95% efficiency) in HeLa cells (Fig. 1A). HURP is known to bundle MTs in vitro, but the mechanism by which HURP modulates K-fiber formation in cells is unknown (35). Therefore, potential interaction partners of HURP were searched using immunoprecipitation and proteomics approaches. Silver-stained gels resolved from immunoprecipitation using HEK293T cell lysates showed that HURP specifically pulled down proteins that have an approximately molecular mass of 150 kDa. Mass spectrometric analysis identified TACC3 as a potential critical interaction partner of HURP (Fig. 1B). Thus, we hypothesized that HURP might regulate K-fiber formation by modulating TACC3. To confirm the interaction between HURP and TACC3, co-immunoprecipitation assays using anti-FLAG beads were performed on prometaphase-synchronized HEK293T cells. Our results demonstrated that FLAG-HURP and HA-TACC3 could interact with each other (Fig. 1C).

To investigate the modulation of TACC3 by HURP, we first studied the localization of TACC3 in both control and HURP-depleted HeLa cells. In control cells, TACC3 (Fig. 1D, top row) was localized along the whole mitotic spindle, whereas in HURP-depleted cells, TACC3 was concentrated on the centromere region with minimal attachment to the mitotic spindle around the chromosome region (Fig. 1D), despite no change in the overall protein expression levels of TACC3 (Fig. 1E). Line scan analysis of fluorescence intensity verified that HURP depletion resulted in a significant loss of TACC3 on the mitotic spindle in the vicinity of the chromosomes (Fig. 1, D and F). We also noted that the relative spindle recruitment of TACC3 to the spindle of MT was significantly reduced in HURP-depleted cells (Fig. 1G). To verify that the intensity distribution change of TACC3 along the spindle is not caused by a change in tubulin distribution, we studied tubulin distribution in WT control and HURP-depleted HeLa cells. We found that the tubulin intensity around the chromosome was not significantly altered relative to the signal around the centrosome (Fig. S1, A and B). To verify that the distribution change of TACC3 is directly regulated by HURP, the dynamics of MT plus ends were studied. Measurement of the microtubule tip-tracking protein EB3-EGFP (+TIPS) movements showed that HURP depletion had no effect on microtubule polymerization rate at the MT plus ends (Fig. S1, C and D and Movies S1 and S2). We also examined spindle recruitment of two other kMT proteins, ch-TOG and CHC, in HURP-depleted HeLa cells. Localization of these two proteins was unchanged by HURP depletion (Fig. S1, E and G), suggesting that HURP modulates TACC3 specifically. Thus, our results suggest that HURP is essential for correct localization of TACC3 along the K-fibers in the vicinity of the chromosomes.

HURP1–625 is the TACC3 interaction region

To understand the interaction mechanism between HURP and TACC3, several fragments with different domain truncation of HURP were constructed. HURP1–278, containing the first 278 amino acids, is responsible for microtubule binding, whereas HURP279–625 contains the G1KAP domain (20, 23). As shown in Fig. 2A, both HURP1–278 and HURP279–625, but not HURP626–846, could interact with the full-length HA-TACC3. However, the interaction of these domains (Fig. 2A, second and third lanes) to TACC3, respectively, was much weaker compared with the full-length HURP (Fig. 2A, lane 5).

We then performed rescue experiments by re-introducing the fragments of HURP into HURP-depleted cells to determine whether these fragments of HURP could rescue the defects of HURP depletion in the distribution of TACC3 along the mitotic spindle. To this end, the localization of TACC3 was examined by line scan profile along the pole-to-pole axis. As shown in Fig. 2B, we found that overexpression of neither HURP1–278 nor HURP279–625 could fully rescue the distribution of TACC3 along the spindle at the chromosome region. Similarly, HURP626–846 could not rescue mislocalization of TACC3. Line profile analysis confirmed that the relative fluorescence intensity around the chromosome region remained below 0.5 absorbance units using these truncated fragments of HURP (Fig. 2C). Only reintroduction of HURP1–625 or full-length HURP could rescue the defects of TACC3 spindle recruitment in HURP-depleted HeLa cells (Fig. 2, B and C). Thus, the relative spindle recruitment of TACC3 verified that HURP1–625 possesses the TACC3-interacting region, which is important for...
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A

B

C

D

E

F

G

15735
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TACC3 localization along the mitotic spindle in the vicinity of chromosomes (Fig. 2D).

To further understand the molecular mechanism, we then aimed to identify the region of TACC3 that is responsible for the interaction with HURP. As shown in Fig. S2A, only FLAG-TACC3<sup>3678–688</sup> failed to pull down full-length HA-HURP. Spindle recruitment of TACC3<sup>3678–688</sup> was also significantly decreased in cells compared with full-length TACC3 (Fig. S2, B and C), suggesting that the TACC3 interaction region of TACC3 is important for its spindle localization and recruitment.

The spindle recruitment of TACC3 has been shown to be regulated by Aurora A phosphorylation, and HURP has also been identified as a substrate of Aurora A (24, 36–40). To study whether Aurora A regulates the interaction between HURP and TACC3, we performed co-immunoprecipitation assays to examine the interaction between TACC3 and Aurora protein kinase A–dead mutants (TACC3<sup>525A</sup> and TACC3<sup>558A</sup>) and HURP. Both TACC3<sup>525A</sup> and TACC3<sup>558A</sup> could interact with HURP (Fig. S2A). On the other hand, the HURP Aurora protein kinase A–dead mutant is also able to interact with TACC3 (Fig. S2D). These results suggest that the interaction between HURP and TACC3 might not be regulated by Aurora protein kinase A phosphorylation.

**HURP interacts with TACC3 in vivo and in vitro**

To study the modulation of TACC3 by HURP further, we performed a bimolecular fluorescence complementation (BiFC) assay. The BiFC assay enables direct visualization of protein–protein interactions in living cells by Venus signals (41, 42). HURP and TACC3 were fused with the nonfluorescent Venus-C (VC) or -N (VN) tag, respectively. These vectors were then co-transfected in HeLa cells, with empty vectors used as controls. Visualization of the Venus signal (green) in the BiFC assay indicated co-localization of TACC3 and HURP along the mitotic spindle (Fig. 3A), demonstrating that HURP interacts with TACC3 <i>in vivo</i> along the mitotic spindle.

To investigate whether TACC3 interacts directly with HURP, we purified His-HURP and GST-TACC3 recombinant proteins from SP9 insect cells and <i>Escherichia coli</i> cells, respectively, and performed protein–protein interaction assays. The results from <i>in vitro</i> GST bead pulldown experiments showed that GST-TACC3 could interact with His-HURP (Fig. 3B and Fig. S3), suggesting that HURP and TACC3 may associate with each other directly in cells during mitosis.

To study the MT binding affinity of the association between HURP and TACC3, we performed MT co-pelleting assays. First, recombinant GST or GST-TACC3 protein was incubated with Taxol-stabilized microtubules to test whether GST-TACC3 could interact directly with microtubules. After incubation, MTs were pelleted through a glycerol cushion by ultracentrifugation. Both supernatants and pellets were resolved by SDS-PAGE and subjected to Western blot analysis. As shown in Fig. 3C, GST or GST-TACC3 alone could not interact with the Taxol-stabilized microtubules directly (Fig. 3C, left panel). On the other hand, HURP was able to bind to Taxol-stabilized microtubules under a similar condition, consistent with its role as a MAP (Fig. 3C, second row). Next, the Taxol-stabilized MTs were incubated with both TACC3 and HURP (Fig. 3C, right panel). In the presence of HURP, even a small amount (0.25–1 μM), TACC3 could now be identified in the pellet, indicating that direct interaction between HURP and TACC3 is important for the attachment of TACC3 to MTs.

To study the structure of the Taxol-stabilized MTs modulated by HURP and TACC3, the supernatants and pellets were collected and imaged by confocal microscopy. In the absence of HURP, the Taxol-stabilized MTs were elongated (Fig. 3D, first and second rows). After incubating MTs with HURP and TACC3 proteins, MTs contained more branches in a larger tangled structure (Fig. 3D, third and fourth rows). The formation of MTs indicates that the association of HURP and TACC3 is involved in bundling and cross-linking of MTs. Therefore, our findings suggest that HURP is important for spindle recruitment of TACC3 <i>in vivo</i> and <i>in vitro</i> and that the interaction between HURP and TACC3 might be crucial for K-fiber formation during mitosis.

**HURP modulates TACC3 to be involved in kinetochore MT assembly**

To investigate the effect of HURP and TACC3 interaction on K-fiber formation, Hela cells were treated with 1 μg/ml nocodazole for 5 h to abolish the nucleation of MTs. The cells then were released into fresh medium to allow regrowth of microtubules and formation of the mitotic spindle. This experiment aimed to mimic cells entering mitosis from the stage of nuclear envelope breakdown. After release from the treatment, microtubules of control cells were quickly nucleated from the centrosome region and the chromosome region and formed asters (Fig. 4A, left). Consistent with previous findings, HURP is required for the repolymerization of MTs around chromosomes (21, 25, 26). In HURP-depleted cells, MTs could only nucleate from the centrosome region (Fig. 4A, right, and Fig. S4A). Quantitative analysis of the cells fixed at 5 min showed that the number of newly formed asters in the chromatin region of control cells (6.47 ± 3.0) is significantly higher than...
K-fiber regulation by HURP and TACC3

A

| HURP    | GRAP   | | | | |
|---------|--------|---|---|---|
| HURP^1-278 | - | - | + | - |
| HURP^279-625 | - | + | - | - |
| HURP^1-625 | - | - | - | + |
| HURP^626-846 | - | - | - | - |

FLAG vector  
FLAG HURP^1-278  
FLAG HURP^279-625  
FLAG HURP^626-846  
FLAG HURP  
HA TACC3

B

| HURP shRNA | GFP | TACC3 | DNA α-tubulin |
|------------|-----|-------|--------------|
| GFP vector | | | |

C

| HURP shRNA | GFP | TACC3 | DNA α-tubulin |
|------------|-----|-------|--------------|
| GFP vector | | | |

D

| HURP shRNA | GFP | TACC3 | Relative spindle recruitment of TACC3 (A.U.) |
|------------|-----|-------|---------------------------------------------|
| GFP        | -   | -     | 1                                           |
| GFP HURP   | -   | -     | 0.78                                        |
| GFP HURP^1-278 | - | - | 0.79                                        |
| GFP HURP^279-625 | - | - | 0.77                                        |
| GFP HURP^1-625 | - | - | 1.09                                        |
| GFP HURP^626-846 | - | - | 1.22                                        |

| HURP shRNA | Control |
|------------|---------|
| GFP        | 0.78    |
| GFP HURP   | 0.78    |
| GFP HURP^1-278 | 0.79    |
| GFP HURP^279-625 | 0.77    |
| GFP HURP^1-625 | 1.09    |
| GFP HURP^626-846 | 1.22    |
that in HURP-depleted cells (0.77 ± 1.2) (Fig. 4B), indicating that HURP is very important for chromatin-dependent MT nucleation.

Based on the observation, we hypothesized that the function of TACC3 on the formation of kMTs may be based on HURP-dependent nucleation of MTs. To confirm this, the localization of TACC3 was examined in nocodazole washout experiments. In control cells, TACC3, as a centrosomal protein, was localized not only at the centrosome region but also around all newly formed microtubules at the chromosome region. However, in HURP-depleted cells, TACC3 could only localize on the spindle poles, even at 10 min after release from nocodazole treatment, and failed to localize on the newly formed spindle around the chromosome region (Fig. 4 and Fig. S4A). Taken together, our findings suggest that HURP is important for recruitment of TACC3 to mitotic spindle assembly round the chromosome and is also critical for the function of TACC3 on chromatin-dependent MT assembly during early prometaphase.

To study bipolar spindle formation by interaction of HURP and TACC3, the fluorescence intensities of HURP and TACC3 on newly nucleated kMTs were analyzed by line profile quantification. As shown in Fig. 4D, our results showed that HURP and TACC3 dynamically associated with microtubules around kinetochores in control cells; however, when HURP was depleted, TACC3 failed to localize around the kinetochores (Fig. 4D). Line profile analysis of fluorescence intensities indicated that the fragment of HURP1–625 that contains the TACC3 interaction region of HURP could rescue the mislocalization of TACC3 on newly nucleated kMTs (Fig. 4E). Furthermore, we found that HURP recruits TACC3 to facilitate K-fiber formation in early prometaphase and that this recruitment might be crucial for successful establishment of chromosome biorientation.

HURP regulates the efficiency of chromosome congression by modulating TACC3

Unstable lateral attachment is a dominant state in early prometaphase before the establishment of biorientation (14). Considering the important function of HURP in regulating the assembly of K-fibers and chromosome congression, we hypothesized that HURP might regulate lateral attachment during early mitosis by modulating TACC3. Depletion of Nuf2, an Ndc80 component, has been shown to disrupt the KT–MT attachment in Nuf2-depleted cell (Fig. 5A). Thus, the kinetochore attachment status was quantified in both control and HURP-depleted cells following transfection of Nuf2 siRNA. Compared with the control cells, depletion of Nuf2 by siRNA successfully abolished kinetochore end-on attachment but did not alter the MT stabilization feature of HURP (Fig. 5A).

Relative spindle recruitment of TACC3 is quantified. Error bars indicate mean ± S.E. (≥25 cells in each group were quantified). *, p < 0.05 (two-tailed t test). Scale bar = 5 μm.

Figure 2. HURP1–625 is the TACC3 interaction region. A, schematic showing the structure of HURP, HURP1–278, HURP279–625, HURP626–846, and HURP626–846. HEK293T cells were synchronized at prometaphase, followed by co-transfection of FLAG-HURP1–278, FLAG-HURP279–625, FLAG-HURP626–846, FLAG-HURP, or FLAS vector control with HA-TACC3. Whole-cell lysates were collected for IP using anti-FLAG magnetic beads. Immunoprecipitated proteins were blotted with anti-HA antibody. The overexpression levels of HURP fragments were detected using an anti-FLAG antibody. HA-TACC3 co-precipitated with FLAG-HURP1–278 and FLAG-HURP279–625, and FLAG-HURP but not FLAG-HURP626–846 and FLAG vector (top lanes). B, fluorescence images of mitotic HeLa cells and HURP shRNA knockdown cells transfected with GFP, HURP1–278, GFP-HURP279–625, GFP-HURP626–846, GFP-HURP1–625, and GFP-HURP. Proteins were labeled with an anti-TACC3 antibody (red), GFP-tagged protein (green), an anti-α-tubulin antibody (magenta), and DNA (blue), respectively. C, illustration of the relative fluorescence intensity generated along the dashed line in B, representing half of the spindle length. D, bar chart showing the recruitment of TACC3 to the spindle MTs in control and HURP-depleted HeLa cells with overexpression of GFP, GFP-HURP1–278, GFP-HURP279–625, GFP-HURP626–846, and GFP-HURP. Relative spindle recruitment of TACC3 is quantified.
chromosomes aligned well and formed a metaphase plate halfway between the spindle poles, with a relative kinetochore–equator distance value of $0.096 \pm 0.06$ (mean ± S.D., $n = 1732$ kinetochores). Double depletion of HURP and TACC3 (0.13 ± 0.12, $n = 1502$ kinetochores) resulted in more severe chromosome misalignment than HURP-depleted (0.11 ± 0.08, $n = 1840$ kinetochores) or TACC3-depleted cells (0.11 ± 0.08, $n = 2016$ kinetochores) (Fig. S6, B and C). Taken together, our findings suggest that HURP and TACC3 co-regulate the status of KT–MT attachment, which is crucial for efficient chromosome congression and establishment of chromosome biorientation in mitotic cells.

Figure 3. HURP interacts with TACC3 in vivo and in vitro. A, bimolecular fluorescence complementation assays using split Venus fragments. HURP was fused with Myc and VC, and TACC3 was fused with Myc and VN. HeLa cells were co-transfected with VN and VC vectors only (VC-vector + VN-vector), VC-HURP and VN-vector, VC-vector and VN-TACC3, or VC-HURP and VN-TACC3. Cells were fixed and stained with an anti-Myc (red) antibody. The fluorescence signal of Venus was detected using a 488-channel (green) laser. DNA was stained with Hoechst 33342 (blue). Scale bar = 5 μm. B, an in vitro co-immunoprecipitation reaction analyzed by Western blotting using GST and HURP antibodies. The GST bead pulldown results indicate that His-HURP interacts with GST-TACC3 but not GST protein (top lanes). C, representative Western blots of the microtubule pelleting experiment. GST, GST-TACC3, and HURP were incubated with 1 μM Taxol-stabilized microtubules either independently or together. The supernatants (S) and pellets (P) were obtained after ultracentrifugation of the samples. Both supernatants and pellets were blotted using TACC3, HURP, α-tubulin, and GST antibodies. No precipitation of GST and GST-TACC3 were observed in pellets (left). GST-TACC3 was observed in pellets in the presence of HURP (right). D, MTs stabilized and bundled by HURP and TACC3 in vitro. The supernatants and pellets collected from MT co-pelleting assays were fixed on glass coverslips. After fixation, α-tubulin was stained with an anti-α-tubulin antibody (red), and HURP was stained with an anti-HURP antibody (green). Scale bar = 10 μm. Enlargements show ×3.5 magnifications. Arrowheads indicate the microtubule bundles.
K-fiber regulation by HURP and TACC3

![Image of K-fiber regulation by HURP and TACC3](image)

**A**

| Time  | Control shRNA | HURP | α-tubulin | Merge | HURP shRNA | HURP | α-tubulin | Merge |
|-------|---------------|------|-----------|-------|------------|------|-----------|-------|
| 3 min |               |      |           |       |            |      |           |       |
| 5 min |               |      |           |       |            |      |           |       |
| 10 min|               |      |           |       |            |      |           |       |

**B**

![Graph showing number of acrosomal microtubule foci](graph)

**C**

| Time  | Control shRNA | HURP | TACC3 | Merge | HURP shRNA | HURP | TACC3 | Merge |
|-------|---------------|------|-------|-------|------------|------|-------|-------|
| 3 min |               |      |       |       |            |      |       |       |
| 5 min |               |      |       |       |            |      |       |       |
| 10 min|               |      |       |       |            |      |       |       |

**D**

| Time  | Control shRNA | HURP | TACC3 | ACA   | Merge |
|-------|---------------|------|-------|-------|-------|
| 3 min |               |      |       |       |       |
| 5 min |               |      |       |       |       |
| 10 min|               |      |       |       |       |

**E**

![Graphs showing relative intensity vs. spindle size](graphs)

**Legend**

- GFP
- TACC3
- ACA

Spindle size (μm) vs. Relative intensity (A.U.)
HURP regulates the congression of laterally attached chromosomes by modulating TACC3

To verify the function of HURP and TACC3 on the congression movement of laterally attached chromosomes, we quantified chromosome movement in Nuf2-depleted cells (control) and HURP/Nuf2 double-depleted HeLa cells. Mean square displacement (MSD) analysis is a commonly used technique for determining the extent of random motion of particles such as kinetochores (45). The shape of MSD can be used to identify the patterns of particle movement if the particle is freely diffusing, transported, or bound and limited in its movement (46, 47).

Thus, to evaluate the extent of random movement of laterally attached kinetochores, the MSD was calculated based on the recorded kinetochore movement trajectories (Fig. 6A and Movies S3–S5). Compared with control cells, the movement of kinetochores in HURP/Nuf2 co-depleted HeLa cells displayed a...
more constrained pattern. Additionally, compared with the control, the MSD curve was almost linear in shape, indicating a random diffusion pattern of kinetochore movement (Fig. 6B).

To compare the apparent diffusing properties of kinetochore movement in control and HURP-depleted cells, the MSD was fitted to a linear plot to calculate the diffusion coefficient. The kinetochores in HURP/Nuf2 co-depleted HeLa cells had a reduced diffusion coefficient \( ((2.38 \pm 0.2) \times 10^{-3} \ \mu m^2/s, n = 45 \text{ kinetochores}) \) compared with control cells \( ((4.32 \pm 0.1) \times 10^{-3} \ \mu m^2/s, n = 46) \), indicating that laterally attached kinetochores could not be transported normally in HURP-depleted cells. To examine the importance of HURP and TACC3 association in this biological process, HURP\(^{1–625}\) was overexpressed in HURP-depleted cells. The TACC3 interaction region of HURP could rescue the phenotype caused by HURP depletion \( ((6.22 \pm 0.2) \times 10^{-3} \ \mu m^2/s, n = 41 \text{ kinetochores}) \) (Fig. 6B).

Moreover, the instantaneous velocity of kinetochore movement significantly decreased in HURP-depleted cells \( ((4.93 \pm 0.2) \times 10^{-2} \ \mu m/s) \) compared with control cells \( ((5.86 \pm 0.2) \times 10^{-2} \ \mu m/s) \), and this could also be rescued by the TACC3 interaction region HURP\(^{1–625}\) \( ((6.08 \pm 0.3) \times 10^{-2} \ \mu m/s) \) (Fig. 6C). Taken together, we show that HURP regulates the congression movement of laterally attached kinetochores by modulating TACC3.

Discussion

We have demonstrated previously that HURP governs chromosome congression in metaphase (19). Here we characterize HURP as an important MAP that functions in the regulation of K-fiber formation in prometaphase.
critical interaction partner of HURP in mammalian somatic cells and demonstrate that this interaction between these two proteins is important for spindle recruitment of TACC3 at the chromosome region. TACC3 is a nonmotor protein localized at the centrosome and along the kMTs (31). BiFC assays confirm that HURP interacts with TACC3 on kMTs in vitro; therefore, this interaction may be important in regulating the stability of kMTs. Although it has been reported that the TACC domain (TACC3638–858) can nucleate microtubule assembly (48), full-length TACC3 is not able to attach to MTs directly in vitro (27, 49). This indicates that the interaction between HURP and TACC3 is essential for TACC3 spindle recruitment. Thus, the modulation mechanism of TACC3 by HURP in mammalian cells is different from the one characterized in Drosophila. Although the Drosophila HURP ortholog Mars associates with dTACC3, Mars could not modulate dTACC3 localization directly (50).

In addition, a direct interaction between these two proteins is confirmed in vitro by co-immunoprecipitation assays. Images of MT morphology observed by MT co-pelleting assay indicate that HURP interacts with TACC3 to regulate the bundling and cross-linking of MTs. Elements present in HURP1–625 are required for the regulation of mitotic spindle recruitment of TACC3 at the chromosome region. Although this region contains the MT binding domain (24), the interaction between HURP and TACC3 is independent of the presence of MTs. HURP1–625 also contains a GKAP domain (23), suggesting a potential function of the GKAP domain in the interaction of HURP and TACC3. Thus, we show that the direct interaction between HURP and TACC3 is important for their function in regulating KMT formation and stability.

TACC3 was shown previously to attach to MTs through a mechanism dependent on CHC and ch-TOG protein (28, 33, 51–54). However, the unaltered localization of CHC and ch-TOG in HURP-depleted cells indicates that the interaction between HURP and TACC3 occurs via a mechanism separate from that described for the interaction between TACC3 and CHC/ch-TOG. Thus, the presence of HURP might enhance the efficiency of TACC3 spindle recruitment by CHC–ch-TOG. TACC3 is thought to play multiple roles in regulating bipolar spindle formation by interacting with different proteins. The TACC3–ch-TOG complex promotes MT polymerization and spindle assembly, whereas the TACC3–CHC–ch-TOG complex is important for kMT cross-linking (32, 34, 55). It is still unclear whether TACC3 functions as an MT polymerizer or MT cross-linker in its interaction with HURP. HURP and TACC3 have also been identified as substrates of important mitotic kinases, such as Aurora A (24, 36–39). However, it seems that the interaction between HURP and TACC3 is not regulated by Aurora A phosphorylation.

Our data indicate that HURP modulates the involvement of TACC3 in the regulation of MT nucleation at the chromosome region. Depletion of HURP induces a significant decrease in the number of the microtubule-organizing centers at the chromosome region, suggesting an important function of HURP in the nucleation of MTs during early mitosis. Depletion of HURP also results in the disappearance of TACC3 in newly formed spindles, suggesting an additional function of HURP in modulating TACC3 localization. TACC3 is localized in the small asters near the kinetochores at the chromosome region for initial kinetochore capture (30). TACC3 has also been shown to be essential for sorting of MTs at the chromosome region with centromal MTs. Although TACC3 interacts with importin β and is regulated by the RanGTP gradient (56), the regulation mechanism of human TACC3 is complicated (57, 58). Therefore, the direct interaction between HURP and TACC3 could be crucial for HURP-mediated recruitment of centromal TACC3 for MT nucleation at the chromosome region. For efficient kinetochore capture, newly nucleated MTs should be quickly sorted with pre-existing MTs to form stable kMTs.

Both HURP and TACC3 play important roles in regulating chromosome congression, and depletion of HURP or TACC3 results in chromosome misalignment. Furthermore, double depletion of HURP and TACC3 causes a synergistic effect on chromosome misalignment. Considering that HURP modulates TACC3 function on the spindle around the chromosomes, the synergistic effect on kinetochore distribution caused by HURP and TACC3 co-depletion could be due to abolishment of TACC3 centrosome function. Our results also suggest that HURP is important for regulating chromosome congression in the lateral attachment status through the modulation of TACC3. Double depletion of HURP and Nuf2 results in a significant decrease in the percentage of laterally attached kinetochores. Thus, the association of HURP and TACC3 plays a significant role in regulating lateral attachment to increase the efficiency of chromosome congression.

HURP depletion affects the relative kinetochore distribution and movement in Nuf2-depleted cells. MSD analysis of the movement trajectories of the laterally attached kinetochore by live-cell imaging showed that kinetochores move more irregularly in HURP-depleted cells. Moreover, the kinetochores in HURP-depleted cells have a much lower diffusion coefficient in their movement, suggesting defects in partial chromosome congression. These defects could be rescued by the fragment of HURP1–625 containing the TACC3 interaction region, suggesting an important function of HURP in TACC3 modulation.

Observation of chromosome movement during prometaphase has identified two kinds of chromosome movement behaviors in mitotic cells: oscillatory movement and sliding (59). These activities are dependent on different types of motor transport (60) and the status of kinetochore attachment. Our previous study identified that HURP modulates the function of Kif18A, a microtubule depolymerizer that accumulates on the bioriented chromosomes, during metaphase (19). In this study, we characterize a new function of HURP during prometaphase when the chromosome is laterally attached. The laterally attached chromosome could be transported by dynein, a minus-end-directed motors localized to kinetochores (17). Moreover, a separate study of partial chromosome congression demonstrated the contribution of two mitotic motors, Kid and CENP-E, whose motor activities are dependent on the stability of MTs (61). Our current finding suggests that HURP and TACC3 regulate laterally attached kinetochore movement by modulating motor activities.

Our study proposes, for the first time, a new mechanism for MAPs to regulate kinetochore capture and chromosome biori-
K-fiber regulation by HURP and TACC3

Figure 7. A model for K-fiber formation and chromosome congression in prometaphase. Top panel, in control cells, HURP modulates TACC3 to regulate K-fiber formation at the chromosome region. As a result, chromosomes become laterally attached and slide along the newly formed stabilized mitotic spindles (left). Finally, chromosomes are bioriented and congress to the spindle equator (right). Bottom panel, HURP depletion results in less spindle recruitment of TACC3, leading to inefficient K-fiber formation and unstable lateral attachment (left). Therefore, in HURP-depleted cells, chromosome biorientation is delayed, with a prolonged mitotic process (right). The arrows represent centrosome-dependent MT nucleation, which is not regulated by HURP.

entation. We show that HURP recruits the centrosomal protein TACC3 to newly nucleated kMTs to increase the proportion of laterally attached kinetochores (Fig. 7). Successful lateral KT-MT attachment promotes kinetochore capture and chromosome biorientation. This study sheds new light on our understanding of MAP regulation of chromosome movement during prometaphase and demonstrates the importance of efficient formation and stability of kMTs to promote chromosome biorientation. Future studies should consider how motor proteins are involved in these cellular processes.

Experimental procedures

Cell culture and synchronization

HeLa and HEK293T cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin and streptomycin at 37 °C and 5% CO₂. SP9 cells were cultured in Sf-900 II serum-free medium (Invitrogen) supplemented with 2% FBS and 1% penicillin and streptomycin at 27 °C. HeLa cells were synchronized to G₁/M phase by incubation in 100 ng/ml nocodazole (Sigma) for 16 h, whereas HEP293T cells were synchronized to prometaphase by incubation in 50 ng/ml nocodazole for 16 h. HeLa cells were arrested in metaphase by release from nocodazole treatment into fresh DMEM containing 10 μM MG132 (Sigma) for 1 h.

Antibodies

The following commercial antibodies were used: rabbit anti-HURP (Abcam, ab70743 and ab70744), mouse anti-TACC3 (Abcam, ab134154), rabbit anti-ch-TAG (Abcam, ab86073), rabbit anti-NuF2 (Abcam, ab176556), rabbit anti-β-tubulin (Abcam, ab6046), mouse anti-Clathrin heavy chain (BD Biosciences, 610499), rabbit anti-β-actin (Santa Cruz Biotechnology, sc-805), anti-c-Myc (Santa Cruz Biotechnology, sc-789), mouse anti-FLAG (Sigma-Aldrich, F1804), mouse anti-α-tubulin (Sigma-Aldrich, T5168), mouse anti-acetylated tubulin (Sigma-Aldrich, T7451), rabbit anti-γ-tubulin (Sigma-Aldrich, T5192), and human anti-calcium-responsive transactivator (CREST) antibody (ImmunoVision).

Plasmid construction

Full-length TACC3, EB3, and CENP-A were amplified from cDNA using Phusion DNA polymerases (Thermo Fisher). TACC3 was cloned into the pXJ40 vector, the Venus-N vector, and the pGEX-4T1 vector. Full-length HURP and related fragments (1–278 aa, 279–625 aa, 1–625 aa, and 626–846 aa) were subcloned into the pFastBac DUAL vector containing a His₆ tag or a pXJ40 vector tagged with GFP, mCherry, FLAG, HA, and photo-activated (PA)-GFP, respectively. EB3 and CENP-A were cloned into the pEGFP-N1 vector. GFP-centrin 1 was a gift from Dr. Maki Murata-Hori. mCherry α-tubulin was obtained from Addgene (49149) and constructed into a pXJ40 vector tagged with PA-GFP, GFP, and HA.

Recombinant protein expression and pulldown experiment

Expression of the recombinant GST-TACC3 protein in the pGEX-4T1 vector was induced with 0.1 mM isopropyl 1-thio-β-D-galactopyranoside at 20 °C overnight, and cells were harvested in lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 2 mM EGTA, and 0.1% Triton X-100 (pH 7.4)) containing protease and phosphatase inhibitors. The His₆-tagged HURP bacmid was purified and transfected into SF9 cells for baculovirus amplification. The cells were infected in a suspension culture at 27 °C for 72 h. Cells were resuspended in His₆ lysis buffer (50 mM NaH₂PO₄, 10 mM imidazole, 150 mM NaCl, and 1% NP-40 (pH 8.0)) containing protease and phosphatase inhibitors. The recombinant proteins were purified, and in vitro GST pulldown experiment were performed as described previously (62). Purified proteins were dialyzed against BRB80 buffer (80 mM Pipes (pH 6.8), 1 mM MgCl₂, and 1 mM EGTA) and stored at −80 °C.

Immunostaining

HeLa cells were grown on coverslips and fixed with pre-cooled methanol for 10 min or 4% paraformaldehyde for 20 min at room temperature. Cells were permeabilized and blocked with PBS-BT (1 × PBS, 0.1% Triton X-100, and 3% BSA) for 45 min at room temperature. Cells were incubated for 1 h at room temperature with primary antibodies diluted in PBS. Cells were labeled for 1 h at room temperature with anti-mouse, anti-rabbit, or anti-human secondary antibodies conjugated with fluor dyes (Invitrogen). DNA was stained with Hoechst 33342 (Invitrogen) for 10 min, and cells were mounted in FluorSave reagent (Calbiochem).

Immunoprecipitation

HEK293T cells synchronized at prometaphase were lysed in mammalian cell lysis buffer (50 mM Hepes, 100 mM NaCl, 1.5 mM MgCl₂, 1 mM EDTA, 1% Triton X-100, and 10% glycerol) with protease and phosphatase inhibitors. For each IP reaction, a total of 10 μl of anti-FLAG M2 beads (Sigma) was added to the cleared cell lysate harvested from a 10-cm cell culture dish and incubated for 3 h at 4 °C.
**Image acquisition and time-lapse microscopy**

For interphase and mitotic cells, a stack of images was collected along the z axis (Z-stack) and merged using a maximum intensity projection. For time-lapse imaging, HeLa cells were seeded onto 35-mm glass-bottom Petri dishes (Ibidi). Regular DMEM was substituted with DMEM without phenol red supplemented with 10% FBS (Invitrogen) for image acquisition at 37 °C. Images were collected using an Ultraview Vox spinning disc confocal system (PerkinElmer Life Sciences) consisting of a Yokogawa high-speed spinning disc CSU-X1 scanner and an Olympus IX81 inverted microscope. The microscope was equipped with an Olympus UPlan SApo ×60/1.20 water lens and an Olympus UPlan SApo ×100/1.40 oil lens. The images were captured with an EMCCD (electron-multiplying charge-coupled device) camera (C9100-50, Hamamatsu) with high resolution and an EMCCD camera (C9100-13. Hamamatsu) with high sensitivity. All parameters used for image acquisition were controlled by Volocity software (PerkinElmer Life Sciences).

**Microtubule co-pelleting assay**

For the microtubule co-pelleting assay, recombinant GST, HURP, and GST-TACC3 proteins (1 μM final concentration each) were added to the reaction mixture containing 2 mM GTP (Sigma), 1× protease inhibitors, 20 μM Taxol, and Taxol-stabilized microtubules in 1× BRB80 buffer. The reaction was incubated at 20 °C for 30 min and pelleted through a 60% glycerol cushion containing 20 μM Taxol and 1× protease inhibitors in BRB80 buffer at 100,000 × g for 20 min at 20 °C. Supernatants and pellets were analyzed by Western blotting and immunofluorescence.

**RNAi**

RNAi using NuF2 siRNA (5'-AACGCAUGCGCUGAAACGC-UAUA-3') and TACC3 siRNA (5'-GGUUCGAAGGUGUG-UAUA-3') or control siRNA sequence (5'-UUCCGGAACGGUGUCACCU-3') oligos was performed as described previously (30). The siRNA oligonucleotides were synthesized by Sigma. Sequences of HURP shRNA were as described previously (18).

**Quantitative analyses of chromosome movement**

MSD analysis was used for assessing the type of chromosome movement in prometaphase cells. To quantify the lateral attached kinetochore movement, HeLa cells were overexpressed with EGF-P–CENP-A and mCherry–β-tubulin and imaged every 5 s for a total of 300 s. Kinetochores were tracked as described previously (19, 63). Only tracks with all 61 time points were analyzed for accurate evaluation of mean square displacement. To avoid an effect from spindle dynamics on displacement of chromosome movement, the tracking results in the original coordination system, Oxy, were transformed to a new coordination system, O’x’y’’. In the new coordination system, the x’ axis is the spindle equator, whereas the y’ axis is the pole-to-pole direction. Thus, the origin of the coordination system is the midpoint between two spindle poles. The angle from the x axis to the x’ axis is θ, and the O’ is (x₀, y₀). Thus, the coordination transformation formula is x = x’cost − y’sint + x₀, y = x’sint + y’cost + y₀. All tracking results were analyzed by a MatLab R2009a (Mathworks) class named msdanalyzer, published in previous literature (43).

**Imaging processing and statistical analysis**

Images acquired by spinning disc confocal microscopy with an Ultraview Vox module were processed using Volocity™ 6.0 (PerkinElmer Life Sciences) and ImageJ (National Institutes of Health, Bethesda, MD). Processing included cropping, setting channel colors, adjusting brightness and contrast, intensity projection, drift correction, and deconvolution. Measurements included intensity, distance, line profile, and particle tracking. Data analysis was performed blind. All data had a normal distribution, with similar variance between all conditions tested. Mann–Whitney U tests were used for comparison of dispersion, and two-sided t tests were used for comparison of averages. Statistical analysis was performed using GraphPad Prism version 5.0 (GraphPad Software, San Diego, CA).

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