Recruitment of the human Cdt1 replication licensing protein by the loop domain of Hec1 is required for stable kinetochore–microtubule attachment

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Cdt1, a protein critical for replication origin licensing in G1 phase, is degraded during S phase but re-accumulates in G2 phase. We now demonstrate that human Cdt1 has a separable essential mitotic function. Cdt1 localizes to kinetochores during mitosis through interaction with the Hec1 component of the Ndc80 complex. G2-specific depletion of Cdt1 arrests cells in late prometaphase owing to abnormally unstable kinetochore–microtubule (kMT) attachments and Mad1-dependent spindle-assembly-checkpoint activity. Cdt1 binds a unique loop extending from the rod domain of Hec1 that we show is also required for kMT attachment. Mutation of the loop domain prevents Cdt1 kinetochore localization and arrests cells in prometaphase. Super-resolution fluorescence microscopy indicates that Cdt1 binding to the Hec1 loop domain promotes a microtubule-dependent conformational change in the Ndc80 complex in vivo. These results support the conclusion that Cdt1 binding to Hec1 is essential for an extended Ndc80 configuration and stable kMT attachment.

The cell-division cycle is the process of complete and precise duplication of the entire genome in S phase followed by accurate chromosome segregation in mitosis. The formation and stability of kMT attachments during mitosis depends on the Ndc80 complex, Ndc80 (hsHec1), Nuf2, Spc24 and Spc25 (refs 1,2). Hec1/Nuf2 and Spc24/25 form dimers respectively that are tethered together at the carboxy termini of Hec1/Nuf2 and the amino termini of Spc24/Spc25 by long α-helical coiled-coil rod domains. In the middle of the rod domain is a ‘hinge’ site produced by a ~40-amino-acid loop in Hec1 that is thought to play a key role in microtubule-binding dynamics and attachment3. Binding partners for the loop region of yeast Hec1/Ndc80 proteins have been identified4,5, but the partners and function of the unique Hec1 loop domain at metazoan kinetochores remains unknown. In this study, we identify the Cdt1 protein as an essential partner for Ndc80 dynamic function through interaction with the Hec1 loop domain.

Cdt1 is required for DNA replication origin licensing, the initial step in genome duplication, which occurs in G1 phase6,7. Proteins involved in origin licensing are not strictly DNA replication factors, however; some licensing proteins have functions in transcription8,9, the DNA-damage response10,11, centrosome duplication12 and mitosis13,14. We report here the previously undiscovered mitotic role for the Cdt1 replication licensing protein that is distinct from its role in DNA replication.

RESULTS

Inhibition of Cdt1 function induces mitotic arrest

Both Cdc6 and Cdt1 proteins are required for replication licensing in human cells, but we found an intriguing difference in the phenotypes of Cdc6-depleted cells when compared with Cdt1-depleted cells. Cdc6-depleted cells arrest primarily in G1 as expected15,16, but Cdt1-depleted cells arrested in both G1 and G2/M, despite the fact that both proteins cooperate in the same DNA replication step, MCM loading (Fig. 1a). The accumulation of G2 cells indicated a possible unique mitotic role for Cdt1 that we set out to test.

To eliminate the possibility that a mitotic phenotype simply reflected replication errors in the preceding S phase, we took advantage of the fact that Cdt1 is actively degraded during S phase after its G1 origin licensing role is complete17 (Fig. 1b). We synchronized cells in early S phase, at which time origins are fully licensed, and released them into medium

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Cells depleted of Cdt1 after S phase do not complete cell division. (a) Normal human fibroblasts (NHF1-htert) were transfected with siRNAs targeting cdc6, cdt1 or GFP (control) messenger RNAs for 72 h. Cells were labelled with BrdU for the final hour and then analysed by flow cytometry for cell-cycle position (left) and by immunoblotting for endogenous Cdc6, Cdt1 and tubulin (right). (b) Diagram of experimental design. Endogenous Cdt1 protein levels normally drop during S phase owing to ubiquitin-mediated proteolysis, and recovery begins in G2 (dotted line). Release from an early S block into cdt1 siRNA transfection medium blocks Cdt1 protein re-accumulation (solid grey line). (c) Immunoblot analysis of endogenous Cdt1 protein in cells synchronized as shown in b; M phase occurs between 9 and 10 h post-release from the second thymidine arrest. (d) Stable HeLa cell lines transduced with empty vector (lanes 1–4) or an siRNA-resistant form of Cdt1 ('Cdt1res' lanes 5 and 6) were synchronized as in b and transfected with either control siRNA targeting GFP (lanes 1 and 2) or with cdt1 siRNA (lanes 3–6). Cells were released from early S phase into nocodazole (Noc.) for 10 h and then either collected (0 h) or released for 2 h into G1 phase, and Cdt1 protein levels were analysed by immunoblotting. (e) Flow cytometric analysis of DNA content of the cells in d. Uncropped images of blots are shown in Supplementary Fig. S9.

-containing control short interfering RNA (siRNA) or cdt1 siRNA. As expected, control cells had very low amounts of Cdt1 in S phase, and Cdt1 re-accumulated in G2 (Fig. 1c, lanes 1–5). Synchronized cells treated with cdt1 siRNA could not re-accumulate Cdt1 in G2, however (Fig. 1c, lanes 7–10). (Cdt1 is phosphorylated in G2 by stress MAP kinases27,28.) Cdt1 depletion during S phase caused no delay in S-phase progression (Supplementary Fig. S1a,b), and by 9 h after release, both control and Cdt1-depleted cells exhibited normal chromosome condensation and/or nuclear envelope breakdown (for example, Fig. 2a and data not shown). This unique experimental approach allowed us to generate cells that underwent a normal G1 and S phase but lacked Cdt1 during G2 and M phase.

We then investigated the ability of Cdt1-depleted cells to transit mitosis to G1 after a nocodazole arrest and release (Fig. 1d,e). Control cells completed cell division, but Cdt1-depleted cells remained arrested with G2 DNA content (Fig. 1c, control siRNA versus cdt1 siRNA). A cell line stably expressing normal Cdt1 with synonymous mutations in the siRNA target site was fully capable of mitotic progression (Fig. 1d,e). Unphosphorylatable Cdt1 (ref. 18) also complemented the cell-division phenotype of Cdt1-depleted cells (Supplementary Fig. S1c,d). Cells depleted of Cdt1 during S phase did not accumulate phosphorylated H2AX by the subsequent G2, a marker of DNA damage, whereas siRNA transfection of asynchronous cultures to deplete either Cdt1 or another licensing protein, Orc6, resulted in the accumulation of phospho-H2AX-positive foci, presumably from incomplete replication and fork collapse (Supplementary Fig. S1e).

Most of the synchronized Cdt1-depleted cells arrested just before metaphase with most chromosomes positioned near the spindle equator. A smaller fraction of cells arrested in prometaphase (Fig. 2a,b and Supplementary Fig. S2a,b). In addition to Cdt1 depletion in synchronized cells, we also microinjected purified anti-Cdt1 antibody (Supplementary Fig. S2c) into HeLa cells expressing GFP-tagged histone H2B during prophase or early prometaphase and conducted live-cell imaging (n = 27). Anti-Cdt1 antibody induced both a severe
Buffer-injected Anti-Cdt1-antibody-injected Control siRNA cdt1 siRNA 70 Luciferase siRNA cdt1

Figure 2 G2-specific Cdt1 inhibition induces mitotic arrest. (a) HeLa cells synchronized in early S phase were released into control (luciferase) or cdt1 siRNA for either 9 h (top and middle panels) or 10 h (bottom panels), followed by fixation and staining with DAPI to label chromosomes (blue), anti-tubulin antibody to label microtubules (red) and anti-Knl1 antibody to label kinetochores (green). (b) Quantification of the results at 10 h in a by mitotic stage; n = 1,500 cells. (c–e) HeLa cells stably expressing GFP–histone H2B were injected with control buffer (c, n = 15) or anti-Cdt1 antibody (d, n = 27). Selected frames of GFP–histone (top panels) and phase-contrast images (bottom panels) are shown. (e) Quantification of the results from the microinjection experiments in c and d. Scale bars, 5 μm. Time is h.min.s. See also Supplementary Movies S1–S4.

delay in chromosome congression and an arrest near metaphase for the entire ~3 h duration of imaging (Fig. 2d,e). Control buffer-injected cells (n = 15) or control anti-mouse IgG-injected cells (n = 8) executed mitosis normally (Fig. 2c,e and Supplementary Movies S1 and S2). Anti-Cdt1 injection during late prometaphase or metaphase (n = 10) resulted in 70% of the cells remaining arrested in that stage for up to 8 h. During this period, cells progressively lost chromosome alignment but did not divide (Supplementary Fig. S2d and Movie S4).

Cdt1 localizes to mitotic kinetochores through interaction with the Hec1 component of Ndc80

The late-prometaphase arrest of Cdt1-depleted cells prompted us to examine Cdt1 localization in mitosis. Remarkably, we detected endogenous Cdt1 co-localization with a known kinetochore protein, Hec1, in prometaphase in both nocodazole-treated PTK2 and HeLa cells (Fig. 3a, and data not shown). Cdt1 depletion by siRNA treatment in S phase eliminated detectable anti-Cdt1 kinetochore staining (Supplementary Fig. S3a). We observed no kinetochore localization of
Figure 3 Cdt1 transiently localizes to kinetochores during prometaphase and metaphase. (a) Nocodazole-treated PTK2 cells were immunostained with anti-Cdt1 antibody and anti-Hec1 antibody. (b–f) LLCPK1 cells at different stages of mitosis were immunostained with anti-Cdt1 antibody and anti-Hec1 antibody. Scale bars, 5 µm.

The Cdt1 inhibitor geminin at any stage of mitosis (Supplementary Fig. S3b). A second anti-Cdt1 antibody also detected endogenous Cdt1 at kinetochores in LLCPK1 cells (Fig. 3b–f). Thus, Cdt1 localizes to kinetochores in control cells at a time coincident with the stage at which Cdt1-depleted cells arrest.

In a search for Cdt1-interacting proteins by two-hybrid screening in yeast, we identified multiple independent clones containing portions of human Hec1 (Supplementary Fig. S4a); the shortest contained Hec1 amino acids 306–642 (Supplementary Fig. S4a,b). To determine whether Cdt1 and Hec1 interact biochemically, we incubated immobilized bacterially expressed GST–Cdt1 with lysates of asynchronous HeLa cells. Endogenous Hec1 associated with GST–Cdt1 but not GST alone (Fig. 4a). Nuf2 was also retrieved from cell lysates by GST–Cdt1 (Fig. 4a), indicating that Cdt1 can associate with the Ndc80 complex, possibly through a direct interaction with Hec1 (based on interaction of the fusions in yeast). We further confirmed the interaction between Cdt1 and Hec1 by reciprocal co-immunoprecipitation of the endogenous proteins (Fig. 4b,c).

To determine whether Hec1 recruits Cdt1 to kinetochores in mitosis, we stained for endogenous Cdt1 in Hec1-depleted PTK2 cells. Hec1 depletion resulted in a profound loss of Cdt1 at kinetochores in nocodazole-treated prometaphase (Fig. 4d, bottom panel and Fig. 4e) and metaphase cells (Supplementary Fig. S4c, bottom panel) when compared with controls (Fig. 4d and Supplementary Fig. S4c,
Figure 4 Hec1 is required for Cdt1 kinetochore localization. (a) A lysate of T98G (human glioblastoma) cells was incubated with beads coated with bacterially produced GST or GST–Cdt1, and the endogenous Hec1 and Nuf2 were detected in the input or bound fractions by immunoblotting. (b) Whole-cell lysates of HeLa cells were incubated with pre-immune serum or anti-Cdt1 antibody, and endogenous Hec1 was detected in the input and immune complexes. (ND: Cdt1 co-migrates too closely to IgG heavy chain for detection in this immunoprecipitate, IP.) (c) Whole-cell lysates of HeLa cells were incubated with control serum or anti-Hec1 antibody; endogenous Cdt1 and Hec1 were detected in the input and immune complexes. (d) Nocodazole-treated PTK2 top panels). We observed no Cdt1 decrease in extracts of Hec1-depleted cells (Supplementary Fig. S4d), and Cdt1 depletion had no effect on Hec1 expression or kinetochore localization (Supplementary Fig. S4e). Other kinetochore proteins, including components of the Mis12 complex, Knl1 and the Zwint–RZZ complex, were also localized normally in Cdt1-depleted cells (Fig. 2a and Supplementary Figs S4e and S8a,b).

Similarly to Cdt1, ORC is essential for replication origin licensing, and the Orc2 and Orc6 subunits in particular have also been reported to localize to centromeres or kinetochores13,14. ORC is required for Cdt1 chromatin localization in G1 (ref. 21), and the Orc6 subunit associates with Cdt1 (Supplementary Fig. S5a; ref. 22). We found that, as for Cdt1, Orc6 immunofluorescence intensity at kinetochores was also substantially reduced in Hec1-depleted cells (Fig. 4f,g). Orc6 depletion had no effect on Hec1 or Cdt1 kinetochore localization, however (Supplementary Fig. S5d,e). Interestingly, depletion of Cdt1 resulted in a ~50% decrease in the intensity of Orc6 kinetochore staining (Supplementary Fig. S5b,c). To compare the reported mitotic defects induced by Orc6 inhibition14 to those produced by depleting Cdt1, we first calculated the mitotic indices of asynchronous cells depleted of Orc6. Orc6 is not degraded in S phase like Cdt1, so we could not employ the same synchronization strategy to remove Orc6 only before mitosis. Orc6 depletion caused a ~50% increase in the number of mitotic cells when compared with controls (Supplementary Fig. S5f) by producing a fourfold increase in the number of telophase cells (Supplementary Fig. S5g). In addition, cells depleted of Orc6 exhibited normal cold-stable kMTs (Supplementary Fig. S5h). These observations support the conclusion13,23 that Orc6 has a role late in mitosis during cytokinesis distinct from the role of Cdt1 during prometaphase or metaphase.

The Hec1 loop domain is required for Cdt1 localization and anaphase onset

Our two-hybrid analysis indicated that the Cdt1-binding region of Hec1 includes a short interruption in the coiled-coil pattern postulated to be a flexible ‘loop’3 (Supplementary Fig. S4a,b). To determine whether...
Figure 5 Cdt1 targeting to kinetochores depends on the flexible loop region of Hec1. (a) Diagram of the Ndc80 complex showing the loop region and the construction of the Hec1 loop replacement mutant, Hec1 LoopMUT (adapted from ref. 38). (b) Endogenous Cdt1 was immunoprecipitated from lysates of asynchronously growing HeLa cells transfected with Hec1-GFP plasmids; ‘Ctrl IP’ indicates the use of normal mouse serum as a control. Hec1-GFP in the bound (Cdt1 IP) and unbound (Supe.) fractions was detected with anti-GFP antibody. (c) PTK2 cells were treated with hec1 siRNA followed by transfection with either wild-type Hec1-GFP or Hec1 LoopMUT-GFP constructs. The cells were treated with nocodazole, then fixed and stained using anti-Cdt1 antibody and anti-GFP antibody. (d) The same as in c, except that HeLa cells were stained with anti-Orc6 and anti-GFP antibodies. (e) The same as in c, except that HeLa cells were stained with anti-tubulin antibody to mark microtubules and anti-GFP antibody to mark Hec1-GFP at kinetochores. (f) Quantification of mitotic stages in Hec1-depleted cells expressing ectopic wild-type Hec1-GFP or Hec1 LoopMUT-GFP stained with DAPI; n = 125 GFP-expressing cells. Scale bars, 5 μm. See also Supplementary Fig. S8 and Movies S5 and S6. Uncropped images of blots are shown in Supplementary Fig. S9.

This domain is required for Cdt1 binding, we replaced the normal loop sequence with a sequence of alternative amino acids of similar length, which we term ‘Hec1 LoopMUT’ (Fig. 5a). A similar sequence provides a flexible linker in a well-studied GFP-PCNA fusion24. Both wild-type Hec1 and Hec1 LoopMUT were tagged with GFP at their C termini. Strikingly, Hec1 LoopMUT-GFP failed to co-immunoprecipitate with Cdt1, whereas wild-type Hec1-GFP bound Cdt1 readily (Fig. 5b, compare lanes 4 and 5). Both GFP-tagged wild-type and LoopMUT Hec1 localized normally to kinetochores in PTK2 and HeLa cells (Fig. 5c,e). We then tested Cdt1 localization in PTK2 cells depleted of endogenous Hec1 but expressing siRNA-resistant versions of wild-type Hec1-GFP or Hec1 LoopMUT-GFP. Cdt1 localized to kinetochores normally in cells expressing wild-type Hec1-GFP (Fig. 5c, top panel), but was strikingly undetectable at kinetochores in cells expressing Hec1 LoopMUT-GFP (Fig. 5c, bottom panel). Orc6 kinetochore targeting was also dependent on the Hec1 loop (Fig. 5d).
HeLa cells expressing only Hec1 LoopMUT–GFP bore striking similarities to Cdt1-depleted cells. Only 14% reached late prometaphase and 86% remained in early to mid-prometaphase (Fig. 5e,f and Supplementary Movie S6 and Fig. S6b), unlike cells expressing wild-type Hec1–GFP (12/13 cells, Supplementary Movie S5 and Fig. S6a). Live-cell imaging of cells expressing only Hec1 LoopMUT–GFP also revealed a 100% mitotic arrest phenotype (n = 29). The phenotypes of Hec1LoopMUT–GFP cells were more severe than Cdt1-depleted cells, which could be due to quantitative differences in the effectiveness of the different siRNAs in synchronized versus asynchronous cultures, defects in spindle structure from Hec1 perturbation or to other functions of the loop apart from Cdt1 binding. The range of phenotypes in both circumstances was similar, however (Supplementary Fig. S6c,d), indicating that the Hec1 LoopMUT phenotype might be largely explained by failure to recruit Cdt1 to kinetochores.

The absence of Cdt1 at kinetochores causes defective kMT attachment and Mad1-dependent arrest

These phenotypes prompted us to probe the spindle-assembly-checkpoint status of arrested cells. In Cdt1-depleted cells, kinetochore localization of the checkpoint protein, Mad1, on aligned chromosomes was 5.2-fold higher than in control cells (Fig. 6a, top and middle panels, and Fig. 6b). We measured a similar 4.5-fold Mad1 increase at aligned kinetochores in cells expressing Hec1 LoopMUT–GFP.
Figure 7 Cdt1 and the Hec1 loop domain are required for stable kMT attachments. (a) Control cells in metaphase, Cdt1-depleted cells and cells expressing Hec1 Loop\textsuperscript{MUT} (in place of endogenous Hec1) in late prometaphase were incubated with ice-cold PBS (which allows retention of only stable kMTs), followed by fixation and staining with anti-tubulin antibody (microtubule, MT) and either anti-Bub1 antibody to mark the kinetochores or anti-GFP antibody for ectopic Hec1, as indicated. (b) Quantification of the fraction of kinetochores making successful contact with kinetochore fibres in Cdt1-depleted or Hec1 Loop\textsuperscript{MUT} cells; \( n = 250 \) kinetochores. (c) Inter-kinetochore (K–K) distances in the indicated cells; \( n = 100 \) kinetochore pairs in each category. \( P < 0.01 \). Data are presented as mean ± s.d. (d) Synchronized HeLa cells treated with either control luciferase or \textit{cdt}1 siRNA, as indicated, were treated with both ZM447439 (Aurora B inhibitor, diagrammed) and MG132 (anaphase inhibitor) for 30 min at 8.5 h after S-phase release, followed by cold treatment and staining with anti-tubulin antibody and anti-Knl1 antibody. (e) Synchronized HeLa cells were co-transfected with both \textit{hec}1 siRNA and either a plasmid encoding unphosphorylatable 9A-Hec1\textsubscript{GFP} (top panels) followed by MG132 treatment for 1/2 h at 8.5 h after S-phase release or plasmid encoding Hec1 Loop\textsuperscript{MUT}\textsubscript{GFP} (bottom panel). The cells were then subjected to cold treatment at 9 h after S-phase release and stained with anti-tubulin antibody and anti-GFP antibody. Scale bars, 5 \( \mu \text{m} \).

versus wild-type Hec1–GFP (Fig. 6a, bottom panel, and Fig. 6c). Kinetochore localization of another checkpoint protein, BubR1, was unaltered by Cdt1 depletion (data not shown). To determine whether Mad1 recruitment fully accounts for the mitotic arrest produced by Cdt1 inhibition, we co-depleted Mad1 with Cdt1. As demonstrated previously\textsuperscript{25}, control cells depleted of Mad1 alone underwent premature anaphase onset (Fig. 6, compare d with f). Importantly, co-depletion of Mad1 with Cdt1 bypassed the mitotic arrest of Cdt1-depleted cells (Fig. 6, compare e with g). We thus conclude that Cdt1 depletion induces persistent Mad1-dependent spindle-assembly-checkpoint signalling. Of further note, synchronized cells co-depleted of both Mad1 and Cdt1 did not produce many anaphase chromosome bridges relative to Mad1-depleted cells. We reasoned that unreplicated DNA segments would not be segregated properly, leading to the production of anaphase chromosome bridges. The absence of increased anaphase bridges reinforces our assertion that Cdt1 is not required during \( S \) phase for complete DNA replication. On the other hand, asynchronous cells depleted of either Cdt1 or Orc6 produced many anaphase chromosome bridges (Supplementary Fig. S7a,b and data not shown).
Figure 8 Cdt1 and the Hec1 loop domain are required for proper Ndc80 conformation in vivo. (a) Diagram of the Ndc80 complex depicting the antibody epitopes used for the Delta analysis. (b) Representative image of a normal mitotic metaphase cell labelled with antibodies to the Hec1 CH domain and the Spc24 head domain. The images to the right show a magnification of the area outlined in the left image. Scale bars, 1 μm. (c) Delta values (corrected for tilt) of mean separation between Spc24 and the Hec1 CH domain (9G3 antibody) in synchronized control or Cdt1-depleted mitotic cells (9 h after S-phase release), or in Rod-depleted cells (mitotic metaphase cells selected from an asynchronous population); n = 84, 47 and 54 kinetochore pairs respectively; (control versus cdt1 siRNA) P < 0.001, (control versus rod siRNA) P = 0.31 (not significant). (d) The same as in c, except that Delta values of separation between Nsl1 (Mis12 complex subunit) and the Hec1 CH domain were determined; n = 74, 41 and 60 kinetochore pairs respectively; (control versus cdt1 siRNA) P < 0.001, (control versus rod siRNA) P = 0.86 (not significant). Data are presented as mean ± s.d. (e) Scale model for the Cdt1–Hec1 Loop domain interaction.

The presence of high levels of Mad1 at kinetochores indicated that loss of Cdt1 or mutation of the Hec1 loop produces kMT attachments that are insufficiently robust. We therefore investigated the cold stability of kMTs by chilling cells at late prometaphase or metaphase before fixing them for immunofluorescence analysis. The fluorescence intensity of kMTs after cold treatment for Cdt1-depleted cells or Hec1 Loop\textsuperscript{MUT} cells was ~50% of controls (Fig. 7a, top and middle panels, and Supplementary Fig. S7c). Quantification of kinetochores that made contact with cold-stable spindle microtubules revealed a 77% decrease in the number of kinetochore fibres in Cdt1-depleted cells and an 84% decrease in Hec1 Loop\textsuperscript{MUT}–GFP cells relative to corresponding controls (Fig. 7b). As another indicator of kMT attachment, we measured the inter-kinetochore (kinetochore–kinetochore) distance of aligned sister kinetochore pairs; 1.4 μm for siRNA-transfected controls and ~1.25 μm for Hec1-depleted cells expressing wild-type Hec1–GFP. In contrast, the kinetochore–kinetochore distance was just 0.96 μm in Cdt1-depleted cells and 1.05 μm in cells expressing Hec1 Loop\textsuperscript{MUT}–GFP (Fig. 7c). These values correspond to a 50–60% loss of
kinetochore stretch because the unstretched centromere length is ∼0.7 μm in cells treated with nocodazole. As in Cdt1-depleted cells, there were no discernible changes in the localization of most other proteins examined in cells expressing Hec1 LoopMUT–GFP (Supplementary Fig. S8b).

To determine whether artificially strengthening microtubule attachments can compensate for Cdt1 loss or mutations in the Hec1 loop domain, we blocked Aurora-B-kinase-mediated Hec1 phosphorylation. Pharmacological Aurora B inhibition or mutation of the 9 phosphorylation sites in the N-terminal Hec1 tail enhances microtubule-binding affinity at this end of the Ndc80 complex. Treating Cdt1-depleted mitotic cells with the Aurora B inhibitor, ZM447439, caused premature anaphase onset similar to control drug-treated cells or Mad1-depleted cells, demonstrating effective Aurora B inhibition (Supplementary Fig. S7f,g). We also coupled our synchronization and Cdt1 depletion protocol with expression of the Aurora-B-resistant Hec1 (9A–Hec1–GFP) combined with endogenous Hec1 depletion. The result of this manipulation was identical to that of Aurora B inhibition (data not shown). To determine whether progression to anaphase in these experiments involved stabilization of kMT attachments, we treated cells with the proteasome inhibitor MG132 to prevent anaphase onset and subjected them to the cold-stability assay. ZM447439-treated or 9A–Hec1–GFP-expressing cells lacking Cdt1 retained stable kMTs (Fig. 7d,e). These results indicated that the absence of Cdt1 at kinetochores results in failure to establish and/or maintain sufficiently strong kMT attachments.

**Interaction of Cdt1 with the Hec1 loop is required for the normal conformation of the Ndc80 complex at kinetochores of bi-oriented chromosomes**

The loop region of Hec1 allows conformational changes within the Ndc80 complex. To determine whether Cdt1 influences this conformation, we employed an in vivo two-colour super-resolution fluorescence microscopy technique by labelling the two ends of the Ndc80 complex (Delta analysis illustrated in Fig. 8a; ref. 26). We used the 9G3 antibody, whose epitope in Hec1 resides next to the N-terminal CH domain, an anti-Spc24 antibody to mark the C-terminal region of the Ndc80 complex and a N-terminal tail of Hec1 make direct contacts with microtubules, and the loop domain is not required for high-affinity binding of Ndc80homai complexes to microtubules, a construct lacking the region that includes the loop. It was therefore surprising that mutation of the Hec1 loop domain, a site ∼18 nm along the Hec1 rod domain from these direct microtubule contacts, had such profound negative effects on the formation of stable microtubule attachments. Clearly the formation of robust attachments in situ requires not only the Hec1 CH domain and N-terminal region, but also Cdt1 interaction with the loop domain.

Recent studies in yeast have shown a requirement for the loop in yeast Hec1/Ndc80 for binding microtubule-binding proteins (MAPs), that is, the Dam1 complex in budding yeast or Dis1/TG in fission yeast. The amino-acid sequence of the loop is not highly conserved, and human Cdt1 has no detectable sequence homology to Dam1 complex members or to Dis1/TG. Nevertheless, Cdt1 could serve an analogous function by interacting with microtubules or an as yet, unidentified microtubule-associated protein (Fig. 8e) or alternatively by inhibiting Aurora B-mediated kinetochore-fibre destabilization.

**A model for Cdt1–Hec1 interaction in mitosis**

The Hec1 loop produces a flexible hinge in the otherwise relatively rigid Ndc80 complex. In the complete absence of microtubules, the average separation, Delta, between the two ends of the Ndc80 complex is only ∼19 nm (ref. 26) in both controls and Cdt1-depleted cells. This...
average distance extends to \(\sim 41\) nm for control metaphase cells when kinetochores achieve a full microtubule complement. In the absence of Cdt1, Ndc80 does not make this full extension, producing Delta measurements \(\sim 68\% - 76\%\) between the two extremes. In contrast, Rod depletion removes the main recruitment factor for the dynein–dyncin complex and disrupts other kMT–binding sites contributed by this microtubule motor.\(^{32,39,40}\) The fact that Ndc80 can adopt the full extended shape in Rod-depleted cells, which, similarly to Cdt1-depleted cells, have unstable kMT attachments\(^{31}\), provides evidence against a general indirect effect of attachment on Ndc80 conformation. We propose that Cdt1 physically links the Hecl1 loop to the microtubule or a microtubule–associated protein, and this extra attachment stabilizes the extended conformation and enhances the microtubule affinity of the Ndc80 complex provided by the Hecl1 CH domain and N-terminal tail.

**METHODS**

Methods and any associated references are available in the online version of the paper at www.nature.com/naturecellbiology

**AUTHOR CONTRIBUTIONS**

D.V. and J.G.C. designed and carried out experiments, analysed data and wrote the manuscript. S.L.D., V. de Vries, M. A. & Bell, S. P. Orc6 is required for the assembly of the replication-precipitating complexes in Xenopus laevis. Nature 404, 622–625 (2000).

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METHODS

Cell culture, transfection and flow cytometry. HeLa (normal and stably expressing histone H2B) and NIH3T3-hTERT cells were cultured in DMEM (Difco or Invitrogen) supplemented with 10% fetal bovine or calf serum (Sigma), 100 U/ml penicillin and 100 mg/ml streptomycin. LLCPK1 cells were grown in DMEM with 5% FBS and PTK2 cells were cultured in MEM-alpha supplemented with 15% FBS. Transfections were performed with a total of 100 nM of each siRNA duplex using Dharmafect 1 reagent (Dharmacon/Thermo Scientific) according to the manufacturer’s guidelines. Stable cell lines expressing Cdt1 have been described previously24. Flow cytometric analysis for cell-cycle position was performed as described previously25. Synthetic siRNAs: cdt1 siRNA and cdk6 siRNA (ref. 16) were synthesized by Invitrogen/Life Technologies, mad1 siRNA was purchased from Dharmacon (SMART pool catalogue no. sc-00825-00-005), rod siRNA from Dharmacon (SMART pool catalogue no. L006829-00-005), hecl siRNA from Dharmacon (SMART pool catalogue no. sc-00825-00-005) and hecl siRNA (ref. 26) 5'-AAAAAGAAAGCCCAUGUCUCAGAAA-3', hec1 siRNA (rat kangaroo) 5'-AAUGAGCGGAUUGCUAAUAUAGT-3' and orc6 siRNA (ref. 14) 5'-AAGAUUUGACAGGACGCUAUCUU-3' were synthesized by Dharmacon.

HeLa cells were synchronized by treatment with 2 mM thymidine for 18 h followed by release for 9 h and then re-treatment with 2 mM thymidine for 18 h. Synthetic duplexed RNA oligonucleotides were transfected into HeLa cells according to the manufacturer’s instructions. Other cell manipulations include 10 μM nocodazole treatment, 3 μM ZM447439 treatment and cold treatment for 10 min with ice-cold PBS.

Antibodies. Guinea pig anti-Cdt1 is described in ref. 41 (diluted 1:5,000 for immunoblots and 1:100 for immunofluorescence staining); rabbit polyclonal anti-Cdt1 was purchased from Santa Cruz Biotechnology (catalogue no. sc-28262). Other primary antibodies used in this study include anti-tubulin monoclonal (1:20,000 for immunoblots and 1:500 for cell staining, Sigma-Aldrich, no. 9026, clone DM1A), anti-dynein IC monoclonal antibodies (1:200 for staining Sigma-Aldrich, no. D5167, clone 70.1), anti-Hecl (1:6,000 for immunoblots and 1:500 for staining, Abcam, no. ab3613, clone 9G3), Nud2 (1:5,000 for immunoblots and 1:200 for staining, Abcam no. ab17058, clone 28-37), anti-GFP polyclonal (1:200 for staining, Invitrogen, no. A6453), monoclonal (1:100 for staining, Chemicon/Millipore, no. MAB3380) or monoclonal (1:5,000 for immunoblots, Clontech, Mountain View, no. 632381, clone JL-8), anti-Cdc6 monoclonal (1:100 for immunoblots Santa Cruz Biotechnology, no. sc-9964 clone 180.2), anti-geminin polyclonal (1:200 for staining, Santa Cruz Biotechnology, no. sc-13015), anti-phospho-H2A.X(Ser139) (1:1,000, for staining, Millipore, no. 05-636, clone JBW301), anti-Mad1 polyclonal (GeneTex, no. GTX109519), Alexa 488-, Rhodamine Red-X-, Cy5- or HRP-labelled secondary antibodies were obtained from Jackson ImmunoResearch. Orc6 polyclonal antibody (1:200) for staining was a generous gift from B. Stillman; anti-Orc6 for immunoblots (1:1,000) was purchased from Santa Cruz Biotechnology (no. sc-32735, clone 3A4). Immunoblots were scanned into Adobe Photoshop and any manipulations for brightness were applied to the entire image; final figures show cropped regions and the uncropped blots are provided as Supplementary Fig. S9.

Immunofluorescence microscopy, live-cell imaging and Delta analysis. Cells were typically fixed for 20 min using 4% paraformaldehyde after permeabilization with 0.5% Triton X-100. For Spc24 staining, 2% paraformaldehyde was used. For dynein IC staining, the cells were fixed for 6 min at −20°C with ice-cold methanol. Cells were rinsed in PHEM buffer (120 mM PIPES, 50 mM HEPES, 20 mM EGTA and 4 mM magnesium acetate, at pH 6.9) before fixation. All of the antibody incubations and washes were also performed in PHEM buffer plus 0.05% BSA. DAPI staining (0.1 μg/ml−1) was for 5 min, and cells were mounted using Prolong Antifade (Molecular Probes). All antibody incubations were conducted at 37°C for 1 h. For image acquisition, three-dimensional stacks were obtained sequentially at 200 nm steps along the z axis through the cell using a high-resolution Nikon confocal microscopy equipped with a Yokogawa CSU10 spinning disc with image magnification yielding a 65 nm pixel size from the camera41 and a ×100/1.4 NA (Planapo) DIC oil-immersion objective (Nikon). Delta analysis to measure separations at high (nanometre) accuracy between protein epitopes labelled with separate colours was carried out as described previously5,26.

For injection, polyclonal anti-Cdt1 antisemur was depleted of antibodies to non-Cdt1 proteins by repeated rounds of incubation with lysates of ultraviolet-treated HeLa cells (which lack Cdt1). These antibody preparations were then concentrated by centrifugation using microconcentrators. Anti-Cdt1 antibody, control IgG or buffer were injected into HeLa cells that stably express a GFP fusion to histone H2B either in prometaphase or in metaphase. Injections were carried out on a Zeiss IM microscope equipped with phase optics and a ×40/0.75 NA (Planfluar) objective at 35°C using a Sage air stage incubator. Injections were 5% cell volume with a Narishige micromanipulator (Narishige USA). Mitotic progression was monitored by live-cell imaging using both phase-contrast and GFP–histone H2B fluorescence analysis with images acquired every two minutes until the end of the duration of imaging.

For live-cell imaging of HeC1-depleted cells, HeLa cells were plated to 50% confluency in a T25 flask and treated with siRNA to HeC1. At 24 h post-siRNA transfection, cells were trypsinized, counted and the wild-type and mutant HeC1 constructs were electroporated along with mCherry–histone H2B expression plasmid using the Lonza Nucleofector apparatus. At 24 h post-electroporation, cells were imaged by time-lapse microscopy every 4 min for 5 h using a Deltavision PersonalDV Imaging System (Applied Precision) equipped with a Photometrics CoolSnap HQ2 camera and a ×60/1.42 NA (Planapo) DIC oil-immersion lens (Olympus).

Protein–protein interaction assays. For two-hybrid screening, the full-length human Cdt1 complementary DNA was inserted into pGBT9 (Clontech). Co-transformants of yeast strain PJ69α (ref. 43) with two cDNA fusion libraries (placental cDNA or thymus cDNA, Clontech) were selected on medium containing 10 mM 3-amino-1,2,4 triazole (Sigma). More than 8 million co-transformants of each library were screened; HeC1 clones were identified from both libraries. GST pull-downs and co-immunoprecipitations were performed essentially as described previously5. GST–Cdt1 was produced in BL21(DE3) purified on glutathione–Sepharose (GE Healthcare) and incubated with cell lysates prepared in buffer 1 (50 mM HEPES at pH 7.2, 33 mM potassium acetate, 0.5 mM EDTA, 0.5 mM EGTA, 0.1% Nonidet P-40, 10% glycerol, protease and phosphatase inhibitors). Cell lysates were prepared in buffer 1 or a 1:1 mix of buffer 1 and CSK buffer (10 mM PIPES, at pH 7.0, 100 mM NaCl, 300 mM sucrose, 3 mM MgCl₂, plus protease and phosphatase inhibitors), digested with micrococcal nuclease before centrifugation to remove insoluble material, and incubated with GST-protein–coated beads for 2 h, or with antibodies for co-immunoprecipitation and protein A beads, washed three times and then released by boiling in SDS–PAGE loading buffer.
Figure S1 Cdt1-depletion during S phase does not perturb replication. (a) HeLa cells transfected with control or cdt1 siRNA after release from double thymidine synchronization described in Fig. 1 were harvested at the indicated time points and analyzed for DNA content by flow cytometry and (b) for Cdt1 protein by immunoblotting. (c) HeLa cells stably expressing normal Cdt1 (WT), or an unphosphorylatable derivative with alanine substitutions at positions S391, T402, T406, S411, and S491 (from Chandrasekaran, et. al., 2011) were synchronized in early S by double thymidine block and released into siRNA as described in Fig.1. Nocodazole was added during S phase to hold cells briefly in early mitosis (0–10 hrs after thymidine release into nocodazole) and then removed to allow cells to divide (2 hrs). Flow cytometric analysis of DNA content is plotted. (d) Immunoblot analysis of Cdt1 protein; the alanine substitutions block the phosphorylation-induced gel mobility shift. Vector, siRNA controls, and WT Cdt1 data are the same as Fig. 1d. (e) Asynchronous HeLa cells treated with control luciferase, orc6, or cdt1 siRNAs (as indicated) or double thymidine synchronized HeLa cells treated with cdt1 siRNA in S and fixed at 10 hrs after S phase release (right-most panels) were stained with DAPI (pseudo-colored green) and anti-phospho-H2A.X antibody (red).
Figure S2 Inhibition of Cdt1 function induces mitotic arrest. Synchronized HeLa cells transfected with control luciferase siRNA (a) or cdt1 siRNA (b) were stained with DAPI (pseudo-colored red) and anti-tubulin antibody (green) at 9 hrs (left panels) or 10 hrs (right panels) after S phase release. (c) Immunoblot detection of Cdt1 using the purified anti-Cdt1 antibody, "GP47". (d) Metaphase HeLa cells stably expressing GFP-histone H2B were injected with the anti-Cdt1 antibody followed by live-cell imaging as in Fig. 2d. Selected frames of GFP-histone H2B (top panels) and phase contrast images (bottom panels) are shown. Scale bars = 5 µm. See also Supplementary Movie S4.
Figure S3 Cdt1, but not its inhibitor geminin, localizes to kinetochores. (a) Double thymidine synchronized HeLa cells treated with control luciferase (top panels) or cdt1 siRNA (bottom panels) were fixed 9 hrs after S phase release and stained with anti-Cdt1 antibody and anti-Hec1 antibody. (b) HeLa cells at the indicated stages of mitosis were immunostained using anti-Geminin antibody and anti-Hec1 antibody to mark kinetochores. Scale bars = 5 µm.
Figure S4 Cdt1 and Hec1 interact. (a) A yeast-two-hybrid library screen of proteins interacting with Cdt1 yielded multiple clones of the kinetochore protein Hec1. The diagram illustrates the Hec1 sequences contained in 5 independent clones isolated (some more than once); the location of the loop domain is indicated. (b) Yeast strain PJ69a was transformed with plasmids encoding fusions to the Gal4 DNA binding domain (“Gal4DB”) and fusions to the Gal4 transcriptional activation domain (“Gal4AD”). The Hec1 protein sequences included in the fusions are listed in superscript; a Gal4AD fusion to the Cdt1 inhibitor geminin serves as a positive control, and negative controls (empty vectors) are indicated by “0.” Serial five-fold dilutions of yeast cultures were spotted onto agar plates containing the indicated concentrations of 3-amino-1,2,4 triazole (“3-AT”) to measure transcriptional activation of the GAL4pr-HIS3 interaction reporter and incubated for 3 days at 30°C before photographing. YPD and SCD-UL control plates contained no 3-AT. (c) Metaphase PTK2 cells treated with hec1 siRNA were stained with anti-Cdt1, anti-Hec1 and anti-ACA antibodies. (d) Lysates from HeLa cells treated with siRNA targeting control GFP, Cdt1 or Hec1 were immunoblotted for Cdt1, Hec1 and tubulin. (e) Synchronized HeLa cells subjected to Cdt1 RNAi during S phase for 9 hrs were stained using anti-Hec1 and anti-Zwint1 antibodies. Scale bars = 5 μm.
Figure S5  Cdt1 facilitates Orc6 localization in mitosis; Orc6 is not required for stable kMT attachments.  (a) A lysate of HeLa cells was incubated with beads coated with bacterially-produced GST or GST-Cdt1, and the endogenous Hec1 and Orc6 were detected in the input or bound fractions by immunoblotting.  Recombinant proteins bound to the beads were detected by Ponceau S staining of the blot; the blot was cut to probe the top for Hec1 and the bottom for Orc6.  (b) Synchronized control and cdt1 siRNA-treated cells were stained for Orc6 and Hec1.  (c) Quantification of Orc6 fluorescence intensity in c; n = 140 kinetochores.  P < 0.01.  (d) Asynchronously growing HeLa cells treated with orc6 siRNA for 3 days as in Prasanth et al., 2002, were stained for Orc6, Hec1, and ACA.  (e) HeLa cells as in d were stained for Orc6, Hec1, and Cdt1.  (f) HeLa cells as in d were stained using DAPI and anti-tubulin antibody, and the percentage of dividing cells (mitotic index) was calculated for three different coverslips from three separate experiments (n= 1500).  (g) The mitotic cells from f were classified into the indicated stages.  (h) Metaphase cells from a and late prometaphase cells from synchronized cultures depleted of Cdt1 were subjected to cold treatment and stained using anti-tubulin antibody and either anti-ACA antibody or anti-Bub1 antibody as indicated.  Scale bars = 5 µm.
Figure S6 Phenotypic comparison of mitotic arrest induced by Cdt1 depletion or Hec1 LoopMUT expression. HeLa cells transiently co-expressing mCherry-histone H2B and either wt Hec1-GFP (a) or Hec1 LoopMUT-GFP (b) after depleting endogenous Hec1 were imaged by time-lapse microscopy every 4 min. Selected frames of mCherry-histone H2B (top panels) and phase contrast images (bottom panels) are shown for each case. (c) HeLa cells were either synchronized in early S phase and released into cdt1 siRNA for 10 hrs (top panels) or treated with hec1 siRNA followed by transfection with the Hec1 LoopMUT construct (bottom panels). Cells were fixed and stained with DAPI to label the chromosomes, anti-tubulin antibody to label microtubules, and either anti-Knl1 antibody (top) or anti-GFP antibody (bottom panels) to label kinetochores. (d) Quantification of the cells arrested in various stages of mitosis after Cdt1 depletion (n = 1500 cells) or Hec1 LoopMUT expression (n = 125 cells). Scale bars = 5 µm.
Figure S7 Cdt1 is required to satisfy the spindle assembly checkpoint. (a) Synchronized HeLa cells treated with siRNAs targeting control luciferase, Cdt1, Mad1, or both Cdt1 and Mad1 as indicated were fixed at 10 hrs after S phase release. For comparison, asynchronously growing HeLa cells were depleted of Cdt1 (bottom right panel). All cells were stained with DAPI (pseudo-colored red) and anti-Knl1 antibody. (b) Percentage of anaphase cells with at least one anaphase chromosome bridge is plotted for the indicated cell populations. (c) Quantification of total tubulin fluorescence to evaluate the loss of kinetochore-microtubule stability in Cdt1-depleted or Hec1 LoopMUT-expressing HeLa cells. n ≥ 10 cells, p < 0.01. (d-g) Synchronized HeLa cells transfected with either control luciferase (d) or cdt1 siRNA (e) were mock treated or treated with 3 µm Aurora B inhibitor ZM447439 (as indicated) at 8.5 hrs post S phase release and fixed immediately (f and g, left panels) or after 9 hrs (f and g, right panels) followed by staining with DAPI (pseudo-colored red) and anti-tubulin antibody. Panels d and e are reiterated from Figure 6. Scale bar = 5 µm.
Figure S8 Neither Cdt1 depletion nor Hec1 LoopMUT expression grossly perturbs kinetochore structure. (a) Double thymidine synchronized HeLa cells subjected to Cdt1 depletion during the 2nd thymidine release for 10 hrs were fixed and stained with an antibody to the Mis12 complex subunit, Nsl1/DC31 and with antibody to Hec1. (b) HeLa cells subjected to Cdt1 depletion or expressing siRNA resistant GFP-Hec1 LoopMUT after the depletion of endogenous Hec1 were stained with antibodies to kinetochore proteins involved in spindle assembly checkpoint and kinetochore microtubule attachment with or without nocodazole treatment as indicated. Pluses and minuses indicate relative staining intensity compared to control cells. Scale bars = 5 µm.
**Figure S9** Un-cropped immunoblots corresponding to Figures 1-8. The regions shown in the figures are outlined with dashed boxes.
Legends for Supplementary Movies

**Movie S1** HeLa cells stably expressing GFP-histone H2B were injected with buffer control in early prometaphase and imaged by time lapse microscopy every 2 min subsequent to injection. The movie was accelerated 800X times to about 6 frames/sec.

**Movie S2** HeLa cells stably expressing GFP-histone H2B were injected with control donkey anti-mouse IgG in early prometaphase and imaged by time lapse microscopy every 2 min subsequent to injection. The movie was accelerated 480X times to about 4 frames/sec.

**Movie S3** HeLa cells stably expressing GFP-histone H2B were injected with anti-Cdt1 antibody in early prometaphase and imaged by time lapse microscopy every 2 min subsequent to injection. The movie was accelerated 840X times to about 6 frames/sec.

**Movie S4** HeLa cells stably expressing GFP-histone H2B were injected with anti-Cdt1 antibody during metaphase and imaged by time lapse microscopy every 2 min subsequent to injection. The movie was accelerated 1700X times to about 7.75 frames/sec.

**Movie S5** HeLa cells transiently co-expressing mCherry-histone H2B and wt Hec1-GFP after depleting endogenous Hec1 by siRNA transfection were imaged by time-lapse microscopy for histone fluorescence every 4 min. The movie was accelerated 912X times to about 3.8 frames/sec.

**Movie S6** HeLa cells transiently co-expressing mCherry-histone H2B and Hec1 LoopMUT -GFP after depleting endogenous Hec1 by siRNA transfection were imaged by time-lapse microscopy histone fluorescence every 4 min. The movie was accelerated 957X times to about 3.5 frames/sec.