Quaternary structure of the human Cdt1-Geminin complex regulates DNA replication licensing

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All organisms need to ensure that no DNA segments are rereplicated in a single cell cycle. Eukaryotes achieve this through a process called origin licensing, which involves tight spatiotemporal control of the assembly of prereplicative complexes (pre-RCs) onto chromatin. Cdt1 is a key component and crucial regulator of pre-RC assembly. In higher eukaryotes, timely inhibition of Cdt1 by Geminin is essential to prevent DNA rereplication. Here, we address the mechanism of DNA licensing inhibition by Geminin, by combining X-ray crystallography, small-angle X-ray scattering, and functional studies in *Xenopus* and mammalian cells. Our findings show that the Cdt1:Geminin complex can exist in two distinct forms, a ''permissive'' heterotrimer and an ''inhibitory'' heterohexamer. Specific Cdt1 residues, buried in the heterohexamer, are important for licensing. We postulate that the transition between the heterotrimer and the heterohexamer represents a molecular switch between licensing-competent and licensing-defective states.

solution structure | X-ray structure | pre-RC | cell cycle

The duplication of chromosomal DNA is an essential process for all organisms and needs to be tightly regulated to preserve genomic integrity. To ensure the timely completion of replication, DNA duplication in eukaryotic cells initiates from thousands of origins of replication. To coordinate origin activation and achieve exactly one complete round of DNA synthesis during S phase, the processes of origin selection and of initiation of DNA replication from these origins are separated. During telophase and early G1, the licensing process marks the origins of replication that can be used during the following S phase, by the stepwise assembly of the prereplicative complexes (pre-RC) on specific chromatin sites (1, 2). Pre-RC assembly culminates in the recruitment of the hexameric minichromosome maintenance complex (MCM) onto chromatin. This process requires the activity of the other pre-RC proteins: the origin replication complex (ORC), Cdc6, and Cdt1 (3–5).

To prevent rereplication, licensing must be temporally limited to late mitosis and G1 phase only. In higher eukaryotes, control of licensing largely occurs by down-regulating Cdt1 activity. This tight temporal control of Cdt1 is achieved by at least two different mechanisms: through ubiquitin-dependent proteolysis at the onset of S phase (6–9) and through the binding of its inhibitor Geminin (10–12). Geminin is present in all metazoans, but apparently not in yeast. It was initially discovered in *Xenopus* in a screen for substrates of the anaphase-promoting complex (APC) and immediately recognized as an inhibitor of pre-RC assembly (10–13). Geminin tightly binds and sequesters Cdt1 in a complex that is unable to recruit MCM to origins. Interestingly, Geminin does not inhibit the interaction of Cdt1 with origins of replication (3), and it has been shown both in vitro and in vivo that Geminin is recruited onto chromatin via Cdt1 (14, 15). Because Geminin is a substrate of the APC, it was suggested that its degradation at the metaphase-anaphase transition represented the event inaugurating the licensing period. However, in the *Xenopus* early embryo, less than half of the endogenous Geminin is degraded at the exit from mitosis, while the remainder becomes inactivated and incapable of inhibiting Cdt1 (16–18). The remaining Geminin gets reactivated in the next cell cycle, through a mechanism that is not well understood, but is dependent on the nuclear import of Geminin, which restores its ability to block Cdt1 (16, 19, 20). Cell-cycle differences in Geminin's ability to inhibit Cdt1 has also been inferred from observations in other organisms (21–24). The properties of Geminin that persists during the licensing period are controversial. The conventional view is that inactivated Geminin is unable to bind Cdt1 and therefore does not prevent licensing (16). However, a more recent study suggests that a complex between Geminin and Cdt1 is competent for licensing and capable to load the MCM helicase onto chromatin (25). In this view, Geminin only inhibits Cdt1 activity when a critical Geminin:Cdt1 ratio is achieved, which occurs after Geminin is reactivated before entry into S phase. Importantly, the fact that Geminin is regulated posttranslationally (8, 17, 26) is probably crucial to allow it to switch rapidly between functional states.

The structure of a mouse $Cdt1^{172–368}$:$Geminin^{79–157}$ complex (*m*tCdt1:*m*tGem) reported by Lee *et al.* (27) showed a heterotrimer between one Cdt1 and two Geminin molecules. The C-terminal coiled-coil of Geminin, known to be important for inhibiting Cdt1 function (10, 28, 29), was not in contact with Cdt1 in that structure. To explain the ability of Geminin to inhibit Cdt1 function, the authors suggested that the essential long C-terminal coiled-coil of Geminin sterically interferes with the C terminus of Cdt1 (whose structure is unknown) to prevent the recruitment of MCM onto chromatin. However, because Geminin colocalizes with Cdt1 onto chromatin (3, 15, 25), and Geminin binding to Cdt1 is not sufficient to prevent MCM interaction with chromatin (14), this mechanism may not be sufficient to explain Geminin function.

In this study, we present the X-ray structure of a truncated human Cdt1-Geminin complex at 3.3 Å resolution. This unique structure, in combination with solution X-ray scattering measurements, shows

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The atomic coordinates and structure factors have been deposited in the Protein Data Bank. www.pdb.org (PDB ID code 2WVR).

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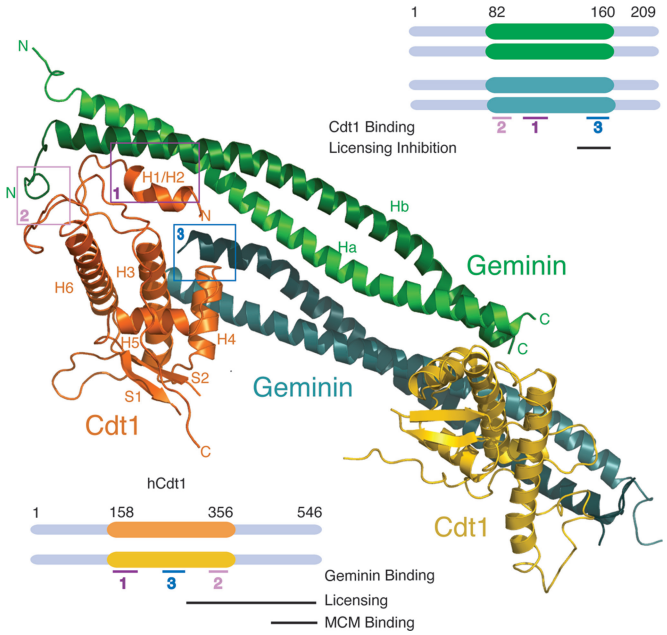

Fig. 1. Structure of the human tCdt1:tGeminin complex. The (2x[Cdt1:2xGeminin]) heterohexamer is shown as a cartoon representation: Cdt1 molecules in orange, Geminin molecules in green and blue shades. The primary, secondary, and the novel tertiary interface regions are boxed, in a magenta-, pink-, and blue-colored box, respectively. We also show a schematic representation of the Cdt1 and Geminin proteins; the 3 interfaces are marked in the sequence and shown with the same color scheme (additional functional regions are shown with labeled bars).

that the Cdt1:2xGeminin heterotrimer reported by Lee *et al.* (27) forms a heterohexamer (2x[Cdt1:2xGeminin]), indicating that the Cdt1-Geminin complex can exist in at least two distinct quaternary forms. The C-terminal coiled-coil of Geminin is essential for heterohexamer formation through a direct interaction with Cdt1. Mutations in the C-terminal coiled-coil, which do not affect its overall length, result in functional differences that correlate well with the quaternary structure of the Cdt1:Geminin complex, arguing against the steric hindrance hypothesis proposed by Lee *et al.* (27) for explaining the mechanism of Cdt1 inhibition by Geminin. Furthermore, we show that Cdt1 residues that are in direct contact with the C-terminal coiled-coil of Geminin are functionally important for licensing in *Xenopus* and mammalian cell lines. We therefore suggest that the heterotrimer and heterohexamer states of the Cdt1:Geminin complex that we describe could represent a molecular switch between licensing-competent and licensing-defective states, affecting the dynamic regulation of the Cdt1-Geminin complex recruited onto chromatin.

Results

A New Atomic Structure of the Human tCdt1:tGeminin Complex. We crystallized a human truncated Cdt1$^{158–356}$:Geminin$^{82–160}$ (*h*tCdt1:*h*tGem) complex in a solution buffered to pH 7.5 and ≈100 mM NaCl (see *Methods*). The phases were determined experimentally to alleviate concerns of model bias [supporting information (SI) Table S1] and yielded an electron density map of excellent quality, at 3.3 Å resolution. The *h*tCdt1:*h*tGem complex shows the same fold as the mouse counterpart (27), with the parallel coiled-coil of *h*tGem resembling the handle of an axe, where *h*tCdt1 is the blade (Fig. 1 and Fig. S1). The primary and secondary Cdt1:Geminin interfaces described in Lee *et al.* (27) are conserved in the human proteins (Figs. 1 and 2 *A* and *B*), but the orientation of the long Geminin "handle" relative to the Cdt1 "blade" is slightly different (Fig. S1). The C-terminal part of the *h*tGem coiled-coil is

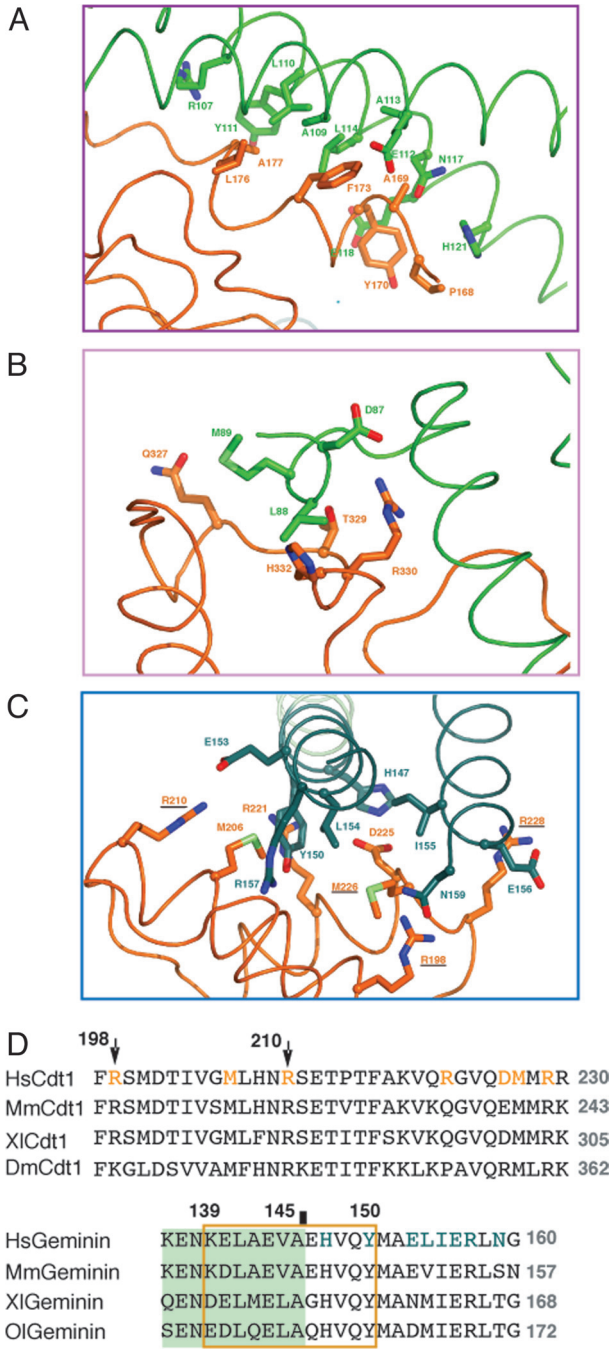

Fig. 2. The tripartite human *h*tCdt1:*h*tGeminin interface. The primary (*A*), secondary (*B*), and tertiary (*C*) interfaces are shown (coloring and bounding boxes as in Fig. 1). Interacting residues are shown as a stick model; carbon atoms follow the cartoon coloring; oxygens, nitrogens, and sulfurs are in red, blue, and green. Important residues are labeled; mutation sites in Cdt1 that are used in this study have labels underlined (the residues deleted in *h*vtGem are grayed out). (*D*) A multiple-sequence alignment of the sequence regions involved in the formation of the novel tertiary interface; residues in orange for Cdt1 and dark green for Geminin are the ones depicted also in (*C*). Arrowheads denote the mutation sites on the Cdt1 tertiary interface, the vertical bar and the green shading box indicate the Geminin deletion construct, and the dark yellow bounding box the region substituted in the grafting mutations (residues 139–150).

in our structure straight and not kinked (Fig. S1), which is in agreement with the structures of the Geminin coiled-coil alone (29, 30) and strongly suggests that the kink in *m*tCdt1:*m*tGem was only

induced by crystal packing, as proposed by Lee *et al.* (27) in the original publication.

Crucially, the C-terminal part of the *h*tGem coiled-coil (residues 145–160), known to be essential for Geminin function (10, 28, 29), is in our structure in close contact with a second molecule of *h*tCdt1, related by crystallographic symmetry. This quaternary arrangement creates a heterohexameric *h*tCdt1:*h*tGem complex (Fig. 1) with a head-to-tail interaction between *h*tCdt1:*h*tGem heterotrimers. In this conformation, a new interface (tertiary interface) (Fig. 2*C*) is generated by residues of both chains of the *h*tGem coiled-coil (H147, Y150, E153, L154, R157 on chain A; I155, E156, N159 on chain B) and of the Cdt1 residues in helices H3 (R198, M206, R210) and H4 (R221, D225, M226, R228). This interface is rich in charged residues (Fig. 2 *C* and *D*), displaying 8 hydrogen bonds and 8 salt bridges; additional stacking of hydrophobic side chains contributes to the interaction and a total area of 1,300 Å^2 is buried upon dimerization of the heterotrimers. The region of Geminin and Cdt1 involved in this interaction has been conserved throughout evolution (Fig. 2*D*), suggesting that it is functionally important.

Next, we wanted to assess whether the heterohexamer exists in solution and exclude the possibility of a crystallographic artifact.

Molecular Shape Analysis of the Cdt1:Geminin Complex in Solution. To study the *h*tCdt1:*h*tGem complex in solution, we used small-angle X-ray scattering (SAXS). The SAXS curves were recorded at sample concentrations of 1.0, 2.3, and 5.6 mg/mL. The experimental molecular mass (MM) of the solute (90 ± 10 kDa) and the radius of gyration (R_g) and maximal distance (D_{max}) values (38 ± 1 Å and 140 ± 10 Å respectively) agree with the heterohexameric conformation (88 kDa, 40 Å, and 130 Å, respectively), but not with the heterotrimeric one (44 kDa, 29 Å, and 98 Å, respectively) (Table S2). Moreover, the scattering curve computed from the model of the heterotrimer deviates significantly from the experiment (χ = 2.7), whereas the heterohexamer yields better agreement both at low and medium scattering angles with χ = 1.7 (Fig. 3*A*). Theoretical scattering curves were computed from possible heterohexameric arrangements generated by alternative crystal symmetry in our structure, and based on the crystal structure by Lee *et al.* (27). The asymmetric unit of the latter structure, either as initially submitted to the Protein Data Bank (PDB ID code 1WLQ), or in its very recent update (2ZXX, which obsoletes 1WLQ and contains a sequence correction mediating an interaction vaguely resembling the tertiary interface we describe) both show possible heterohexamers. All of these models, however, fit the experimental data much worse than the heterohexamer we present (Fig. S2 and Table S2), suggesting that they do not represent actual conformations of the complex in solution. Moreover, a low-resolution shape generated from the SAXS data ab initio using a dummy residues model (31) correlates well with the new heterohexamer, both for P2 and P1 reconstructions (Fig. 3*B*).

Finally, SAXS measurement on the full-length human Cdt1:Geminin complex at concentrations of 1.4 and 3.1 mg/mL indicated a molecular mass of 165 ± 15 kDa, which is not conclusive, but interestingly suggests an average value between the expected MM of the full-length heterohexamer (218 kDa) and the heterotrimer (109 kDa). This is consistent with the idea that the full-length complex could exist in both hexameric and trimeric forms in the experimental conditions used for the SAXS analysis in solution (SAXS is unable to resolve such dynamic equilibriums).

Geminin Residues 145–160 Affect Formation of the Cdt1-Geminin Heterohexamer. To validate the hexameric model, we designed a construct of Geminin excluding residues 145–160 that should be essential for the quaternary assembly. The human Cdt1$^{158–356}$Geminin$^{82–145}$ (*h*tCdt1:*h*vtGem) complex was expressed and purified under identical conditions as *h*tCdt1:*h*tGem and analyzed by SAXS, at sample concentrations of 2.8 and 4.9 mg/mL. The overall parameters of *h*tCdt1:*h*vtGem (MM = 37 ± 3 kDa, R_g = 28.5 ± 0.5 Å, D_{max} =

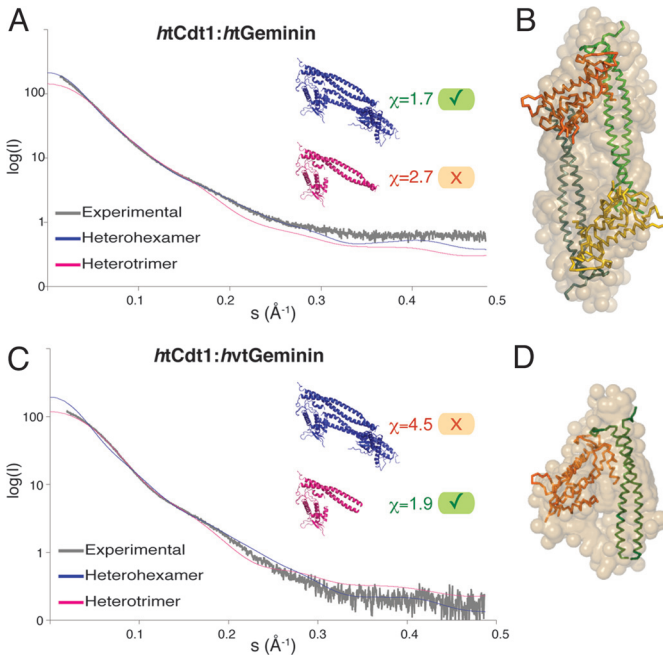

Fig. 3. Solution analysis of *h*tCdt1:*h*tGeminin and *h*tCdt1:*h*vtGeminin by SAXS. (*A*) The experimental SAXS profile (log intensity as a function of the momentum transfer) of *h*tCdt1:*h*tGem (gray curve) is compared with the theoretical scattering curves calculated from the corresponding crystallographic models of the complex as a heterohexamer (blue line and cartoon) and a heterotrimer (magenta line and cartoon), respectively. (*B*) The ab initio calculated SAXS model of *h*tCdt1:*h*tGeminin (depicted as light gray spheres) is superimposed to the crystal structure of *h*tCdt1:*h*tGeminin heterohexamer (Cα trace colored as in Fig. 1). (*B* and *D*) The same as (*A*) and (*B*) but for *h*tCdt1:*h*vtGeminin.

90 ± 10 Å, respectively) were significantly smaller than those for *h*tCdt1:*h*tGem and are compatible with the values calculated for the complex heterotrimeric conformation (Table S2). Furthermore, the computed curve from the heterotrimer fits the experimental data from the deletion mutant much better than the heterohexamer (χ = 1.9 and 4.5 respectively; Fig. 3*C*). Finally, low-resolution shapes generated from the SAXS data ab initio (31) also validate the above observations (Fig. 3*D*). Taken together, these findings suggest that heterohexamer formation is dependent on the tertiary interface formed by Geminin residues 145–160.

Structurally Important Geminin Residues 145–160 Affect Licensing Inhibition. Next, we used Geminin mutants to determine whether the tertiary interface, and consequently heterohexamer formation, was likely to be important for inhibition of Cdt1 activity. If full inhibition of Cdt1 is dependent on formation of the heterohexamer, then amino acids from the tertiary interface, either from Geminin or Cdt1, should affect the degree of inhibition. If, in contrast, Geminin's ability to inhibit Cdt1 is purely dependent on steric hindrance with unresolved regions of Cdt1 in the heterotrimer [as originally suggested by Lee *et al.* (27)], then only the length of Geminin's coiled-coil, but not its sequence, should matter.

To test the function of Geminin variants we measured their ability to inhibit licensing of sperm DNA in a *Xenopus* egg extract. We first compared the inhibitory activity of *h*tGem (which can support heterohexamer formation together with Cdt1) with that of *h*vtGem (which cannot support heterohexamer formation). As expected, *h*tGem was able to inhibit licensing over a wide range of concentrations (Fig. 4*A*); in contrast, *h*vtGem could not inhibit licensing activity even at the highest concentrations (Fig. 4*A*). We next constructed chimaeras of Geminin in which the C-terminal

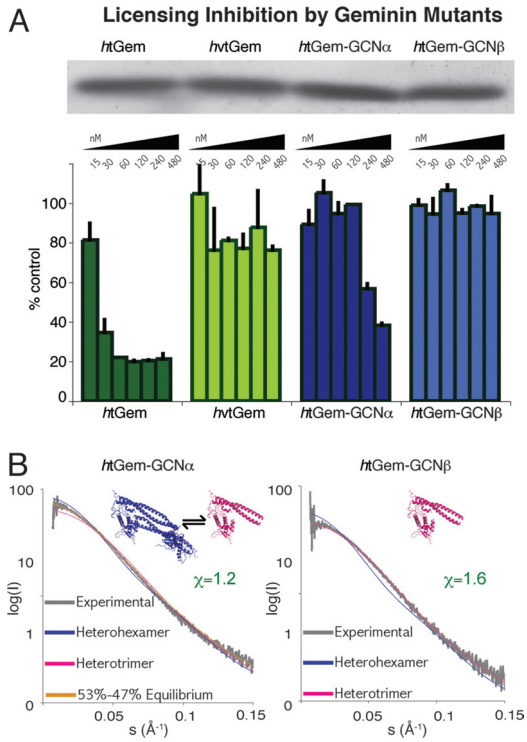

Fig. 4. Correlation between Geminin mutants activity and quaternary arrangement of the Cdt1:Geminin complex. (*A*) *h*tGem, *h*vtGem, *h*tGem-GCN4α, and *h*tGem-GCN4β were tested for their ability to inhibit DNA replication in *Xenopus* egg extracts. For each construct, 6 concentrations were tested: 480, 240, 120, 60, 30, and 15 nM; a Coomassie blue-stained gel was used for loading control and is shown. For the graph, 100% replication was adjusted based on buffer-treated extracts, and error bars represent standard errors of the mean. (*B*) The experimental SAXS profile of *h*tCdt1:*h*tGem-GCN4α (*Left*) and *h*tCdt1:*h*tGem-GCN4β (*Right*) are compared with the theoretical scattering curves calculated from the heterohexamer and heterotrimer. Only the low-angle regions are shown for clarity; the coloring scheme is as in Fig. 3 *A* and *D*.

coiled-coil residues 139–150 were substituted either by the 12-residue helical linker used by Lee *et al.* (27) (residues 251–262 of yeast GCN4, giving a construct we call *h*tGem-GCN4α) or by the adjacent heptad repeat (residues 264–275 of yeast GCN4, giving a construct we call *h*tGem-GCN4β). These two constructs should have identical length and helical properties (Fig. S3 *a* and *b*). They are also fairly similar to the corresponding Geminin coiled-coil (Fig. S3 *c* and *d*), although they appear more well-packed and robust compared with Geminin; thus if the Lee *et al.* (27) hypothesis for the importance of rigidity outside sequence context is correct, they should both result in Geminin with similar—or more potent—inhibitory properties. However, though *h*tGem-GCN4α was inhibitory only at higher concentrations albeit not as potent as *h*tGem, its sister chimera *h*tGem-GCN4β showed no detectable inhibitory activity (Fig. 4*A*). When the same four constructs as regard to the C-terminal variations, but harboring N-termini extending to residue 28 (right after the Geminin destruction box, *h*ΔDB-tGem, *h*ΔDB-vtGem, *h*ΔDB-tGem-GCN4α, *h*ΔDB-tGem-GCN4β), were tested in the same assay, they showed a similar trend (Fig. S4). This argues that rigidity and length of the coiled-coil are not sufficient to specify Geminin functionality, and additional factors, such as sequence context and orientation, must be taken into account.

Although there is no observed interaction between the mutated Geminin residues and the Cdt1 regions involved in the first two interfaces, it could be argued that the differences in function could merely be a result of Geminin's variants inability to interact with Cdt1 via the primary and secondary interfaces. To rule out this possibility, we measured the affinity of all Geminin versions used in this study to Cdt1, in a surface plasmon resonance experiment. A truncated variant of Cdt1 slightly larger than *h*tCdt1, spanning residues 158–396, was immobilized and the different Geminin versions were used as analytes. Because Cdt1 is immobilized, no heterohexamers can form; Geminin can interact with Cdt1 only by the primary and secondary interfaces. The observed differences were small; k_D's were the same within standard error, and differences were less than 2-fold for all constructs (Fig. S5). Thus, the binding affinity between Cdt1 and Geminin mutants cannot account for the observed differences in activity. We have also checked the ability of *h*ΔDB-tGem, *h*ΔDB-vtGem, *h*ΔDB-tGem-GCN4α, and *h*ΔDB-tGem-GCN4β to interact with the full-length Cdt1 and form a complex in *Xenopus* egg extracts. We were able to pull down all these four versions of Geminin by a His-tagged Cdt1, with similar efficiencies, indicating similar interaction properties (Fig. S6). It must also be noted that *h*ΔDB-tGem and *h*tGem, where the same C terminus is now compared to N-terminal variations, exhibit similar licensing activity (Fig. S7) and nearly identical binding affinities to Cdt1 (Fig. S5). This suggests that extreme N- or C-terminal regions of Geminin do not significantly affect the high-affinity interaction between Geminin and Cdt1, which is mediated by the primary and secondary interfaces in the trimer.

Next, we wanted to show if the function of the sister chimeras is correlated with their propensity for heterohexamer formation.

The Function of Geminin Mutants Correlates with the Quaternary Structure of the Cdt1:Geminin Complex. For that reason, we created complexes between *h*tCdt1 and the two Geminin fusion mutants used for the experiments above (*h*tGem-GCN4α and *h*tGem-GCN4β) and measured them in a SAXS experiment at concentrations of 1, 2, and 4.7 mg/mL for *h*tCdt1:*h*tGem-GCN4α, and 1, 2, and 3.1 mg/mL for *h*tCdt1:*h*tGem-GCN4β; only the 2 mg/mL curve was used for the analysis (Fig. 4*B*). The *h*tCdt1:*h*tGem-GCN4β complex, harboring the Geminin chimera that could not inhibit licensing in the *Xenopus* assay, appears as a clear trimer, similar to the noninhibitory, trimeric, but shorter in Geminin length, *h*tCdt1:*h*vtGem. The *h*tCdt1:*h*tGem-GCN4α complex, which harbors a Geminin version that inhibits licensing with low efficiency compared with the hexameric *h*tCdt1:*h*tGem, did not fit well the SAXS data either as a trimer or a hexamer, but did fit well as an almost 50/50 equilibrium between the two forms, fully consistent with its "intermediate" functional profile.

Consistent with this interpretation, we also show that full-length (DDB) Geminin, which forms hexamers with a lower affinity than *h*tGem (see above), also has a reduced ability to inhibit Cdt1 activity (Fig. S7).

These findings show that Geminin residues 145–160 affect licensing inhibition and that the ability of Geminin to mediate formation of a heterohexamer with Cdt1 correlates with its ability to inhibit the licensing activity of Cdt1.

Cdt1 Residues Buried in the Cdt1-Geminin Hexamer Are Important for Licensing. Should heterohexamer formation be essential for full inhibition of Cdt1 by Geminin, Cdt1 residues buried in the tertiary Geminin–Cdt1 interface that is formed upon hexamerization are likely to be of functional importance for Cdt1. To corroborate this notion further, we mutated residues in the Cdt1 tertiary interface region (198–228) and analyzed their function. A human wtCdt1 fragment spanning residues 158–546, and tertiary interface mutants, were tested for their ability to restore licensing activity to a *Xenopus* egg extract immunodepleted of Cdt1. Two mutants, the evolutionary conserved R210A and R198A/R210A, showed severely impaired DNA licensing activity (Fig. 5*A*), indicating a direct role of these residues in Cdt1 function. Interestingly, mutations at residues R210 and R198/R210, which had a clear impaired biological function, destabilized the heterohexamer conformation as indicated by analytical size-exclusion chromatography (Fig. S8).

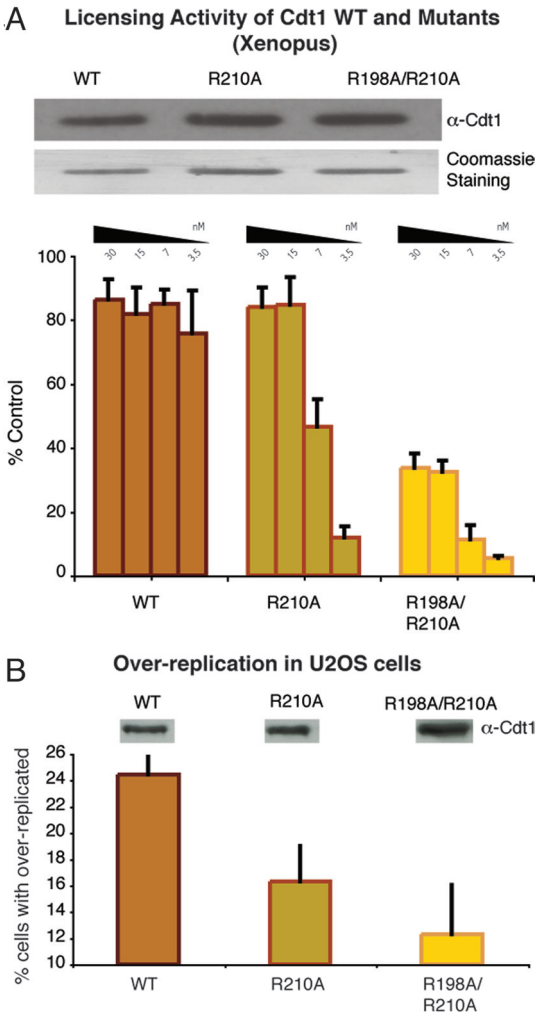

Fig. 5. Functional significance of tertiary interface Cdt1 mutants. (*A*) WT human Cdt1 fragment 158–546 and 2 mutants (R210A and R198A/R210A) were tested for their ability to rescue DNA replication in Cdt1-depleted *Xenopus* egg extracts. For each construct, four concentrations were tested: 30, 15, 7, and 3.5 nM. One hundred percent represents replication in non–immune-depleted extracts. Error bars represent standard errors of the mean. Equal loading of Cdt1 constructs was verified by Coomassie staining and Western blot analysis with anti-His antibody. (*B*) Rereplication in U2OS cells with reduced cyclin A activity is different when wtCdt1 or the 2 interface mutants (R210A, R198A/R210) are overexpressed. The panel reports the mean and the standard error of the mean of 4 independent experiments; the baseline 10%, representing the mean amount of cells with rereplicated DNA in cells with reduced cyclin A.

Our findings therefore suggest that regions in both Geminin and Cdt1 that form the tertiary interface are important for both biological activity and heterohexamer formation.

Validation in Mammalian Cell Lines. In animal cells, overexpression of Cdt1 induces DNA re-replication (19, 20, 32–35), DNA fragmentation (36), chromosomal instability (37), and ATM/ATR-dependent checkpoint activation (20, 36). We therefore tested in U2OS cells wtCdt1 and the same two inactive mutants as in the *Xenopus* experiments for their ability to induce DNA rereplication, chromosomal damage, and checkpoint activation. Transient overexpression of wtCdt1 in U2OS cells induces a strong inhibition of cell-cycle progression, with a prominent G2 arrest as measured by FACS analysis (Fig. S9). The G2 arrest indicates that wtCdt1 overexpression caused activation of the ATM DNA damage checkpoint pathways, as expected in the presence of functional p53 (32, 37). The two mutants displayed a reduced ability to block cell-cycle progression and to induce such a checkpoint response (Fig. S9), suggesting an impairment of their DNA licensing function, consistent with their behavior in the *Xenopus* licensing assay. Because overexpression of wtCdt1 in U2OS cells did not produce DNA rereplication, we opted to test the effect of Cdt1 deregulation in a silenced Cyclin A background (38). Consistent with all assays above, we could show that wtCdt1 overexpression induced overreplication in U2OS cells, whereas the mutants induced rereplication to a significantly lesser extent (Fig. 5*B* and Fig. S10).

Thus we conclude that arginines 198 and 210 of Cdt1 have an important role in licensing.

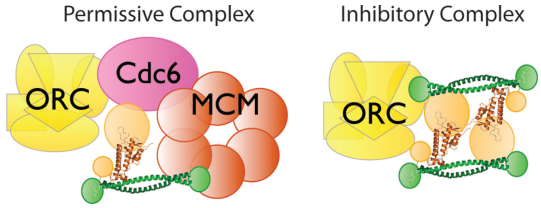

Fig. 6. Proposed model for the mechanism of DNA licensing inhibition by Geminin.

Discussion

Our findings show that the Geminin C-terminal coiled-coil residues 145–160 mediate head-to-tail dimerization of *h*tCdt1:*h*tGem heterotrimers and suggest this is important for the ability of Geminin to inhibit Cdt1 activity.

The Geminin residues involved in the tertiary interface were already previously shown to be important for function: truncation mutants of human Geminin missing residues 145–160 are unable to interfere with DNA synthesis (29), and in *Xenopus*, residues 140–160 (corresponding to residues 132–152 in the human protein) are essential to inhibit chromatin replication (10). Our additional experiments in *Xenopus*, using *h*tGem, *h*vtGem, and the two chimera coiled-coil graft proteins *h*tGem-GCN4α and *h*tGem-GCN4β, further support the functional role of these Geminin residues, but also correlate them with the quaternary structure of the Cdt1:Geminin complex. Lee *et al.* (27) showed that a chimerical Geminin protein with a portion of the coiled-coil replaced by a sequence from GCN4 (*h*tGem-GCN4α) still inhibited licensing, suggesting that the length of the coiled-coil was central, not its exact sequence. However, we show here that this construct is in fact a relatively poor inhibitor of Cdt1, and that a graft of identical sequence length (*h*tGem-GCN4β) almost abolishes Geminin's ability to inhibit Cdt1. The ability of Geminin to inhibit licensing in these chimeras correlates with the propensity of the chimera complexes to form a heterohexamer or a heterotrimer, but not with the ability of the chimerical Geminin to bind Cdt1 at the primary and secondary interfaces.

Moreover, upon heterohexamer formation, Geminin shields some Cdt1 residues. We have shown that at least two of these residues—namely, the evolutionary conserved arginines 198 and 210—are important for licensing. Previous experiments support our findings: residues 243–311 in *Xenopus* (corresponding to 167–236 in the human protein) were shown to be crucial for MCM loading and Cdt1 licensing activity (39). The established in vivo functionality of residues that participate in the tertiary interface, both in Geminin and in Cdt1, argue that hexamer formation is critical for full inhibition of Cdt1 by Geminin.

Based on our data, we propose a molecular model for Geminin activity that involves an equilibrium between a heterotrimer and a heterohexamer, whose relative abundance is regulated during the cell cycle (Fig. 6). Because the tertiary interface appears relatively weak in vitro, when compared with the tight primary and secondary

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interfaces, it offers a good candidate for inhibition in a more flexible (analog) manner than that of a simple (digital) steric hindrance mechanism. The heterohexamer would then represent an inhibitory complex, which prohibits DNA licensing, with the residues in the tertiary interface of Cdt1 hidden and unable to engage in replication licensing. The heterotrimer, where the same Cdt1 residues are exposed and free to engage in replication licensing (by promoting MCM chromatin association) would represent a permissive complex that allows licensing even in the presence of Geminin. The Cdt1-Geminin complex thus likely functions as a conformational switch, where the equilibrium between defined quaternary states regulates activity. This notion is also conceptually in line with the proposal by Mechali and coworkers (25) that a licensing switch exists, represented by an active form (A-complex), able to promote controlled licensing, and an inactive one (I-complex) that blocks further licensing activity during S, G2, and M phases.

The regulation of the suggested equilibrium between heterotrimer and heterohexamer during the cell cycle poses an intriguing question. The formation of the inhibitory (heterohexamer) complex could be explained by the increase of local concentration of Cdt1 and Geminin, e.g., mediated by Cdt1:Geminin recruitment to chromatin (14, 15). It is unfortunately hard to associate quantitatively our in vitro observations to the concentrations used in the *Xenopus* experiments: Many additional events occur in the egg extract, the mechanism of Cdt1-mediated MCM loading, necessary for licensing to occur, is dependent on many factors other than the affinity of Cdt1:Geminin hexamer formation. The disassembly of the inhibitory complex and its conversion to the permissive heterotrimer would require a different hypothesis. A good candidate is posttranslational modifications, which could regulate the equilibrium toward a heterotrimer. Interestingly, in mitotic cell extracts, at a stage when Cdt1 has to engage in replication licensing, both Cdt1 and Geminin are present as slower migrating forms, as a result of hyperphosphorylation (8, 26), and Geminin is inactivated upon exit from metaphase through a CDK- and ubiquitination-dependent mechanism (17). One could speculate that at mitotic exit phosphorylation and/or ubiquitination of the inhibitory heterohexamer promotes reformation of the permissive heterotrimer, as a requirement for triggering commitment to the next cell cycle.

Methods

Summary. The Cdt1$^{158-356}$:Geminin$^{82-160}$ complex was overproduced in *E. coli* and purified by two successive chromatography steps. Crystals of the complex were grown by free interface diffusion, and the structure of the Cdt1$^{158-356}$:Geminin$^{82-160}$ complex was determined by the MAD method using a selenomethionine substituted sample. The final model was refined to R_{work}/R_{free} of 24.0%/30.7% at 3.3 Å resolution (Table S1). SAXS data was processed with the ATSAS suite (40). Licensing assays in *Xenopus* egg extracts were performed as described in Ferenbach *et al.* (39). Further details for all assays are available as *SI Methods*.

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