during the response to specific changes in the extracellular environment. All of the known targets for these two activators were confirmed, and functional modules were discovered that are regulated directly by these factors.

Expression analysis with DNA microarrays allows investigators to identify changes in mRNA levels in living cells, but the inability to distinguish direct from indirect effects limits the interpretation of the data in terms of the genes that are controlled by specific regulatory factors. Genome-wide location analysis provides information on the binding sites at which proteins reside through the genome under various conditions in vivo, and will prove to be a powerful tool for further discovery of global regulatory networks.

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Inhibition of Eukaryotic DNA Replication by Geminin Binding to Cdt1

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In all eukaryotic organisms, inappropriate firing of replication origins during the G_2 phase of the cell cycle is suppressed by cyclin-dependent kinases. Multicellular eukaryotes contain a second putative inhibitor of re-replication called geminin. Geminin is believed to block binding of the mini-chromosome maintenance (MCM) complex to origins of replication, but the mechanism of this inhibition is unclear. Here we show that geminin interacts tightly with Cdt1, a recently identified replication initiation factor necessary for MCM loading. The inhibition of DNA replication by geminin that is observed in cell-free DNA replication extracts is reversed by the addition of excess Cdt1. In the normal cell cycle, Cdt1 is present only in G_1 and S, whereas geminin is present in S and G_2 phases of the cell cycle. Together, these results suggest that geminin inhibits inappropriate origin firing by targeting Cdt1.

Eukaryotic cells maintain a tight control on the initiation of DNA replication to ensure that chromosome duplication in S phase and chromosome segregation in M phase strictly alternate with each other so that daughter cells do not receive too many or too few chromosomes. Fusion of cells in the G_2 phase of the cell cycle to those in S phase revealed that the G_2 nuclei were unable to replicate their DNA, although conditions in the heterokaryon were permissive for replication in the S phase nuclei (*1*). This observation led to the hypothesis that G_2 nuclei lack a factor essential for replication initiation and/or possess a negative factor that prevents replication initiation. For the initiation of DNA replication in eukaryotes, the origin recognition complex (ORC) binds to initiator elements and recruits the CDC6 and Cdt1 proteins to chromatin in the G_1 phase of the cell cycle. The latter, in turn, are required to recruit the MCM complex to form the pre-replication complex (pre-RC). After origin firing at the beginning of S phase, the pre-RC is dissociated and is not re-assembled until the cell passes through mitosis into the G_1 phase of the next cell-cycle. As factors essential for replication initiation were discovered, it became clear that some of them become limiting in G_2 nuclei (*2*, *3*). In particular, CDC6 is exported out of the nucleus (or proteolysed in yeast) as S phase proceeds, while the MCM proteins are phosphorylated and become dissociated from the chromatin (or exported out of the nucleus in yeast). In addition, the elevated activity of cyclin-dependent kinase (cdk) in the G_2-M phase of the cell cycle blocks re-replication by preventing assembly of pre-RC even when initiation factors such as CDC6 are artificially expressed or stabilized in G_2 nuclei (*4–6*). Therefore, the absence of initiation factors and the presence of negative factors prevent a second round of origin firing until cells pass through mitosis.

Geminin was identified in a screen for proteins that are degraded in mitotic extracts from *Xenopus* eggs and was shown to inhibit DNA replication by preventing the loading of MCM complex on chromatin (*7*). The target of geminin in this inhibitory reaction was unclear, but its expression in S and G_2 led to the suggestion that it provides an additional mechanism to prevent re-replication in the normal cell cycle. To identify the target of geminin, we raised antibodies to bacterially expressed His6-geminin (Fig. 1A) (*8*). Immunoblotting of extracts from 293T cells with this antibody recognized the 33-kD geminin protein in cell extracts (Fig. 1A). Immunoprecipitation of ^{35}S-methionine labeled cell extracts indicated that the 33-kD geminin protein co-immunoprecipitated with cellular proteins of 65 and 130 kD (Fig. 1B) (*9*). Re-immunoprecipitation with antibody to geminin under denaturing conditions precipitated geminin but not the 65- and 130-kD polypeptides (Fig. 1B). These results suggest that the 65- and 130-kD polypeptides were present in the native anti-geminin immunopre-

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cipitate because of their association with geminin and that these polypeptides could be the target of geminin action.

A new replication initiation factor, Cdt1 or double-parked, was recently identified as being essential for DNA replication in *Schizosaccharomyces pombe*, *Xenopus laevis*, and *Drosophila melanogaster* (*10*–*12*). Inactivation of the gene or immunodepletion of the protein from cell extracts results in failure to recruit the MCM complex on chromatin despite the loading of ORC and CDC6. Because pre-RC formation was blocked at the step of MCM loading either by depletion of Cdt1 or by addition of geminin, we hypothesized that geminin interacts with and inhibits Cdt1. However, the published sequence of human Cdt1 predicted a protein of about 45 kD (*12*) that was not present in the geminin immunoprecipitate. Rescreening the expressed sequence tag (EST) database for homology to the *Xenopus* Cdt1 gene identified a cDNA clone for human Cdt1 that was extended at the NH_2-terminus by 145 amino acids and coded for a protein of about 65 kD (*13*). The GenBank accession number for this sequence is AF321125. With the new addition, the human Cdt1 protein is similar to *Xenopus* Cdt1 all the way to the NH_2-terminus (Fig. 2A) (46% identity and 60% similarity). An antibody raised against bacterially expressed protein containing amino acids 238 to 546 of Cdt1 recognized a single protein with a molecular mass of 65 kD upon immunoblotting of 293T cell extracts (Fig. 2B) (*14*). The size of the protein corresponds to the predicted size of Cdt1 with the

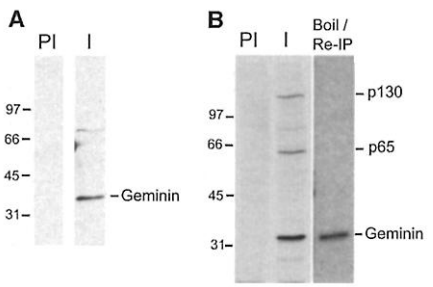
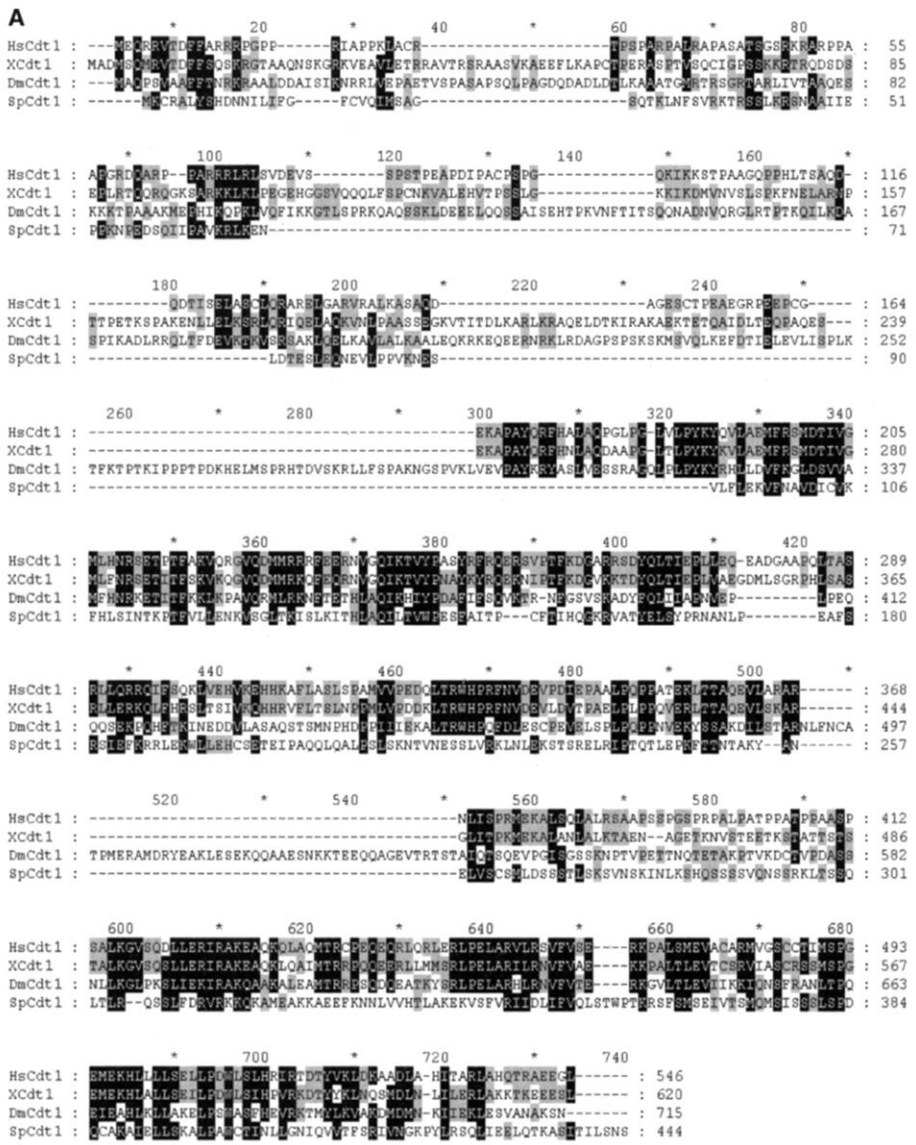


Fig. 1. Human geminin is associated with proteins of 65 and 130 kD. (**A**) Immunoblotting of cell lysates with antibody to geminin (I) recognizes a 33-kD protein while the preimmune sera does not (PI). (**B**) Immunoprecipitation of ^{35}S-methionine labeled cell extracts with antibody to geminin and its corresponding preimmune sera followed by fluorography of the immunoprecipitate. The anti-geminin immunoprecipitate was boiled in 1% SDS, diluted 10-fold, and reimmunoprecipitated with antibodies to geminin to show that the antibody recognized only the 33-kD geminin protein under denaturing conditions.



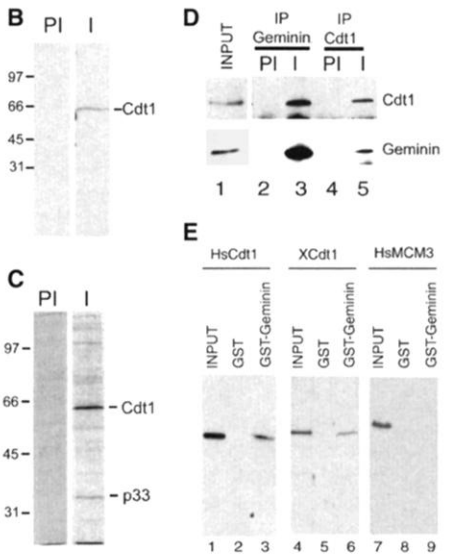


Fig. 2. Human Cdt1 protein is a 65-kD protein that coimmunoprecipitates with geminin. (**A**) Alignment of the complete human Cdt1 sequence with that of *Xenopus*, *Drosophila*, and *S. pombe* Cdt1 homologs. Human Cdt1 from amino acid residues 145 to 546 has been published (*12*). (**B**) Antibody to human Cdt1 (I) recognizes a 65-kD protein upon immunoblotting of 293T cell lysates whereas the preimmune control (PI) does not. (**C**) Immunoprecipitation of ^{35}S-methionine labeled cell extracts with antibody to Cdt1 (I) and the corresponding preimmune sera (PI) followed by fluorography of the immunoprecipitate. (**D**) Immunoprecipitation of 293T cell lysates with either antibodies to geminin or Cdt1 followed by immunoblotting with antibodies to Cdt1 and geminin. (**E**) GST-geminin specifically associates with human and *Xenopus* Cdt1 but not human MCM3. Cdt1 or MCM proteins were produced by in vitro transcription and translation and were tested for their ability to bind either GST or GST-geminin in a pull-down assay on glutathione agarose beads. For each set, the input lane contains 5% of the amount of labeled protein incubated with GST or GST-geminin. The labeled proteins were visualized by SDS-PAGE and fluorography.

NH_2-terminal extension reported here. Immunoprecipitation of ^{35}S-methionine–labeled cell extracts revealed that the 65-kD Cdt1 protein was coimmunoprecipitated with a 33-kD cellular protein (Fig. 2C) (*9*).

The presence of a 33-kD polypeptide in the Cdt1 immunoprecipitate and a 65-kD polypeptide in the geminin immunoprecipitate led us to test whether geminin (33 kD) and Cdt1 (65 kD) associate with each other in extracts of asynchronously growing cells. Immunoprecipitation of cellular proteins with antibody to geminin followed by immunoblotting of the precipitate with antibody to Cdt1 revealed that Cdt1 was present in the geminin immunoprecipitate (Fig. 2D, lane 3). Conversely, immunoprecipitation of cell extracts with antibody to Cdt1 and immunoblotting the precipitate with antibody to geminin revealed that geminin was present in the Cdt1 immunoprecipitate (Fig. 2D, lane 5). The ratio of geminin to Cdt1 in the Cdt1 immunoprecipitate (lane 5) is similar to their ratio in the input cell extract (lane 1), arguing for a very efficient interaction between geminin and Cdt1 in extracts of 293T cells. The association of Cdt1 and geminin could also be reproduced in vitro. Glutathione *S*-transferase (GST)–geminin purified from bacteria specifically associated with full-length human and *Xenopus* Cdt1 produced in rabbit reticulocyte lysates by in vitro transcription and translation but not to similarly produced human MCM3 (Fig. 2E) (*15*).

Because the geminin-Cdt1 interaction could be re-created in vitro, we turned to an in vitro system of DNA replication to test whether geminin inhibits replication initiation by targeting Cdt1 (*16*). In this system, sperm chromatin is incubated in cytosolic egg extract to assemble pre-RCs. The pre-RCs are then induced to initiate replication through the addition of a nucleoplasmic extract (NPE). Because replication in this system typically uses only *Xenopus* replication factors, it was first necessary to show that human geminin and human Cdt1 could function in this system. In Fig. 3A, we show that bacterially expressed GST-geminin but not GST alone interacts with XCdt1 from egg cytosol. In Fig. 3B, we show that human GST-Cdt1 is capable of associating with sperm chromatin when added to *Xenopus* extracts (*16*). To examine geminin's ability to inhibit DNA replication through Cdt1, sperm chromatin was incubated with cytosolic egg extract that was supplemented with geminin (*8*), Cdt1 (*17*), or both, and the proteins that associated with the chromatin were examined by immunoblotting. As reported previously for *Xenopus* geminin (*7*), recombinant human geminin disrupted pre-RC assembly by inhibiting the loading of XMCM4 onto chromatin without affecting the loading of XORC2 (Fig. 3C, lane 4) (*16*). Strikingly, when recombinant human Cdt1 was added with the geminin, the binding of MCM to chromatin was restored (Fig. 3C, lane 5). To ask whether addition of human Cdt1 rescues formation of functional pre-RCs, the pre-RCs assembled in Fig. 3C were mixed with nucleoplasmic extract, and replication was measured. As expected from previous results (*7*), pre-RCs assembled in the presence of geminin did not support DNA replication (Fig. 3D, lanes 3 and 7) (*16*). However, when human Cdt1 was also present during pre-RC formation, replication was restored (Fig. 3D, lanes 4 and 8). Therefore, the inhibition of pre-RC formation and DNA replication by geminin is counteracted by the presence of excess Cdt1. Together with the physical interaction of geminin and Cdt1 (Figs. 2 and 3A), these results strongly suggest that geminin blocks DNA replication by targeting Cdt1.

If geminin targets Cdt1 to inhibit re-replication, it is expected to be expressed soon after Cdt1 function is executed in the cell cycle. Pre-RCs are assembled in G_1 and are dissociated soon after origin firing in S phase, providing a finite window of time during which Cdt1 has to be active (*18*, *19*). HeLa cells were released from a mitotic cell cycle block and were harvested at 3-hour intervals as they progressed synchronously through G_1 and S, and extracts were blotted for cyclin A, Orc2, Cdt1, and geminin (Fig. 4) (*20*). The time of expression of cyclin A suggests that S phase began about 12 to 15 hours after release from the nocodazole block. Cdt1 protein was detected in G_1 and early S, consistent with its role in pre-RC formation, whereas its expression dropped off in G_2. The absence of Cdt1 in G_2 nuclei is, therefore, another mechanism by which the cell prevents re-replication. Conversely, geminin appears in S phase and persists through G_2 into mitosis, disappearing as cells enter G_1. Cdt1 and geminin are coexpressed in S phase at about the time when geminin can inhibit Cdt1 function without impairing the normal replication initiation process. The excess of geminin in late S and G_2 suggests that even if Cdt1 were expressed in G_2 nuclei, geminin would act as a negative factor to prevent the reinitiation of DNA replication. Therefore, the pattern of expression of geminin in the cell cycle is consistent with it being a second negative factor in G_2 that prevents re-replication by targeting Cdt1.

In summary, our results identify Cdt1 as the target of geminin. The results also indicate that Cdt1 and geminin are separately regulated such that both the absence of Cdt1 in G_2 and the appearance of geminin in S help prevent re-replication of the chromosomes. Clearly, evo-

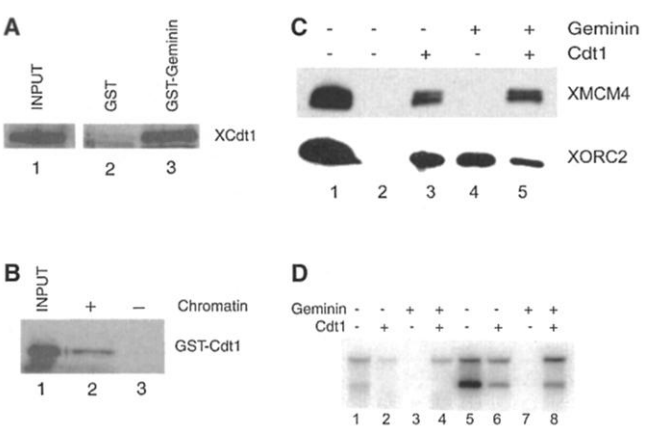


Fig. 3. Cdt1 reverses inhibition of DNA replication by geminin. (**A**) GST-geminin interacts with XCdt1 from *Xenopus* egg extracts. *Xenopus* egg extracts were incubated with either GST (lane 2) or GST-geminin (lane 3) in a pull-down assay on glutathione agarose beads and were immunoblotted with antibodies to XCdt1. Lane 1 shows XCdt1 in the *Xenopus* egg extract. (**B**) Human GST-Cdt1 associates with sperm chromatin in *Xenopus* extracts. After incubation of GST-Cdt1 with *Xenopus* egg extracts with (lane 2) or without (lane 3) sperm chromatin, the chromatin was isolated and immunoblotted with antibodies to Cdt1. Lane 1 shows GST-Cdt1 in the input. (**C**) After incubation in *Xenopus* egg extracts, the chromatin was isolated and recruitment of ORC and MCM to the chromatin was visualized by immunoblotting with the indicated antibodies. The replication reactions contained the following additions: buffer (lane 1), GST-Cdt1 (400 nM, lane 3), His6-geminin (100 nM, lane 4) and His6-geminin (100 nM) + GST-Cdt1 (400 nM) (lane 5). No sperm chromatin was added to the negative control reaction in lane 2. (**D**) DNA replication in *Xenopus* egg extracts with addition of buffer (mock, lanes 1 and 5), GST-Cdt1 (400 nM, lanes 2 and 6), His6-geminin (100 nM, lanes 3 and 7), and His6-geminin (100 nM) + GST-Cdt1 (400 nM) (lanes 4 and 8). Products of the replication reaction were separated on a 0.8% agarose gel and were visualized by autoradiography. Lanes 1 through 4 and 5 through 8 correspond to the replication reaction 30 and 60 min after addition of NPE, respectively.

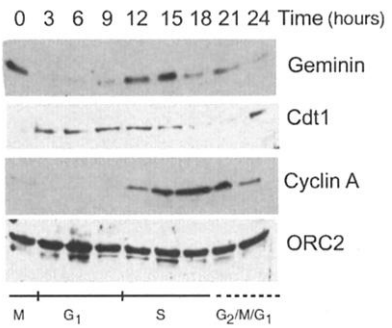


Fig. 4. Expression of Cdt1 and geminin proteins during the HeLa cell cycle. Cells released from a nocodazole block were harvested at the indicated time points, and the levels of Cdt1 and geminin were visualized with immunoblotting. Cyclin A (S phase specific) was used to estimate progression of the cells through the cell cycle. ORC2 protein levels were used as a loading control because ORC2 levels do not vary through the cell cycle.

lution has selected for multiple parallel pathways to ensure that a replicated chromosome does not erroneously reinitiate replication until after chromosome segregation is completed in mitosis. Cdks are believed to prevent replication initiation by phosphorylating CDC6 to target it either for proteolysis (in yeasts) or for nuclear export (in higher eukaryotes) (*6*, *21*–*23*). In addition, phosphorylation of MCM proteins by cdk is concordant with their dissociation from chromatin (*3*). However, in response to checkpoint activation, e.g., after DNA damage, cdk activity is repressed to block cell cycle progression until DNA repair is completed (*24*, *25*). The decrease in cdk activity could allow inappropriate pre-RC assembly on already fired origins, particularly because higher eukaryotes do not degrade CDC6 at the G_1-S transition. Therefore geminin, a second negative factor for DNA replication in S and G_2, may have evolved in higher eukaryotes to prevent re-replication in the event that cdk activity is lowered during a checkpoint response. It is also interesting that geminin targets a different initiator protein, Cdt1, in comparison to the CDC6 or MCM targeted by cdks. This diversity of targets prevents escape from re-replication control through mutation in the gene of a single target protein.

Note added in proof: While this manuscript was under review, we learned that Tada *et al.* have attained similar results (*27*).

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8. Full-length human geminin (amino acids 1 to 209) was polymerase chain reaction (PCR) amplified from IMAGE clone #727990 using primers containing a Nde I site in the 5′ primer and a Bam HI site in the 3′ primer. The PCR product was digested with Nde I and Bam HI and was ligated to pET14b (Novagen, Madison, WI) that had been cut with the same enzymes. This plasmid was transformed into BL21(DE3), and protein expression was induced by adding isopropyl-β-D-thiogalactopyranoside (IPTG) to a final concentration of 1 mM. The cells were lysed by sonication and His6-tagged geminin purified over a nickel–nitrilotriacetic acid (nickel-NTA agarose) column (Qiagen, Valencia, CA) according to the manufacturer's instructions. Fractions containing human geminin were pooled and dialyzed against a buffer composed of 50 mM tris, pH 8.0, 50 mM NaCl, and 10% glycerol. Rabbit antibodies against this protein were generated (Cocalico Biologicals, Reamstown, PA) and reacted to the recombinant His6- geminin.
9. For metabolic labeling with ^{35}S-methionine, 293T cells were incubated for 2 hours in media lacking methionine. 293T cells were then incubated for two additional hours in media containing Express ^{35}S Protein Labeling Mix (200 μCi) (NEN Biochemicals, Boston, MA). Extracts were prepared by lysing the cells in a NP-40 lysis buffer containing 50 mM tris, pH 8.0, 0.1% Nonidet P-40, 150 mM NaCl, 50 mM NaF, 1 mM Na_3VO_4, 5 mM EDTA, 2 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, pepstatin A (2 μg/ml), leupeptin (2 μg/ml), and aprotinin (5 μg/ml). Immunoprecipitations were done as described in (*26*), were washed five times in NP-40 lysis buffer, and were analyzed using SDS–polyacrylamide gel electrophoresis (SDS-PAGE) and fluorography.
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14. The plasmid expressing a fragment of Cdt1 (residues 238-546) was constructed by ligating the Msc I–Not I fragment of Image clone #24767 (AF070552) between the Not I and Bam HI sites of pET28a (Novagen). The protein was then expressed in the bacterial strain BL21(DE3), solubilized in 8M urea, and purified over a nickel-NTA agarose column as described by the manufacturer (Qiagen). Rabbits were immunized with this antigen (Cocalico Biologicals), and the antibody was shown to react specifically to His6-Cdt1 (238-546).
15. The plasmid expressing full-length GST-tagged geminin was constructed by PCR amplifying full-length geminin containing a 5′ Bam HI site and a 3′ Xho I site (restriction sites were contained in the primer) and the cloned into pGEX 5X-3 between the Bam HI and Xho I sites. The fusion protein was then expressed in bacteria and purified according to the manufacturer's instructions (Pharmacia Biotech, Piscataway, NJ). In vitro transcription and translation reactions were performed using the Promega TNT System (Madison, WI). Pull-down assays on glutathione agarose beads were done as described previously (*26*).
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17. Full-length human Cdt1 was PCR amplified from clone BE018212 using PCR primers containing a 5′ Eco RI site and a 3′ Not I site. This PCR product was cloned into pGEX 5X-1 between Eco RI and Not I and expressed in bacteria. The GST-fusion protein was purified according to the manufacturer's direction (Pharmacia).
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20. HeLa cells were synchronized in mitosis using nocodazole (40 ng/ml) for 24 hours and then were released into drug-free media. Whole cell extracts were prepared every 3 hours until 24 hours after release from the mitotic block. The extracts were then immunoblotted with antibodies to geminin (1:2000), Cdt1 (1:2000), cyclin A (1:2000), or ORC2 (1:2000).
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Distinct Roles for TBP and TBP-Like Factor in Early Embryonic Gene Transcription in *Xenopus*

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The TATA-binding protein (TBP) is believed to function as a key component of the general transcription machinery. We tested the role of TBP during the onset of embryonic transcription by antisense oligonucleotide–mediated turnover of maternal TBP messenger RNA. Embryos without detectable TBP initiated gastrulation but died before completing gastrulation. The expression of many genes transcribed by RNA polymerase II and III was reduced; however, some genes were transcribed with an efficiency identical to that of TBP-containing embryos. Using a similar antisense strategy, we found that the TBP-like factor TLF/TRF2 is essential for development past the mid-blastula stage. Because TBP and a TLF factor play complementary roles in embryonic development, our results indicate that although similar mechanistic roles exist in common, TBP and TLF function differentially to control transcription of specific genes.

The TATA-binding protein (TBP) is often considered an essential component of the general transcription machinery, being involved in transcription by all three eukaryotic RNA polymerases. TBP is essential in yeast, binds to a variety of TATA boxes, and is recruited to TATA-less promoters via protein-protein interactions [reviewed in (*1*)]. Recently, however, TBP-related factors have been identified in metazoans. *Drosophila* has two such proteins (TRF1 and TRF2). TRF1 plays a role in the transcription of *tudor* and