A key step in the initiation of eukaryotic DNA replication is
the binding of the activator protein Cdc45 to promote MCM
helicase unwinding of the origin template. We show here that
the c-myc origin DNA unwinding element-binding protein,
DUE-B, interacts in HeLa cells with the replication initiation
protein Treslin to allow Cdc45 loading onto chromatin. The
chromatin loading of DUE-B and Treslin are mutually depen-
dent, and the DUE-B–Treslin interaction is cell cycle–regulated
to peak as cells exit G1 phase prior to the initiation of replication.
The conserved C-terminal domain of DUE-B is required for its
binding to TopBP1, Treslin, Cdc45, and the MCM2-7 complex,
as well as for the efficient loading of Treslin, Cdc45, and TopBP1
on chromatin. These results suggest that DUE-B acts to identify
origins by MCM binding and serves as a node for replication
protein recruitment and Cdc45 transfer to the prereplication
complex.

The c-myc replication origin DNA unwinding element-binding
protein, DUE-B, was identified in a yeast one-hybrid screen
using the c-myc origin DNA-unwinding element (DUE)2 as bait
(1). The crystal structure of the DUE-B N-terminal 159–amino
acid domain closely resembles the structures of aminoacyl
tRNA deacylases conserved from bacteria to higher eukaryotes
and displays tRNA deacylase activity (1, 2). The remaining
50–amino acid C-terminal domain of DUE-B is unstructured
but is highly conserved between vertebrates. Reflecting its
bipartite nature linking translation and replication, in Xenopus
egg extracts DUE-B binds to the MCM2-7 complex and to mul-
tiple members of the aminoacyl tRNA synthetase complex (3).

An ectopic DUE derived from the (ATTCT)n, repeat of the
SCA10/ATXN10 locus (OMIM 603516) restored origin activity
to an inactivated c-myc replication origin that was mutated by
deletion of its DUE. In vivo, DUE-B and Cdc45 bound to the
endogenous c-myc DUE, to a spontaneously expanded SCA10/
ATXN10 DUE, and to the reactivated SCA10/c-myc origin
DUE at a single-copy ectopic locus (4). DUE-B is essential for
Cdc45 loading and replication of sperm chromatin in Xenopus
egg extracts and co-immunoprecipitated from these extracts
with TopBP1 and Cdc45. In egg extracts, mutation of the phos-
phorylation target sites in the unstructured C terminus of
DUE-B blocked Cdc45 loading and inhibited DNA replication
(5).

In Xenopus egg extracts, DUE-B binds to the heterohexam-
eric MCM complex. Cdc7-dependent DUE-B phosphorylation
decreased MCM binding, whereas PP2A-dependent dephos-
phorylation increased MCM binding (5). Immunodepletion of
DUE-B reduced chromatin binding of TopBP1, blocked the
loading of Cdc45, stopped origin unwinding (RPA loading), and
inhibited replication initiation (4). Consistent with the effects
of phosphorylation-dependent binding of DUE-B to MCMs,
asparte mutation of the DUE-B C-terminal phosphorylation
sites decreased MCM binding, whereas alanine mutation of
these sites increased MCM binding in vitro and in HeLa cells.
Alanine mutation of the DUE-B C terminus phosphorylation
sites also dominantly blocked Cdc45 loading and inhibited cell
division in vivo.

The replication protein Ticrr (TopBP1-interacting and rep-
llication regulator) was identified in a zebrafish cell-cycle
genetic screen (6) and shown to be the same as Treslin, initially
defined as a TopBP1-interacting protein in nuclei formed in
Xenopus egg extracts and required for DNA replication (7).
Treslin/Ticrr (hereafter referred to as Treslin) is considered to
be a vertebrate ortholog of the yeast Sld3 protein. Treslin is
required for CMG (Cdc45–MCM–GINS) assembly (8, 9) and
interacts with the first two BRCT repeats of the human Dpb11
ortholog, TopBP1. Nevertheless, the limited amino acid
sequence homology between Treslin and Sld3 (8) raises the
possibility that not all Sld3 functions are conserved in Treslin.

Treslin and TopBP1 are required for Cdc45 loading onto
chromatin (10, 11). The binding of Treslin to chromatin in
Xenopus egg extracts occurs with similar timing as the binding
of TopBP1, although the binding of Treslin and TopBP1 are
independent of one another. Prior to the initiation of DNA
replication, Treslin associates with TopBP1 in a Cdk2-depend-
ent manner (12, 13) that is enhanced by the CDK stimulating
proteins CKS1 and CKS2 (14). The TopBP1–Treslin interac-
tion is necessary for Cdc45 loading, as well as for TopBP1 acti-
vation of the ATR/Chk1 checkpoint pathway (15).
Here we show that the C-terminal domain of DUE-B is required to interact with Treslin and that this interaction is essential for the loading of Treslin, DUE-B, and Cdc45 on chromatin. The binding of DUE-B to Treslin is cell cycle-dependent and peaks late in G1 phase, shortly before the initiation of DNA replication. This interaction is inhibited by replication stress. Similar to the situation in egg extracts, TopBP1 depletion does not affect Treslin binding to chromatin but blocks Cdc45 chromatin loading.

**Results**

*Sequential chromatin binding of DUE-B, Treslin, and Cdc45*

DUE-B binding to sperm chromatin in *Xenopus* egg extracts is preceded by TopBP1 binding and followed by Cdc45 binding (4). To examine the chronology of replication protein binding as cells enter S phase, HeLa cells were released from a nocodazole-induced mitotic block, and protein binding to chromatin was analyzed by Western blotting. TopBP1 and DUE-B were detectable on chromatin at T = 3 h (Fig. 1A) and increased during the M/G1 transition (T = 3–6 h). DUE-B and TopBP1 bound with similar kinetics slightly before Treslin and substantially before Cdc45 loading during G1/S (T = 9–12 h). These results are consistent with previous observations that DUE-B binds to sperm chromatin or plasmid DNA before Cdc45 or RPA in *Xenopus* egg extracts and that DUE-B immunodepletion blocks subsequent Cdc45 and RPA loading onto sperm chromatin (4).

**DUE-B and Treslin are required to load Cdc45 on chromatin**

The similarity in the time courses of DUE-B, Treslin, and TopBP1 binding to HeLa chromatin and the requirement for each of these proteins to load Cdc45 onto chromatin in egg extracts prompted us to ask whether Treslin and DUE-B cooperate in Cdc45 loading. As shown in Fig. 2A, siRNA knockdown of DUE-B blocked the loading of Treslin and Cdc45 onto HeLa chromatin; conversely, knockdown of Treslin inhibited the loading of DUE-B and Cdc45 (Fig. 2B). We note as well that knockdown of Treslin did not decrease the level of Cdc45 in whole cell extracts, but it decreased the amount of Cdc45 detected in soluble nuclear extracts. In contrast, knockdown of DUE-B did not substantially affect the amount of Cdc45 in the soluble nuclear extract.

The yeast ortholog of Treslin, Sld3, has been shown to bind to Cdc45 when the two proteins are overexpressed, if the interaction is stabilized by chemical cross-linking (16). Inasmuch as Treslin has also been reported to bind to Cdc45 in human cell extracts (13), the present results suggest that Treslin may interact with Cdc45 in the nucleoplasm independent of DUE-B binding, retaining Cdc45 in the nucleus.

**TopBP1 is required to load Cdc45, but not DUE-B or Treslin, onto chromatin**

TopBP1 and its yeast ortholog Dpb11 are scaffolding proteins involved in DNA replication and in the response to DNA damage (17–20). Immunodepletion of DUE-B from *Xenopus* egg extracts blocked Cdc45 loading and significantly reduced TopBP1 binding to sperm chromatin but did not completely eliminate TopBP1 binding (4). Because TopBP1, DUE-B, and Cdc45 can interact directly (4), we tested whether TopBP1 knockdown would also block Cdc45 loading. The results of Fig. 3 confirm that knockdown of TopBP1 blocks Cdc45 loading on chromatin. In contrast, neither Treslin loading nor DUE-B loading onto chromatin was affected by TopBP1 knockdown. These results are consistent with the observation that Treslin and TopB1 bind independently to chromatin (7) and further imply that TopBP1 binds to DUE-B after chromatin loading.

**Cellular levels of TopBP1 are stabilized by DUE-B expression**

During the S phase, TopBP1 binds to human Treslin that has been phosphorylated on S1000 by S-phase CDK2 (13, 21). This binding is required for Cdc45 loading on chromatin and for replication initiation, although the mechanism by which these proteins identify prospective replication initiation sites is not known. The binding of TopBP1 to CDK-phosphorylated Treslin is also necessary for activation of the ATR/pChk1 DNA damage response (15, 22). We therefore tested whether the
down-regulation of the chromatin binding of Treslin by DUE-B depletion would also inhibit the UV-induced DNA damage response.

HeLa cells were treated with DUE-B siRNA and exposed to increasing doses of UV light (Fig. 4). Surprisingly, knockdown of DUE-B significantly destabilized cytosolic TopBP1 (lanes 7–9). In control cells, the binding of TopBP1 to chromatin was not detectably affected by UV, as has been observed previously in HeLa cells (17). When the levels of TopBP1 were reduced by DUE-B knockdown, a small increase of TopBP1 in the cytosolic and chromatin fractions could still be detected with increasing UV dose (lanes 7–12). In parallel with the destabilization of TopBP1, the ATR/pChk1 response to DNA damage was substantially blunted. In line with the decrease in total cell TopBP1, we observed diminished recovery and re-entry into S phase after UV treatment of DUE-B–depleted cells (Fig. 4B). Similar effects on TopBP1 levels were observed following DUE-B knockdown in HaCaT cells (Fig. S1). Because DUE-B is required to maintain cellular levels of TopBP1, but DUE-B knockdown does not reduce nuclear Cdc45, it appears that the interaction of Treslin with DUE-B or TopBP1 is not necessary for the nucleoplasmic localization of Cdc45 but is necessary for the subsequent step of chromatin loading.

Complex formation between DUE-B and Treslin

DUE-B and Cdc45 are recruited selectively to the DUEs of the c-myc and SCA10 replication origins and to mutant c-myc origins reactivated by heterologous DNA unwinding elements (ATTCT)27 and (ATTCT)28 but not to inactive c-myc origins containing a DNA-unwinding element deletion or (ATTCT)8 or (ATTCT)13 DUE substitution. Additionally, DUE-B has been shown to interact with Cdc45 and TopBP1 (4). Because our data indicate that Treslin and DUE-B are mutually dependent for chromatin loading, we tested whether Treslin might also be part of a complex that loads DUE-B and Cdc45 onto chromatin (Fig. 5). Indeed, immunoprecipitation of FLAG-tagged DUE-B expressed in HeLa cells or His6-tagged DUE-B from HeLa A1 cells (4) co-precipitated endogenous Treslin. Conversely, epitope-tagged Treslin SF (23) co-precipitated with endogenous DUE-B and TopBP1. Co-precipitation of Treslin and DUE-B was insensitive to ethidium bromide concentrations that disrupt protein–DNA binding (50 μg/ml (24)) (Fig. 5D); hence DNA is not essential to enable the interaction between DUE-B and Treslin.

The N-terminal domain of DUE-B (amino acids 1–159) shows strong evolutionary conservation from bacteria to metazoans. Intriguingly, the C-terminal 50–amino acid domain of DUE-B is highly conserved only among metazoans (1) and has a disordered structure that is not visualized in the DUE-B crystal structure (2). The C-terminal domain of DUE-B is essential for Cdc45 loading in Xenopus egg extracts and onto HeLa chromatin (5), whereas the C terminus deletion mutant of DUE-B (ΔCT DUE-B) does not bind to Cdc45 or TopBP1 (4). Considered together, these observations suggested that the C terminus of DUE-B may also be required for the chromatin loading of Treslin and for the Treslin–DUE-B interaction. As shown in Fig. 6A, expression of an siRNA resistant form of ΔCT DUE-B could not rescue the chromatin loading of Treslin when WT DUE-B was knocked down. Moreover, ΔCT DUE-B was deficient in co-immunoprecipitation of Treslin (Fig. 6B), and Treslin could not pull down ΔCT DUE-B (Fig. 6C).
**Figure 4. Knockdown of DUE-B depletes cells of TopBP1 and inhibits the DNA damage response.** A, control and DUE-B–depleted HeLa cells were treated with increasing doses of UVC light and assayed for cytoplasmic and chromatin-bound protein content after 18 h. pChk1, Ser-345–phosphorylated Chk1. B, control and DUE-B–depleted HeLa cells were treated with increasing doses of UVC light and assayed by flow cytometry for cell-cycle recovery.

**Figure 5. Interaction of DUE-B and Treslin.** A, FLAG-tagged DUE-B was expressed in HeLa cells and immunoprecipitated with FLAG antibody beads, and the immunoprecipitates were assayed for DUE-B and Treslin pulldown. B, HeLa A1 cells stably express His-tagged DUE-B. HeLa and HeLa/A1 cell lysates were incubated with Ni–NTA beads, and the precipitates were analyzed as in A. C, FLAG-tagged Treslin was expressed in HeLa cells, and lysates were immunoprecipitated with FLAG antibody beads. The immunoprecipitates were assayed for DUE-B and Treslin pulldown. D, lysates from HeLa cells as in A were treated with ethidium bromide and immunoprecipitated with FLAG antibody beads. The immunoprecipitates were assayed for DUE-B and Treslin pulldown.
DUE-B binds avidly to the MCM2-7 complex (5), and this interaction is inhibited by Cdc7-dependent phosphorylation of the DUE-B C terminus. Alanine mutation of the C-terminal Cdc7 consensus phosphorylation sites had a dominant-negative effect on Cdc45 loading and DNA replication in Xenopus egg extracts and on Cdc45 loading and colony formation of HeLa cells (5). To test whether phosphorylation of the DUE-B C terminus affected the binding of Treslin, the C-terminal phosphorylation sites were mutated to alanine or aspartate (Fig. 6).

Although the ΔCT DUE-B mutant could not be pulled down by Treslin, both the S/T-A and S/T-D mutants efficiently interacted with Treslin. We conclude that the C terminus of DUE-B is essential for Treslin binding, but this interaction is not affected by DUE-B C-terminal phosphorylation.

The interaction between DUE-B and Treslin is cell cycle–dependent

The time-course similarity and interdependence of DUE-B and Treslin chromatin binding suggested that the interaction between DUE-B and Treslin could be subject to cell-cycle regulation. To test this possibility, we used HeLa T-Rex cells containing a doxycycline-inducible FLAG–DUE-B construct. FLAG–DUE-B was expressed during a nocodazole block and immunoprecipitated at time points following drug release. Overexpression of DUE-B did not detectably alter the cell cycle or mitotic block (Fig. 7A). However, co-precipitation of DUE-B and Treslin was maximal between 5 and 9 h after release of the block, during the G1/S-phase transition (Fig. 7B). These results indicate that the interaction between DUE-B and Treslin is cell cycle–regulated and that this interaction peaks in anticipation of replication origin firing.

The chromatin loading of DUE-B, Treslin, and Cdc45 is suppressed during replication stress

Activation of the ATR/Chk1 pathway during replication stress blocks CDK2 phosphorylation of Treslin and the interaction of Treslin with TopBP1, which is required for Cdc45 loading onto chromatin (12). To determine the effect of replication stress on the interaction of DUE-B with Treslin and TopBP1, the cells were treated with low doses of hydroxyurea or aphidicolin to slow replication fork progress without inducing fork collapse. The binding of DUE-B to Treslin or TopBP1 was

Figure 6. The C-terminal domain of DUE-B is essential for interaction with Treslin. A, DUE-B was knocked down by siRNA in HeLa cells, and Treslin loading on chromatin was assayed by Western blotting after siRNA-resistant His6-WT DUE-B or His6-ΔCT DUE-B was expressed. B, His6-WT DUE-B or His6-ΔCT DUE-B were stably expressed in HeLa A1 cells, and proteins pulled down by Ni–NTA beads were analyzed by Western blotting. C, WT DUE-B and ΔCT DUE-B, DUE-B S/T-A, and DUE-B S/T-D C-terminal mutants were expressed by doxycycline induction in HeLa T-Rex FLAG-Treslin cells. Proteins were pulled down with FLAG antibody beads and analyzed by Western blotting. *, DUE-B degradation products; note the decreased immunostaining with the loss of DUE-B C terminus residues.
DUE-B and Treslin interact to load Cdc45

Figure 7. Cell-cycle regulation of DUE-B interaction with Treslin. HeLa T-Rex FLAG–DUE-B cells were simultaneously induced with doxycycline. A, cells were released from both drugs and analyzed by flow cytometry. B, soluble cell lysates were immunoprecipitated at the indicated time points. FLAG–DUE-B was pulled down with FLAG antibody beads, and the co-immunoprecipitated proteins were analyzed by Western blotting. Note that FLAG–DUE-B is strongly expressed for the duration of the experiment after the removal of doxycycline.

decreased by treatment of cells with HU (Fig. 8A) or aphidicolin (Fig. 8C). In parallel, the chromatin loading of DUE-B, Treslin, and Cdc45 was also inhibited by these replication stressors (Fig. 8, B and D), although the limits of Western blotting quantitation preclude stating that the effects are quantitatively identical. In contrast, however, there was no detectable decrease in the binding of TopBP1 to chromatin. These data suggest that the loss of the DUE-B–Treslin interaction during replication stress is involved in the decreased loading of Cdc45.

Discussion

The loading of Cdc45 to the pre-RC is a decisive step to initiate unwinding by the eukaryotic replicative MCM helicase (25). In Saccharomyces cerevisiae Cdc45 loading requires Sld3 binding to the scaffolding protein Dpb11, the ortholog of human TopBP1. In humans, the CDK-dependent interaction of TopBP1 with Treslin is a conserved requirement for Cdc45 loading. Also conserved is the inhibition of Treslin–TopBP1 binding to replication origins bound by Treslin and Cdc45, whereas DUE-B–independent binding of TopBP1 to chromatin is more dispersed. In this model, DUE-B–dependent binding may target Treslin–TopBP1 interaction for Cdc45 loading (7), we find that knockdown of TopBP1 blocks Cdc45 loading onto chromatin but does not interfere with DUE-B loading. In contrast, immunodepletion of DUE-B from Xenopus egg extracts blocked Cdc45 loading and significantly but incompletely reduced TopBP1 binding to sperm chromatin (4). The current results imply that DUE-B knockdown strongly destabilizes the total cellular levels of TopBP1. However, these results in conjunction with those in Xenopus egg extracts suggest that TopBP1 may bind to chromatin in both DUE-B–dependent and independent means. In line with the requirement for TopBP1 activation of the ATR/Chk1 pathway, DUE-B knockdown also blunts Chk1 phosphorylation and the S-phase DNA damage checkpoint in response to UV light.

We speculate that DUE-B–dependent binding may target TopBP1 to replication origins bound by Treslin and Cdc45, whereas DUE-B–independent binding of TopBP1 to chromatin is more dispersed. In this model, DUE-B binding to MCM2-7 double hexamers localized at sites where the DUE of chromatinized DNA is susceptible to distortion (25–27) would serve to recruit TopBP1 and Treslin–Cdc45 to the initiation site.

We have previously shown that DUE-B, TopBP1, and Cdc45 interact physically and functionally. In the Xenopus egg extract system, DUE-B depletion did not alter the levels of TopBP1, although the loading of Cdc45 and TopBP1 onto sperm chromatin was inhibited (4). Here, we have shown that DUE-B can be co-immunoprecipitated with TopBP1 and Treslin from soluble cell extracts. The interaction between DUE-B and Treslin is cell cycle–dependent and peaks in parallel with the entry of cells into S phase. We note as well that although TopBP1 and Treslin bind to DUE-B, the time course of TopBP1 binding to chromatin and of the DUE–Treslin interaction differ. In view of the independent binding of Treslin and TopBP1 to chroma-
tin, these results suggest that the interactions of DUE-B, Treslin, TopBP1, and Cdc45 are temporally separable. The co-immunoprecipitation of DUE-B and Treslin depends on the 59–amino acid C-terminal tail of DUE-B that is unique to vertebrates. This region is also required for the interaction of DUE-B with TopBP1 and Cdc45 (4). Remarkably, this relatively short C-terminal domain is also necessary for binding of DUE-B to the MCM complex in soluble *Xenopus* egg extracts, and this binding is inhibited by Cdc7-dependent phosphorylation (5). Mutation of the C-terminal serine and threonine residues to alanine blocked Cdc45 loading and origin unwinding in DUE-B–depleted egg extracts and HeLa cells and dramatically enhanced DUE-B binding to MCMs.

The DUE-B C-terminal alanine mutant exerted a dominant-negative effect on cell proliferation (5). In contrast, the DUE-B C-terminal aspartate mutant, which does not bind to MCMs, did not have a detectable effect on proliferation in the presence of WT DUE-B. We propose, therefore, that the DUE-B C-terminal domain binds to MCMs and acts as a replication origin landing site for Treslin, TopBP1, and Cdc45. Crystallography of DUE-B showed that the C-terminal domain of DUE-B is unstructured; however, the dimerization of DUE-B through its N-terminal region (2) suggests that DUE-B, Treslin (28), and TopBP1 (29) dimers may load two molecules of Cdc45 to the double hexameric MCM complex of the pre-RC. Similar to the effect of replication stress on the interaction of Treslin with TopBP1 (23), we also show that the interaction of DUE-B with Treslin and TopBP1 is weakened by mild replication stress. These observations reinforce the conclusion that the interactions of DUE-B, TopBP1, and Treslin are involved in origin activation by Cdc45.

Taken together, our data suggest a model (Fig. 9A) in which PP2A-dependent dephosphorylation of DUE-B during the M/G1 phase leads to origin binding of DUE-B (4) through its C terminus to the MCM2-7 component of the prereplication complex (pre-RC) (1, 5). In temporally separable steps, Treslin and Cdc45 bind to the C terminus of DUE-B (Fig. 9B and C).

The S-phase kinases Cdc7 and CDK2 act to promote subsequent steps in origin activation (30). In *S. cerevisiae* the Cdc7 functions before S-CDK (31), whereas CDK2 acts before Cdc7 in *Xenopus* egg extracts suppressed or immunodepleted for these kinases (21, 32). Following this scheme, Cdc7-dependent phosphorylation of DUE-B (1, 5) leads to its release from the origin (Fig. 9D), after which CDK2 phosphorylation of Treslin establishes a binding site for TopBP1 (Fig. 9D).

The observations that TopBP1 binds to chromatin before DUE-B (Fig. 1) and that immunodepletion of DUE-B from egg extracts only partially blocks TopBP1 chromatin loading (4) suggest that chromatin-bound TopBP1 may redistribute to origins. In this scenario, TopBP1 degradation in DUE-B–depleted egg extracts or cells (Fig. 4 and Fig. S1) may explain the partial decrease in TopBP1 loading onto chromatin observed previously (4). We speculate that the interaction between Treslin and TopBP1 changes the association of Cdc45 with the pre-RC, enabling replication initiation.

**Experimental procedures**

**Cell culture**

HeLa cells and their derivatives were maintained as adherent cultures in DMEM containing 10% newborn calf serum with antibiotic/antimycotic solution (Corning). A1 (stable His-tagged WT DUE-B) and HisSA-tagged ΔCT DUE-B stable cells have been previously described (4, 5). HeLa T-Rex “Flp-In” cells were maintained in DMEM containing 10% newborn calf serum and selected with blasticidin (5 μg/ml). HeLa A1 and stable HisSA-tagged ΔCT-DUE-B cells were cultured in DMEM containing 10% normal calf serum with G418.

To create tetracycline-inducible stable cell lines expressing DUE-B or Treslin, 80% confluent HeLa T-Rex Flp-In cells

**Figure 8. Replication stress inhibits DUE-B interaction with Treslin.** HeLa T-Rex FLAG–DUE-B cells were treated with doxycycline to induce expression of FLAG–DUE-B and HU (A) or aphidicolin (C). Soluble lysates were pulled down with FLAG antibody beads, and the co-immunoprecipitated proteins were analyzed by Western blotting. HeLa cells were treated with HU (B) or aphidicolin (D), and chromatin-bound proteins were visualized by Western blotting.
were transfected using Lipofectamine 2000 (Invitrogen) with pcDNA5/TO/FRT plasmids carrying DUE-B or Treslin (a generous gift from William Dunphy) along with the pOG44 FLP recombinase vector (a kind gift from Geoffrey Wahl) in a 1:9 ratio in a 6-well plate. Twenty-four hours after transfection, the cells were transferred into a 15-cm plate and allowed to recover. The following day selection was started with hygromycin (100 μg/ml) for 15 days until distinct colonies appeared. Single colonies were picked and analyzed by Western blotting after inducing the protein of interest with doxycycline (1 μg/ml). To synchronize cells at the late G2/mitotic (M)–phase boundary, the cells were incubated in complete medium supplemented with 50 ng/ml nocodazole (Sigma) for 18–22 h. Following treatment cells were “shaken off,” washed with PBS, and replated in DMEM with 10% fetal bovine serum.

Induction of HeLa T-Rex cells was done by treatment with doxycycline (1 μg/ml) for 24 h along with treatment with nocodazole for synchronization. Plasmid DNA and siRNA were transfected by using Lipofectamine 2000 (Invitrogen) by following the manufacturer’s protocol.

**Protein expression**

The subcellular protein fractionation kit from Thermo Scientific (product no. 78840) was used for fractionation of soluble nuclear and chromatin-bound proteins. Protein expression in cell lysates was determined by SDS-PAGE and Western blot-
ting analysis. Anti-human β-actin, and anti-FLAG antibodies were purchased from Sigma. Rabbit anti–DUE-B antibodies (WRSU1) were raised in rabbits against a His<sub>6</sub>-tagged recombinant human DUE-B expressed in bacteria. WRSU4, rabbit anti-XOrc2 antibody was raised in rabbits against baculovirus-expressed recombinant *Xenopus* Orc2. Both the WRSU1 and WRSU4 antibodies have been previously described (1). WRSU5, rabbit anti-human TopBP1 antibody was raised in rabbits against *Escherichia coli* expressed recombinant human TopBP1 (4). WRSU1, 4, and 5 antibodies can recognize proteins from both human and *Xenopus laevis*. Anti-human Cdc45 antibodies were purchased from Bethyl Laboratories. An anti-His<sub>6</sub> antibody was purchased from Abcam. Anti-Chk1 phosphoserine 345 antibody was purchased from Cell Signaling. Anti-Treslin antibody was a kind gift from William Dunphy.

**Co-immunoprecipitation (co-IP)**

All steps were carried out on ice or at 4 °C. The cells were lysed in high salt IP lysis buffer (20 mM Hepes-KOH, pH 7.4, 500 mM NaCl, 0.5% Triton X-100, 5 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, 0.1 mM sodium vanadate, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml chymostatin (Sigma), and 1× protease inhibitor mixture (Sigma)). For His<sub>6</sub>-tagged protein pull-downs, 10 mM imidazole was also included in the IP lysis buffer. The cells were sonicated for 5 s, and supernatant was collected by centrifugation at 16,000 × g for 10 min. The supernatant was diluted with 20 mM Hepes-KOH (pH 7.4) to adjust the NaCl concentration to 150 mM. The supernatant was again collected by centrifugation at 16,000 × g for 5 min. Lysates were precleared with protein G–agarose beads for 1 h after equilibration of beads with diluted IP lysis buffer. 4 mg of total protein was used for each IP experiment (4% of total protein for input) and IP was carried out for 4 h at 4 °C with appropriate beads (Ni–NTA (nickel charged nitrilotriacetic acid) beads for His<sub>6</sub>-tagged proteins) (Qiagen) and red Anti-FLAG M2 beads (FLAG-tagged proteins) (Sigma).

Proteins bound to the beads were washed with IP wash buffer (20 mM Hepes-KOH, pH 7.5, 150 mM NaCl, 0.15% Triton X-100, 1.5 mM EDTA, 1 mM DTT, 10 mM β-glycerophosphate, 1 mM NaF, and 0.1 mM sodium orthovanadate) (four times). For His<sub>6</sub>-tagged proteins, 50 mM imidazole was also added to IP wash buffer. After wash steps, FLAG-tagged proteins were eluted with FLAG elution buffer (5 μg/ml FLAG peptide (Sigma), Tris-buffered saline, 700 mM NaCl). His<sub>6</sub>-tagged proteins were eluted by incubating beads with SDS sample buffer at 95 °C for 5 min. Samples were separated by SDS-PAGE, and proteins were detected by Western blotting.

**Flow cytometry**

To analyze cell-cycle progression, DNA in cells was stained with propidium iodide and measured by flow cytometry. About 1 million cells were used for analysis by flow cytometry. Adherent HeLa cells were washed with PBS, trypsinized from their plate, and pelleted by centrifugation at 500 × g at room temperature for 5 min. The cell pellet was washed once with PBS followed by another spin at 500 × g at room temperature for 5 min. The cell pellet was completely suspended in 1 ml of ice-cold 70% ethanol and fixed at −20 °C for at least 20 min. Fixed cells were pelleted by centrifugation at room temperature (500 × g, 5 min), washed once with PBS, and suspended in 1 ml of PBS. RNase A (25 μg/ml, Sigma) was added and incubated at 37 °C for 20 min. Propidium iodide (50 μg/ml) was added to the PBS solution and incubated in the dark at 4 °C for 30 min. Stained cells were directly analyzed using a BD Accuri C6 flow cytometer under the FL2-A channel. Flow cytometry data were analyzed using the FCS Express 4 software.

**Plasmid constructions**

Human DUE-B and its mutants with a C-terminal His<sub>6</sub> tag in pcDNA3.1 have been previously described (5). N-terminal FLAG-tagged DUE-B and its mutants were created by site-directed mutagenesis to insert BamHI and NotI sites by PCR and cloned into pcDNA3.1 for transient expression or into pcDNA5/TO/FRT to create tetracycline/doxycycline inducible cell lines. siRNA-resistant plasmids were constructed using Gibson assembly (33), and restriction digestion in pcDNA3.1. pcDNA5/TO/Treslin-SF (S-protein and 3× FLAG tag) (23) was a generous gift from William Dunphy.

Site-directed mutagenesis was carried out by using Phusion high-fidelity DNA polymerase (NEB M0530S). PCR products (500 ng) were digested by DpnI to remove template plasmid and passed through DNA Clean and Concentration columns (Zymo) to remove buffer components. PCR fragments were digested and ligated into the vector of choice.

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