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Report

Regulation of DNA Replication through Sld3-Dpb11 Interaction Is Conserved from Yeast to Humans

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Summary

Cyclin-dependent kinases (CDKs) play crucial roles in promoting DNA replication and preventing rereplication in eukaryotic cells [1–4]. In budding yeast, CDKs promote DNA replication by phosphorylating two proteins, Sld2 and Sld3, which generates binding sites for pairs of BRCT repeats (breast cancer gene 1 [BRCA1] C terminal repeats) in the Dpb11 protein [5, 6]. The Sld3-Dpb11-Sld2 complex generated by CDK phosphorylation is required for the assembly and activation of the Cdc45-Mcm2-7-GINS (CMG) replicative helicase. In response to DNA replication stress, the interaction between Sld3 and Dpb11 is blocked by the checkpoint kinase Rad53 [7], which prevents late origin firing [7, 8]. Here we show that the two key CDK sites in Sld3 are conserved in the human Sld3-related protein Treslin/ticrr and are essential for DNA replication. Moreover, phosphorylation of these two sites mediates interaction with the orthologous pair of BRCT repeats in the human Dpb11 ortholog, TopBP1. Finally, we show that DNA replication stress prevents the interaction between Treslin/ticrr and TopBP1 via the Chk1 checkpoint kinase. Our results indicate that Treslin/ticrr is a genuine ortholog of Sld3 and that the Sld3-Dpb11 interaction has remained a critical nexus of S phase regulation through eukaryotic evolution.

Results and Discussion

CDK-Dependent Interaction between Treslin/Ticrr and TopBP1

Eukaryotic DNA replication initiates from multiple replication origins, which each fire just once per cell cycle. To achieve this, the Mcm2-7 helicase is loaded around double-stranded DNA during G1 phase as an inactive double hexamer [1]. This loading reaction is inhibited by CDK and, therefore, can only occur during G1 phase when CDK activity is low. The helicase is then activated during S phase by formation of the CMG complex, which requires a set of loading factors including Sld2, Sld3, and Dpb11. In budding yeast, this step is promoted by CDK phosphorylation of Sld2 and Sld3. This dual role for CDK ensures that replication origin firing is limited to once per cell cycle [2].

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We were interested in understanding how CDK regulates initiation in mammalian cells and began by identifying binding partners of the breast cancer gene 1 (BRCA1) C terminus (BRCT) repeats in the mammalian ortholog of Dpb11, TopBP1. We found a fragment of a human open reading frame (C15orf42), subsequently identified as Treslin and ticrr [9, 10], in a two-hybrid screen for TopBP1-interacting proteins. Analysis of the Treslin/ticrr sequence identified a domain conserved across eukaryotic species, the *S*ld3-*T*reslin/ticrr *d*omain (STD) [11]. Although the Treslin/ticrr protein is large (1910 aa), the TopBP1-interacting fragment contained amino acid residues 790–1172, which lie downstream of the STD, similar to the position of the essential CDK sites relative to the STD in budding yeast Sld3 (Figure 1A). Figure 1B shows that this two-hybrid interaction (growth on –His plates) occurs with a construct containing BRCT repeats 0–5 of TopBP1 as well as a construct containing BRCT0–3. In yeast, CDK phosphorylation of Sld3 generates a binding site for the tandem BRCT repeats 1 and 2 in Dpb11. BRCT1 and 2 of TopBP1 that are contained in the BRCT0–3 construct are orthologous [12] to Dpb11's BRCTs 1 and 2 and are essential for replication [10], suggesting that BRCT1/2-mediated CDK-dependent Sld3 binding could be conserved. Consistent with this hypothesis, GST-TopBP1-BRCT0/1/2 could bind full-length Treslin/ticrr from cell lysates (Figure 1C). Lambda phosphatase treatment of the extract, which caused Treslin/ticrr to migrate slightly faster in sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), eliminated interaction with TopBP1 (Figure 1C, lanes 1 and 2). Phosphorylation of extract proteins with recombinant cyclinA/Cdk2 caused Treslin/ticrr to migrate more slowly in SDS-PAGE and greatly enhanced interaction with TopBP1 (lanes 3 and 4) (note that interaction with Treslin/ticrr is not evident in lane 3 because of the short exposure time compared to lane 1). This series of experiments suggests a CDK-dependent interaction of Treslin with TopBP1 via BRCT0/1/2. To examine interactions between full-length proteins, we immunoprecipitated TopBP1 from stable cell lines expressing Treslin/ticrr. Figure 1D shows that the anti-TopBP1 antibody did not coimmunoprecipitate Treslin/ticrr from lambda phosphatase-treated extracts but did coimmunoprecipitate Treslin/ticrr after treating extracts with CDK. BRCT0/1/2 of TopBP1 has previously been shown to bind the 9-1-1 complex component Rad9 when phosphorylated at S387 [13]. A phosphorylated synthetic peptide derived from Rad9 (pRad9) was able to bind TopBP1-BRCT0/1/2 and to elute full-length Rad9 from a complex with TopBP1-BRCT0/1/2 (see Figure S1 available online). Figure 1D shows that the phosphorylated Rad9 peptide, but not an unphosphorylated control (Rad9), also eluted Treslin from its complex with TopBP1.

The CDK-dependent interaction was reconstituted with purified cyclinA/Cdk2, GST-TopBP1(0/1/2), and a fragment of Treslin/ticrr containing amino acid residues 790–1172 generated in reticulocyte lysate by in vitro translation (Figure 1E). Figure 1E also shows that a fragment comprising amino acid residues 790–1056 but not 1014–1172 interacted with TopBP1 in a CDK-dependent manner.

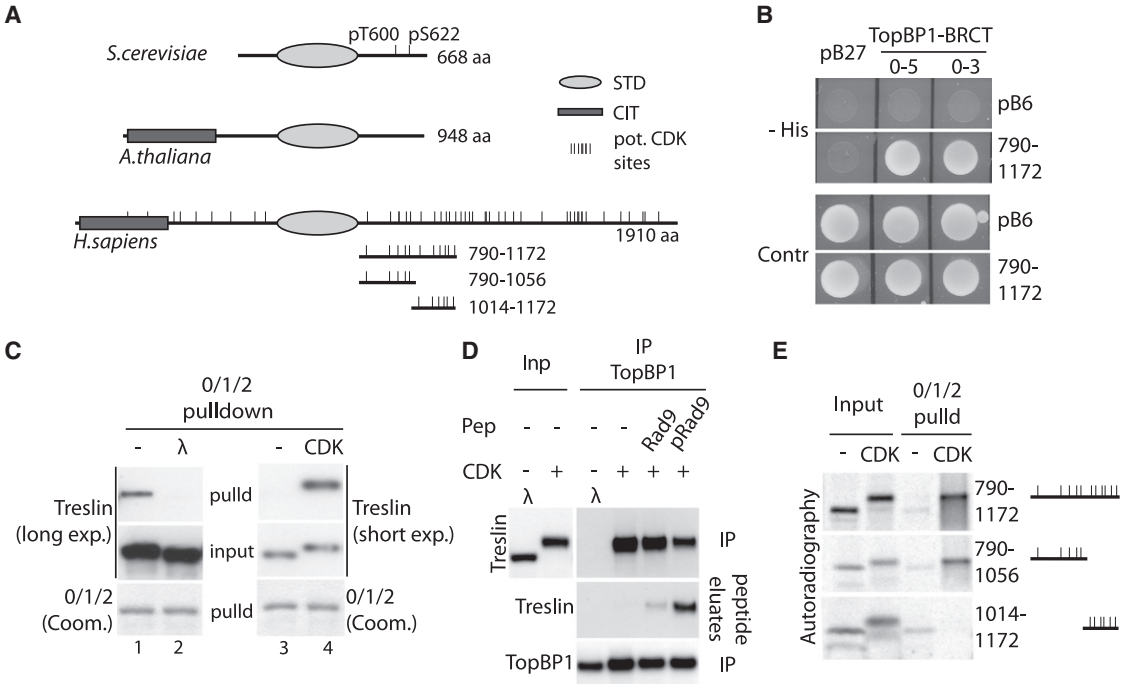

Figure 1. CDK-Induced Interaction of Treslin/Ticrr with TopBP1-BRCT0/1/2 Is Dependent on Treslin Phosphorylation between Amino Acids 790 and 1056

(A) Schematic indicating conservation between human and putative *Arabidopis thaliana* Treslin/ticrr as well as yeast Sld3. All three proteins share an Sld3/Treslin domain (STD) [11]. A region conserved *in T*reslins (CIT) is only present in metazoans. pT600/pS622 indicates that Dpb11-binding Sld3 cyclin-dependent kinase (CDK) sites are situated C-terminal of the STD. Forty-six potential CDK sites (S/T-P motifs) conserved between human and mouse Treslins are shown. Fragments of Treslin C-terminal of the STD: amino acids 790–1172 (used in [B], [E], and Figure 2B), 790–1056, and 1014–1172 (both 1E).

(B) Treslin-790–1172 interacts with TopBP1-BRCT0–3 in yeast two-hybrid analysis. For this yeast two-hybrid analysis, amino-terminally LexA-tagged TopBP1-1–549 (BRCT0–3) or 1–766 (BRCT0–5) and amino-terminally Gal4-tagged Treslin-790-1172 were used. pB27 and pB6 served as empty vector controls.

(C) Interaction of full-length Treslin/ticrr with TopBP1-BRCT0/1/2 is reduced by phosphatase and enhanced by Cdk2 treatment. AcGFP-Flag-Treslin was pulled down from lysates of transiently transfected HEK293T cells using GST-TopBP1-BRCT0/1/2 (amino acids 1–360) immobilized on glutathione sepharose. The following abbreviations are used: λ, lambda phosphatase; CDK, cyclinA/Cdk2; 0/1/2, GST-TopBP1-BRCT0/1/2 (amino acids 1–360); Treslin immunoblot: anti-GFP; exp., exposure; Coom., Coomassie staining.

(D) Phosphorylation-dependent interaction between Treslin/ticrr and endogenous full-length TopBP1 is mediated by TopBP1-BRCT0/1/2. A competition of binding experiment using Rad9 peptides and coimmunoprecipitates of Treslin-wild-type (WT) with endogenous TopBP1 is shown. Stable HeLa-Kyoto cells expressing AcGFP-Flag-Treslin were used. The following abbreviations are used: IP, immunoprecipitation; Pep, peptide; Rad9/pRad9, nonphospho/phospho-S387-Rad9 peptides; CDK, cyclinA/Cdk2; λ, lambda phosphatase; immunoblots: Treslin, anti-GFP; TopBP1, anti-TopBP1.

(E) CDK phosphorylation sites of Treslin/ticrr required for association with TopBP1-BRCT0/1/2 are situated between amino acids 790 and 1056. In vitro-translated Treslin fragments containing the indicated amino acids were pulled down using TopBP1-BRCT0/1/2 in the presence or absence of exogenous Cdk2. The following abbreviations are used: CDK, cyclinA/Cdk2 BRCT0/1/2, GST-TopBP1-BRCT0/1/2 (amino acids 1–360); Treslin fragments: amino acids 790–1172, 790–1056, and 1014–1172, respectively; Inp, input; autoradiography, detection of ^{35}S-Met-labeled in vitro-translated Treslin.

See also Figure S1.

Identification of Conserved CDK Sites Responsible for Interaction

In parallel with these interaction studies, we sought to identify CDK phosphorylation sites equivalent to Sld3 T600 and S622, which have been conserved in Sld3/Treslin/ticrr family members. Out of 70 potential CDK sites in human Treslin/ticrr (46 sites conserved between humans and mouse; Figure 1A), we found two sites, T969 and S1001, which were each embedded in short sequence motifs that are conserved among family members (Figure 2A). These sites are contained within the smallest fragment shown to interact with TopBP1 in Figure 1E. We next tested the relative importance of these two phosphorylation sites. Figure 2B shows that mutation of T969 to alanine in the context of the in vitro-translated Treslin fragment eliminated CDK-induced association with TopBP1-BRCT0/1/2 whereas the S1001-to-alanine mutation resulted in a significant reduction in interaction efficiency. The migration of wild-type and mutant Treslin/ticrr fragments in SDS-PAGE were reduced upon CDK treatment to a similar extent, indicating that phosphorylation of CDK sites outside T969 and S1001 (there are 11 potential CDK sites on the fragment) can occur and contribute to the reduced migration of TopBP1. These phosphorylations, however, do not promote efficient TopBP1 binding, providing an internal control for the specificity of the interaction. To analyze further the sequence requirements for this interaction, we examined the ability of TopBP1 to bind biotinylated synthetic peptides. Figure 2C shows that recombinant TopBP1 (0/1/2) could be pulled down with a Treslin/ticrr peptide containing sequences around T969 when T969 was phosphorylated, but not when T969 was unphosphorylated, showing that phosphorylation of T969, rather than the threonine residue itself, is critical for the interaction. Moreover, mutation of conserved residues at position −1 or −3 relative to T969 (see Figure 2A, asterisks) eliminated TopBP1 binding (Figure 2C). Similarly, TopBP1 (0/1/2) bound to a Treslin/ticrr peptide containing sequences around S1001 when