DUE-B, A NEW HUMAN DNA REPLICATION PROTEIN,
IS THE FUNCTIONAL HOMOLOG OF S. CEREVISIAE SLD3

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By

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I HEREBY RECOMMEND THAT THE thesis PREPARED UNDER MY SUPERVISION BY Jianhong Yao ENTITLED DUE-B, a New Human DNA Replication Protein, is the Functional Homolog of S.cerevisiae Sld3 BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science

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ABSTRACT

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DNA unwinding elements (DUEs) are commonly found at DNA replication origins. The DUE binding protein (DUE-B) is crucial for the initiation of DNA replication in eukaryotes. The unique 59 amino acid C-terminal part of DUE-B shares nearly 50% similarity with yeast the C-terminus of Sld3. DUE-B plays a key role in eukaryotic DNA replication because it is required for the loading of Cdc45, the MCM helicase activator, on chromatin. Here we show that DUE-B, just like yeast Sld3, binds to Cdc45 and TopBP1 through its C-terminus in Sf9 cells and in vitro. We also show that DUE-B, Cdc45 and TopBP1 form a heterotrimeric complex in vitro. The mass spectrometric data show that dominant negative Sf9 DUE-B is not phosphorylated but functional HeLa DUE-B is phosphorylated. All these data suggest that human DUE-B is a functional homolog of yeast Sld3.
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INTRODUCTION

Complete and accurate DNA replication is a basic and essential process for all living organisms. In eukaryotic cells, the precision of this process depends on conserved mechanisms that restrict DNA replication to once and only once per cell cycle. The DNA replication process includes two steps. The first is called initiation, which recruits the replication machinery or “replisome” to replication origins. The second step is elongation, which subsequently duplicates DNA by action of the replisomes (Bell and Dutta, 2002; Sclafani and Holzen, 2007). The main sites of regulation of DNA replication are the recruitment and initiation processes. The replisome is loaded or recruited to the origin by the protein complexes that recognize the initiator protein, origin recognition complex (ORC), bound to origin chromatin. Then DNA helicases, mini-chromosome maintenance complexes (MCMs), unwind the DNA helix, and polymerases copy the DNA (Kelly, 2000; Nasheuer, 2003; Sclafani and Holzen, 2007).

**Eukaryotic DNA replication**

The first step in the initiation of DNA synthesis is identifying the sites or origins where DNA replication will begin. In lower organisms, DNA replication origins often share conserved functional elements. The best-characterized origins are those in budding yeast which were originally identified as so-called autonomously replicating sequences (ARS) based on their ability to allow extrachromosomal plasmid replication (Marahrens and Stillman, 1992; Newlon, 1996). Although replication origins are easy to identify in
unicellular organisms, it is difficult to identify replication elements in metazoan species (Aladjem and Fanning, 2004; Gilbert, 2004). In metazoan cells, the initiation of DNA replication can occur at multiple sites within initiation zones (DePamphilis, 1999; Kobayashi et al., 1998; Dierov et al., 2004). The origins of replication direct the formation of many protein complexes leading to the assembly of bidirectional DNA replication forks. These events are started by formation of the pre-replication complex (pre-RC) at origins from late M to G1 phase when CDK (cyclin-dependent kinase) activity is low. Once the pre-RC is formed, this complex is activated by at least two kinases CDK (cyclin dependent kinase) and DDK (Dbf4-dependent kinase) and additional replication factors to form the pre-initiation complex (pre-IC). The pre-IC unwinds DNA and recruits multiple eukaryotic DNA polymerases while unwinding DNA to start DNA replication at S phase (Kelly and Brown, 2000; Bell and Dutta, 2002; Dierov et al., 2004; Sclafani and Holzen, 2007). The best understood initiation of eukaryotic DNA replication is in yeast (figure 1).

The formation of pre-replication complex (pre-RC)

A set of conserved protein factors work to license genomic sites for replication initiation in all eukaryotes. Importantly, this licensing is finely regulated to ensure the genome duplicates itself only once during each cell division cycle in normal cells.

For replication to occur, the origin recognition complex (ORC) first recognizes and binds to origins of replication (Diffley, 2001; Krude et al., 1997; Nasheuer et al., 2002). ORC is a six-subunit complex (Orc1-6) that acts as the primary eukaryotic replication initiation factor. In budding yeast, ORC binds to the conserved ARS
Figure 1. Branched pathway model of pre-initiation complex assembly in yeast. (Upper pathway) ORC-dependent pre-RC assembly and Mcm10 loading. (Lower pathway) Pre-IC assembly.
consensus sequence (ACS), and it works as an AAA+-ATPase (Bell and Stillman, 1992). The best understood activity of ORC is its binding to DNA (Bell et al., 1992; Rao et al., 1995). The discovery of ORC homologs in higher eukaryotic organisms has confirmed this complex to be a conserved component of the replication initiation machinery (Carpenter et al., 1996; Saxena et al., 2004; Remus et al., 2005).

The next step is to load the DNA helicase onto the replication origin. This recruitment needs at least two proteins, Cdc6 (cell division cycle 6) and Cdt1 (Cdc6 and Cdc10 dependent transcript 1) (Sclafani and Holzen, 2007). In late mitosis to early G1 phase, the interaction of ORC and replication origin DNA functions as a platform for other replication proteins to load (Pollok et al., 2003). The first protein that loads on the platform is Cdc6 (Coleman et al., 1996; Neuwald et al., 1999). Cdc6 was first identified in *S. cerevisiae* as an AAA+ ATPase and has been demonstrated to play a crucial role in the assembly of the pre-RC at a step after ORC and before Mcm2-7 proteins (Hartwell, 1973; Bell and Dutta, 2002). The binding of Cdc6 to ORC makes the ORC-DNA complex more stable and specific for licensing chromatin for replication (Speck et al., 2005 and 2007).

Cdt1 cooperates with Cdc6 to load the DNA helicase, Mcm2-7 (mini-chromosome maintenance) complex, during G1 phase of the cell cycle. The Heilin group recently reported that human Cdt1 binds to Cdc7 and recruits Cdc45 to chromatin at S phase (Ballabeni et al., 2008). Cdt1 was initially identified in fission yeast as a cell-cycle-regulated gene (Hofmann et al., 1994), and is essential for both nuclear localization and loading of MCM on chromatin (Maiorano et al., 2000; Tanaka et al., 2000; Hofmann et al., 2002). Overexpression of Cdt1 leads to ectopic pre-RC formation and re-replication
of DNA (Ricke et al., 2004). The level of Cdt1 activity is controlled by binding of
geminin to form an inactive complex (Lutzman et al., 2006) and degraded by
proteosome in a replication dependent manner (Arias and Walter, 2006; 2007; Aparicio,
et al., 2006). It is well demonstrated that regulation of the activity of Cdt1 is important
for maintaining genomic integrity (Arias and Walter, 2006; 2007).

It is believed that the MCM complex is recruited to the DNA replication origin by
ORC after Cdc6 and Cdt1 bind to the ORC-DNA structure, and functions as a DNA
helicase. MCMs are composed of six subunits (Mcm2-7), which are conserved in
eukaryotes (Forsburg, 2004; Lei 2005). Lutzmann et al. reported that Mcm9, a new
member of Mcm2-7 family, binds to Cdt1 and is also required for the recruitment of the
Mcm2-7 helicase onto chromatin (Lutzman et al., 2008). All six members are AAA+
ATPases with similarity to prokaryotic DNA helicases. All are required for initiation and
elongation during DNA replication. MCMs helicases unwind the DNA double strands
during the chromosomal replication and move with the replication fork. (Sclafani and
Holzen, 2007; Blow et al., 2008). After the MCMs loading on the replication origin, the
pre-replication complex is formed in G1 phase, and the replication origin is licensed and
waits for additional signal for origin firing.

Once the pre-RC forms, re-replication is prevented until the next G1 phase. Cells
limit replication initiation to once per cell cycle by several pathways. One example is the
CDK-mediated prevention of re-replication. The increase of CDK activity in the G1/S
transition phase inactivates the loading of the pre-RC components, Cdc6, MCM and Orc2,
to prevent re-initiation (Nguyen et al., 2001). Cdc6 is phosphorylated and /or exported
from the nucleus during S phase to prevent re-initiation at replication origins
(Alexandrow et al., 2004). MCMs are released from chromatin after phosphorylation (Ishimi et al., 2001), and phosphorylated Orc2 is inactivated in fission yeast (Vas et al., 2001). The second pathway involves the regulation by geminin, the inhibitor of Cdt1. Because constitutive expression of Cdt1 results in re-replication (Yanow et al., 2001; Arias et al., 2006), the regulation of Cdt1 by geminin inhibits the Cdt1-mediated loading of MCMs and thus prevents DNA re-replication. The PCNA-dependent and Cul-Ddb1<sup>Cdt2</sup>-dependent Cdt1 proteolysis destroy the Cdt1 and prevent re-replication in interphase (Arias and Walter, 2006; 2007). Another mechanism to prevent re-replication involves chromatin conformation. It has been suggested that the conformation of local chromatin has an effect on both the activity and timing of origins (Bell and Dutta, 2002).

The formation of pre-initiation complex (pre-IC)

The assembly of the pre-RC in late mitosis and early G1 phases is not sufficient for DNA synthesis. The pre-RC requires to be rearranged during S phase by recruiting at least two protein kinases, the cyclin-dependent kinase (CDK) and the Dbf4 or Drfl-dependent Cdc7 kinase (DDK) (Saxena and Dutta, 2004), and other additional replication proteins, MCM10, Cdc45, TopBP1, Sld2, Sld3 and GINS, to form the pre-initiation complex (pre-IC) (Bell and Dutta, 2002;). The pre-IC forms only after the activation by CDK and DDK at the G1/S transition (Nougarede et al., 2000; Walter, 2000).

MCM10 is characterized as a factor required for efficient initiation of DNA replication (Merchant et al., 1997). The Mcm10 protein associates with the pre-RC complex through Mcm2-7 before Cdc45 recruitment and replication origin unwinding (Kawasaki et al., 2000; Ricke and Bielinsky, 2004). Mcm10 is also required for
continued replication fork progression at times when ORC and Cdc6 are dispensable from replication complex (Piatti et al., 1996; Kawasaki et al., 2000). The MCMs helicases are inactive until Cdc45 protein binds to the origin. Cdc45 is a crucial factor for the initiation of DNA replication (Aparicio et al., 1999; Tercero et al., 2000). The binding of TopBP1 to the pre-RC is required for the recruitment of Cdc45 onto chromatin (Hashimoto et al., 2003). Two other proteins in yeast, Sld2 and Sld3, are also required for loading Cdc45. The putative Sld2 homolog, RECQL4, has been identified in metazoans, but so far, no Sld3 homolog has been found in higher eukaryotes (Matsuno et al., 2006; Sangrithi et al., 2005). All three proteins, Cdc45, TopBP1 and Sld3 will be introduced later, since they are highly related to my thesis. The large multiprotein complex that is formed at this step is the pre-IC (Zou et al., 1998). The pre-IC does not exist for very long, and replication begins as soon as the pre-IC is formed (Bell and Dutta, 2002).

**Initiation of DNA replication**

The CDK- and DDK- dependent recruitment of Cdc45 and GINS to the replication origin coincides with the conversion of the pre-RC to a pre-IC. Upon pre-IC formation, the activated MCMs helicases unwind the DNA at the replication origin (Walter and Newport, 2002; Pacek and Walter, 2004; Dutta et al., 2008). The eukaryotic single-stranded binding protein, RPA, coats the unwound DNA and stabilizes the single-strand as a template for DNA synthesis. Although the exact order of binding of RPA, GINS, PCNA and polymerases is unclear, data from yeast and *Xenopus* egg extracts suggest that RPA is required for loading of DNA polymerases (Zou et al., 2000; Mimura et al., 2000; Walter et al., 2000).
The GINS complex is composed of Sld5, Psf1, Pls2 and Psf3 proteins, and is required for DNA replication by stimulating the processivity of DNA polymerase epsilon (Kubota et al., 2003; Seki et al., 2006; Sclafani and Holzen, 2007). The stable binding of Cdc45 and MCMs also requires the GINS complex during S phase (Labib et al., 2007). The large complex consisting of Cdc45, Mcm2-7 and GINS activates the DNA helicase activity of MCM proteins, which triggers the unwinding of DNA at replication origins. The subsequent recruitment of DNA polymerase alpha allows synthesis of a short RNA primer and initiation of DNA synthesis. The loading of PCNA (proliferating cell nuclear antigen) requires DNA polymerase alpha (Mimura et al., 2000). PCNA functions as a processivity factor for DNA polymerases delta and epsilon for DNA elongation.

Although the comprehensive model of the initiation of DNA replication is well established, additional factors have still been identified recently. Noc3 and Yph1 in yeast (Zhang et al., 2002; Miklereit et al., 2001; Du et al., 2002), Mcm9 in higher eukaryotes (Lutzmann et al., 2005; 2008); Mcm8 in human cells (Gozuacik et al., 2003; Johnson et al., 2003; Volkeing et al., 2005) and ABAP1 in plants (Masuda et al., 2008) are novel factors that have been proved to affect the initiation of DNA replication. The primary components required for the initiation of DNA replication are largely conserved throughout eukaryotic organisms. However, the existence of metazoan-specific replication factors, such as Mcm8 and geminin, may be required for maintaining the large genomes in higher eukaryotes.
The initiation proteins TopBP1, Sld3 and Cdc45

**TopBP1 (topoisomerase IIβ binding protein 1)**

Human TopBP1 was initially identified as a protein interacting with DNA topoisomerase IIβ (Yamane et al., 1997). TopBP1 possesses eight BRCA1 carboxy-terminal (BRCT) domains that are structurally and functionally conserved throughout eukaryotic organisms and are commonly found in proteins involved in DNA repair or cell cycle checkpoints (Callebaut and Mornon, 1997). The BRCT domain is a phosphoprotein binding domain involved in cell cycle control (Yu et al., 2003; Manke et al., 2003). The homologues of TopBP1 are known as Dbp11 in *Saccharomyces cerevisiae*, Rad4/Cut5 in *Schizosaccharomyces pombe*, Mus101 in *Drosophila melanogaster* and Xmus101/Cut5 in *Xenopus laevis* (Schmidt et al., 2007). Human TopBP1 plays a key role in various aspects of DNA metabolism, such as cell survival, DNA replication, resistance to DNA damage and checkpoint control, chromatin remodeling and transcriptional regulation (Valerie et al., 2005; Garcia et al., 2005; Uta et al., 2007).

**DNA replication** Yeast Rad4/Cut5 is known to be required for the initiation of DNA replication. The Forsburg group showed that loading of pre-initiation complex components, such as Cdc45 (XCdc45 indicates *Xenopus* Cdc45; SCdc45 indicates *S. cerevisiae* and *S. pombe* Cdc45, because their sequences are highly conserved.) and RPA, and the DNA polymerases are dependant on Rad4/Cut5 (Dolan et al., 2004). Both *Xenopus* Xmus101/Cut5 and *Drosophila* Mus101 are also shown to have the essential roles in DNA replication (Hashimoto et al., 2003; Van Hatten et al., 2002). Xcut5 is also proved to be required for assembly of pre-ICs and loading of DNA polymerases in *Xenopus* egg extracts (Garcia et al., 2005). According to the model established in
Xenopus egg extracts, Xcut5 associates with chromatin in two distinct modes. In mode 1, before XCdc45 loading, Xcut5 binds weakly to the chromatin in a S-CDK-independent manner, and is required for XCdc45 binding and conversion of the pre-RCs to pre-ICs. This binding is for the initiation of DNA replication. In mode 2, after pre-ICs are formed and DNA replication is initiated, Xcut5 binds the chromatin tightly in a S-CDK-dependent manner. This binding is required for checkpoint function (Hashimoto et al., 2003). In mammalian cells, although TopBP1 is mainly studied in the DNA damage response, many groups proved that it also plays a role in the initiation of DNA replication. TopBP1 mRNA and protein levels both increase during S phase, which suggest that TopBP1 is involved in DNA replication (Yamane et al., 2002; Liu et al., 2004; Jeon et al., 2007). Makiniemi et al. reported that human TopBP1 interacts with polymerase epsilon just like its Xenopus and yeast homologous (Makiniemi et al., 2001). TopBP1 is also proven to be required for recruitment of Cdc45 to origins of DNA replication during G1/S transition (Schmidt et al., 2007; Jeon et al., 2007). The interaction of Dpb11 and CDK phosphorylated Sld2 and Sld3 is the minimal requirement for CDK-dependent activation of DNA replication in budding yeast (Zegerman et al., 2007; Tanaka et al., 2007). Together, these data suggest that the function of helicase activation and replisome loading of TopBP1 orthologues in DNA replication is conserved throughout evolution.

**DNA damage detection and checkpoint activation** As mentioned before, TopBP1 is mainly studied in the DNA damage response. Saka et al. first reported that fission yeast Rad4/Cut5 is required for prevention of mitotic entry in response to DNA replication blocks (Saka et al., 1993), and the Araki group proved the similar function of
Dbp11 in budding yeast (Araki et al., 1995). After that, a more direct connection was found between Dbp11 and Cut5 with the respective Rad9/Rad1/Hus1 (9–1–1) complexes, which recruit Chk1 to stalled replication forks for activation by ATR (Wang et al., 2002; Furuya et al., 2004). All these evidences demonstrate that TopBP1 is involved in DNA damage detection and checkpoint activation. In mammalian cells, the level of TopBP1 reduced with siRNA results in a defect in phosphorylation of Chk1 (checkpoint 1) and other ATR (ATM and Rad3-related) substrates (Burrows et al., 2008) and cells exhibited ultraviolet sensitivity as well as increased cell death by apoptosis (Van Hatten et al., 2002; Yamane, et al., 2002). Yamane et al. demonstrated that TopBP1 and BRCA1 have overlapping functions in the G2/M DNA damage checkpoint to activate the Chk1 kinase (Yamane et al., 2006). The mechanisms that regulate TopBP1 access to ATR-ATRIP (ATR-interacting protein) are complex but include post-translational modifications and recruitment of TopBP1 independently of ATR-ATRIP to sites of replication stress or DNA damage (Delacroix et al., 2007; Lee et al., 2007; Yoo.et al., 2007). Furthermore, TopBP1 physically interacts with ATR through an ATR activation domain (AAD) and activates ATR through ATRIP and a PIKK regulatory domain (ATR PRD) (Kumagai et al., 2006; Mordes et al., 2008).

Transcriptional regulation Several BRCT-repeat containing proteins have been proven to act in transcriptional coactivation (Shimizu et al., 2001; Saka et al., 1997; Liu et al., 2003; Yu et al., 2003). The C-terminal BRCT repeating domains of BRCA1 have been proven to be involved in the activation of transcription (Ouchi et al., 1998). Several groups reported that TopBP1 interacts with transcription factors, such as human papillomavirus (HPV) E2 protein, the E2F1 transcription factor and the POZ-domain
factor Miz1 and TopBP1 can function as a transcriptional regulator (Makiniemi et al., 2001; Boner et al., 2002; Liu et al., 2004; Herold et al., 2002; Garcia et al., 2005).

**Cdc45 (cell division cycle 45)**

Cdc45 is a critical factor for the initiation and elongation of DNA replication (Zou et al., 2000; Tercero et al., 2000). It is highly conserved among all eukaryotes and knockout of this gene causes embryonic death in mice (Yoshida et al., 2001). Cdc45 moves with the replication fork and functions as a helicase cofactor of MCMs (Sclafani et al., 2007). Cdc45 is required for loading of the replisome, DNA polymerases and RPA (Aparicio et al., 1999).

Cdc45 was first identified in a screen for cold-sensitive cell division cycle (cdc) mutants and when grown at the restrictive temperature cdc45-1 cells arrest late in G1 with a haploid DNA complement (Moir et al., 1982). Therefore, Cdc45 is required for the early step, the initiation of DNA replication, in DNA synthesis (Hennessy et al., 1991). Hardy provided strong evidence that Cdc45 plays a role in initiation of DNA replication (Hardy, 1997). In vitro, depletion of XCdc45 in *Xenopus egg extracts* inhibited the loading of DNA polymerase alpha on chromatin (Mimura et al., 1998). Similarly, mutants of SCdc45 prevented the assembly of DNA polymerases at origins of replication, as reported in *S. cerevisiae* and human cells (Zou et al., 2000; Aparicio et al., 1999; Kukimoto et al., 1999). Further studies in yeast and *Xenopus* showed that Cdc45 is also required for the elongation step of DNA replication (Hopwood et al., 1996; Minura et al., 2000; Tercero et al., 2000). Cdc45 origin association corresponds well with the time of activation of the actual replication machinery, which suggests that Cdc45 may serve as a bridge between origins and the replisome (Aparicio et al., 1999; Masuda et al., 2003;
Pacek et al., 2006). The main function of Cdc45 is to activate MCM complex (Masuda et al., 2003). In vitro, purified MCMs-Cdc45-GINS complexes from budding yeast and Drosophila have DNA helicase activity (Pacek et al., 2006; Gambus et al., 2006; Moyer et al., 2006).

In vitro experiments performed in *Xenopus egg extracts* show that XCdc45 loads after the pre-RC formation and before DNA unwinding and polymerase association (Minura et al., 2000; Walter et al., 2000). The loading of Cdc45 on the replication origin may occur in two modes. One is a weak CDK-independent association and another is a stable CDK-dependent association (Aparicio et al., 1997; Aparicio et al., 1999; Kelly and Brown, 2000). Cdc45 has been found to not only associate with DNA polymerases (Kukimoto et al., 1999; Spiga et al., 2004), but also interacts with ORC, RPA and the MCMs (Zou et al., 2000; Kamimura et al., 2001; Uchiyama et al., 2001). The association of Cdc45 with polymerases and MCM helicases suggests that it is a helicase cofactor and helps to unwind the DNA template (Bell and Dutta, 2002; Sclafani and Holzen, 2007). Once assembled at the origin, Cdc45, like MCM proteins, colocalizes with polymerases at the replication fork (Aparicio et al., 1999; Zou et al., 2000). Cdc45 is synthesized in late G1 phase, loads and functions in S phase, and is then ubiquitylated by the APC/C$^{Cdh1}$ E3 ligase and degraded during the next early G1 phase (Pollok et al., 2007). Cdc45 is much less abundant than MCM proteins, which implies that Cdc45 may be a limiting factor for the initiation of DNA replication (Zou et al., 1998; Apricio et al., 1999; Minura et al., 2000; Edwards et al., 2002).
**Sld3 (synthetically lethal with Dpb11-1)**

Sld3 encodes a 77 kDa protein and its predicted amino acid sequence has similarities with those of the budding yeast YOR165w ORF and Psl3 in *S. pombe*. Sld3, together with Sld2 and Sld5, was first identified by screening synthetic lethal mutations with Dpb11-1 (Araki et al., 1998). Sld3 is required for the initiation of DNA replication, and Sld3 knockout yeast strains are inviable. Sld3 and Cdc45 binds early in S phase to early firing origins and late in S phase to late firing origins. (Kamimura et al., 2001; Nakajima et al., 2002).

Sld3 and Sld2 are essential CDK substrates and their phosphorylation is the minimal requirement for CDK-dependent activation of the initiation of DNA replication in budding yeast (Masumoto et al; 2002; Tanaka et al., 2007). Sld3 has 12 CDK phosphorylation motifs. Three Ser/Thr sites, T600, T609 and S622, of Sld3 are important for DNA replication (Zegerman et al., 2007; Tanaka et al., 2007). In budding yeast, phosphorylation of Sld3 at T600 and S622 by CDK is crucial for binding to the C-terminal BRCT3 region of the TopBP1 homolog Dpb11, which is crucial for polymerase loading in cooperation with the Sld3-SCdc45 complex (Araki et al., 1995; Masumoto et al., 2002; Tanaka et al., 2007; Zegerman et al., 2007). DNA replication can occur in both Sld3- and Sld2-S-CDK bypass mutants.

Sld3 has been shown to bind SCdc45 directly in both budding yeast and fission yeast (Kamimura et al., 2001; Nakajima et al., 2002). Sld3, together with Dpb11, MCM10 and GINS, are required for the stable loading of SCdc45 on chromatin at S phase (Mendez and Stillman, 2003). Using a protein cross-linking agent, Kamimura et al. observed that Sld3 and SCdc45 form a complex throughout the cell cycle (Kamimura et
al., 2001). A similar result has been demonstrated in fission yeast (Nakajima et al., 2002). The complex of Sld3 and SCdc45 associates with origins by interaction of Cdc45 and MCM protein, at least in S phase (Kamimura et al., 2001). Different to GINS that is required for stable association of SCdc45 with origin during S phase, Sld3 is required for the initial recruitment of SCdc45 and GINS (Kamimura et al., 2001; Kanemaki et al., 2006). Sld3 also binds to other replication proteins. In budding yeast, Sld3 binds to GINS in a two-hybrid assay and it is required for the loading of GINS on origin (Takayama et al., 2003).

Metazoan homologs of Cdc45, Sld2 (RecQL4), and Dpb11 (TopBP1) have already been isolated, but so far no Sld3 homolog has been found, which are shown in the following table. The main reason is that Sld3, even within fungi, is very poorly conserved. S. pombe Sld3 shares only 14% identity and 24% similarity with S. cerevisiae Sld3. The C-terminal regions of both proteins share 10% identity and 60% similarity. But Cdc45 is well conserved from yeast to higher eukaryotes and the N-terminal BRCT repeats of Dpb11 are conserved in its metazoan homolog TopBP1 (Makiniemi et al., 2001; Yamamoto et al., 2000). All the evidences suggest that Sld3, like other important replication proteins, such as Cdc45 and TopBP1, should have its functional metazoan homolog in DNA replication.

**The homology of yeast Cdc45, Dpb11 and Sld2 in metazoan.**

<table>
<thead>
<tr>
<th>Yeast</th>
<th>Metazoan</th>
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<tr>
<td>Cdc45</td>
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<td>Dpb11</td>
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<td>Sld2</td>
<td>RecQL4</td>
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<tr>
<td>Sld3</td>
<td>DUE-B?</td>
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DUE-B (DNA unwinding element binding protein)

In higher eukaryotic organism, there is no consensus sequence, like ACS in budding yeast, for ORC binding. A common characteristic of eukaryotic origins of replication is the presence of an area of helical instability known as the DNA unwinding element (DUE) (Liu et al., 2003; Natale at al., 1993). To identify proteins that might regulate the origin of higher eukaryotes, our lab used a yeast one-hybrid assay with the DUE from the c-myc origin as bait. A 23.4 kDa protein was found and named the DNA unwinding element binding protein (DUE-B) (Casper et al., 2005).

Human DUE-B has 209 amino acids including an N-terminus head (150 amino acids) and C-terminus tail (59 amino acids). DUE-B was isolated as a homodimer and it is highly conserved throughout evolution. The crystal structure shows that the N-terminus of DUE-B, similar to its homolog in bacteria, archae and yeast, which do not have the C-terminus ~59 amino acids, has strong homology to tRNA proofreading enzymes (Soutourina et al., 2000) and actually it was annotated as human histidyl-tRNA synthetase (Meng et al., 2002; Ferri-Fioni at al., 2001, 2006; Hussain et al., 2006). However, no other tRNA editing enzymes have an Mg\(^{2+}\) ion in the active site (Kemp et al., 2007). The C-terminal part is not visible in the structure and is dynamically disordered (Kemp et al., 2007). Suprisingly, the C-terminus of DUE-B shares 35-54% similarity to Sld3 proteins in yeast (Fig 2).

In vivo, DUE-B localizes to the nucleus and binds to chromatin. Purified DUE-B from HeLa cells possesses ATPase activity and this ATPase activity is requires the Thr 81 residue (Kemp et al., 2007). DUE-B was proposed to be involved in regulating replication initiation because chromatin immunoprecipitation assay results showed DUE-
B to be bound near the *c-myc* replicator DUE in a cell cycle dependent manner (Casper et al., 2005; Ghosh et al., 2006). DUE-B also appeared to be preferentially phosphorylated in cells arrested in early S phase. DUE-B can be phosphorylated by Casein kinase (CK2), and DUE-B loading on chromatin can be inhibited by p27, the CDK inhibitor, in vitro (Chowdhury, unpublished data). Immuno-depletion of DUE-B in *Xenopus* egg extracts inhibits DNA replication and DNA replication can be restored by adding back recombinant DUE-B from HeLa cells (Casper et al., 2005). SiRNA of DUE-B delayed entry into S phase and promoted cell death in HeLa cells (Casper et al., 2005). Dominant negative DUE-B inhibits DNA replication before RPA loading and after pre-RC formation in *Xenopus* egg extracts (Casper et al., 2005). Immuno-depleted DUE-B in *Xenopus* egg extracts eliminated Cdc45 loading and strongly reduced TopBP1 loading on chromatin (Chowdhury, unpublished data). All these results suggested that DUE-B plays an essential role in replication initiation.

As mentioned before, the metazoan Sld3 has not been found. Although DUE-B sequence has a low percent of homolog to Sld3, this could not exclude the possibility that DUE-B is the functional homolog of Sld3 because Sld3 is poorly conserved in evolution. Human DUE-B shares 4% identity and 14% similarity with *S. cerevisiae* Sld3. C-terminal of DUE-B shares nearly 50% similarity with *S. cerevisiae* Sld3 (Figure 2). DUE-B has a unique 59 amino acid C-terminus presented only in vertebrates, which is critical for binding double strand DNA. The C-terminal T154 and S179 of DUE-B are homologous to Sld3 essential phosphorylation sites (Fig 3). As we know, DUE-B is a member of the D-tyrosyl-tRNA deacylases from yeast and bacteria (Kemp et al., 2007; Ferri-Fioni et al., 2001; Lim et al., 2003). It also functions as an ATPase. So DUE-B may be a
Figure 2. Alignment of amino acid sequences of C-terminus of S. cerevisiae Sld3 and human C-terminus of DUE-B.

Identical residues are marked with an asterisk and conservative changes with double or single dots. The homologous phosphorylation sites by CDK are marked with red.
**Figure 3. Conserved sequence motifs in the DUE-B C-terminus region.**

Potential phosphorylation sites indicated in bold red.
multifunctional esterase (Kemp et al., 2007). DUE-B also has many similar functions and characteristics as Sld3. DUE-B is a CK2 substrate and can be phosphorylated during the cell cycle. Our working model (Figure 4) proposes that DUE-B is required for Cdc45 and TopBP1 loading and DNA replication origin firing (Chowdhury et al., unpublished data; Casper et al., 2005; Ghosh et al., 2006). So the work to be reported below is to test the hypothesis that DUE-B can bind Cdc45 and TopBP1 through its C-terminus, in a manner that is homologous to Sld3 binding to Cdc45 and Dpb11 in yeast. (Kamimura et al., 2001; Araki et al., 1995; Tanaka et al., 2007; Zegerman et al., 2007).
Figure 4. Branched pathway model of pre-initiation complex assembly in mammalian cell.
(Upper pathway) ORC-dependent pre-RC assembly and Mcm10 loading. (Lower pathway) Proposed reactions 1-6. Rx. 1: TopBP1 binds to Cdc45. Rx. 2: binding of DUE-B to TopBP1/Cdc45 to form a ternary complex. Rx. 3: ORC-, Cdc7- dependent loose association of the the TopBP1/DUE-B/Cdc45 (TDC) complex at early firing origins. Rx. 4: CDK2-dependent phosphorylation of DUE-B leads to tight binding of DUE-B at the origin and transfer of Cdc45 to the pre-RC, forming the pre-IC. Rx. 5: Activation of the MCM complex and GINS binding lead to origin unwinding, dissociation of phosphorylated DUE-B and TopBP1. Rx. 6: PP2A removes inhibitory phosphates from a soluble factor (DUE-B) to allow subsequent Cdc45 loading. Dephosphorylated DUE-B binds TopBP1/Cdc45 released from early firing origins/forks for loading at late firing origins.
MATERIALS AND METHODS

Cell culture

The insect Sf9 and High Five cells were obtained from Invitrogen. The Sf9 cells were used for making recombinant baculoviruses and High Five cells used for expression and purification of recombinant Cdc45, TopBP1, DUE-B and C-terminal truncated DUE-B proteins. Sf9 cells were maintained in either Grace’s insect medium supplemented with yeastolate, lactalbumin, pluronic acid, and 10% fetal bovine serum or in the serum-free medium Sf-900 II SFM (Invitrogen). High Five cells were cultured in Grace’s insect medium supplemented with yeastolate, lactalbumin, pluronic acid, and 10% fetal bovine serum. Both media included the antibiotic gentamicin (10 µg/ml), except during transfections. Cells were grown as adherent cultures.

Generation of recombinent Cdc45 and TopBP1 baculoviruses by Bac-to-Bac baculovirus expression system

Transformation

The Cdc45 and TopBP1 cDNA were cloned into Baculovirus vector pFastBAC™. The pFastBAC-Cdc45 construct (from Dr. Kukimoto) has a His-tag at the N-terminus of the protein and pFastBAC-HF-XTopBP1 (from Dr. Dunphy) has His and HA tags at the N-terminus and Flag tag at C-terminus. The two constructs were transformed into E.coli component cells DH10Bac, bought from Invitrogen, and selected for ampicillin-resistant
transformants. The DH10Bac competent cells were thawed on ice. Approximately 1ng pFastBAC-Cdc45 and pFastBAC-HF-XTopBP1 plasmids (in 5µl) were added to the competent cells and gently mixed into the cells by tapping the side of tube. The mixtures were incubated on ice for 30 min. Then the mixtures were heat shocked by transferring to 42° C water bath for 45 sec and then the mixture was put on ice for 2 mins. 900 µl S.O.C. medium was added to the mixture then the mixture was put in a shaking incubator at 37° C with medium agitation for 4 hr. Cells were serially diluted by using S.O.C. medium to $10^{-1}, 10^{-2}$ and $10^{-3}$. 100 µl of each dilution was spread on the plates with 50 µg/ml ampicillin. The plates were incubated for 24 hr-48 hr at 37° C.

**Isolation of recombinant bacmid DNA**

Eight white clones were picked and streaked to fresh plates to verify the phenotype. After incubating overnight at 37° C, a single colony, confirmed as having white phenotype on plates containing Bluo-gal and IPTG, was set up in liquid culture for isolation of Cdc45 and TopBP1 recombinant bacmid DNA. A single, isolated bacterial colony was inoculated into 2 ml LB medium supplemented with 50 µg/ml kanamycin, 7 µg/ml gentamicin, and 10 µg/ml tetracycline. The culture was grown at 37° C overnight with shaking at 250 to 300 rpm. 1.5 ml of the culture was transferred to a 1.5 ml micro centrifuge tube and the recombinant bacmid DNA was isolated by using Plasmid Midi Kit (Omega). The bacmid DNA was analyzed by agarose gel electrophoresis to confirm the presence of high molecular weight DNA.
Analyzing recombinant bacmid DNA by polymerase chain reaction (PCR)

The Cdc45 and TopBP1 bacmid DNA were verified by PCR analysis. The M13 Forward and M13 Reverse primers were used for identifying Cdc45 bacmid DNA and the TopBP1 upper and TopBP1 lower primers for TopBP1 bacmid DNA. The primers sequences were the following:

M13 Forward: 5‘ GTTTTCCCAGTCACGAC 3’
M13 Reverse: 5‘ CAGGAAACAGCTAGAC 3’
TOPBP1 upper: 5‘ AAGCCCATTCTTCTTCTTCTTTG 3’
TOPBP1 lower: 5‘ AGGGAGTGCTCTGTGTGCCTGTT 3’

The reactions contained in each sample (50 µl):

10x PCR buffer: 5 µl
MgCl₂: 50 µM
dNTP: 200 µM
P1/P2: 25 pmol each/50 µl
Taq DNA polymerase: 2.5 units/50 µl (Qiagen)
DNA template: 0.1µg

The PCR Cycle was performed as below:

95° C: 5 min (1x)
95° C: 1 min (34x)
55° C: 1 min (34x)
72° C: 2 min (34x)
72° C: 7 min (1x)
PCR products were subjected to 1% agarose gel and stained with ethidium bromide to visualize the DNA.

**Restriction enzyme digestion**

The PCR products of Cdc45 bacmid DNA were digested with the restriction enzyme Pst I (Biolab). The restriction enzyme Bam HI (NEB) was used to digest the PCR products of TopBP1 bacmid DNA. Each 20 µl restriction reaction contained 1 µg PCR product and 10 units restriction enzyme (RE). The PCR product and RE mixture was incubated in 37° C for at least 2 hr and then subjected to 1% agarose gel.

**Transfection of Sf9 cells with recombinant bacmid DNA**

$9 \times 10^5$ cells in 2 ml of Sf-900 II SFM containing 50 units/ml penicillin, 50 µg/ml streptomycin were seeded in each well of a 6-well plate. Cells were allowed to attach at 27° C for at least 1 hr. The following solutions were prepared. **Solution A:** For each transfection, 5 µl of mini-prep Cdc45 or TopBP1 bacmid DNA was diluted into 100 µl Sf-900 II SFM without antibiotics. **Solution B:** For each transfection, 6µl CellFectin Reagent (Invitrogen) was diluted into 100 µl Sf-900 II SFM without antibiotics and mixed thoroughly by inverting the tube 5-10 times before removing the sample for transfection. The two solutions were combined and mixed gently and incubated for 15 to 45 min at room temperature. Cells were washed once with 2 ml of Sf-900 II SFM without antibiotics. For each transfection, 0.8 ml of Sf-900 II SFM was added to each tube containing the lipid-DNA complexes. The diluted lipid-DNA complexes were mixed gently and put onto cells. Cells were incubated for 5 hr in a 27° C incubator. The
transfection mixtures were removed and 2 ml of Sf-900 II SFM containing antibiotics was added. Cells were incubated in a 27 °C incubator for 72 hr. The virus from cell culture medium at 72 hr post-transfection was harvested. The Cdc45 and TopBP1 baculoviruses were stored at 4° C, protected from light. For long term storage, fetal bovine serum (FBS) was added to a final concentration of 2% and stored at –70° C. For amplifying viral stocks, High Five cells were infected with Cdc45 and TopBP1 P1 baculoviruses at a Multiplicity of Infection (MOI) of 0.01 to 0.1. The following formula was used:

\[
\text{Innoculum required (ml): } \text{desired MOI (pfu/cells) x (total number of cells)} \\
\text{Titer of viral innoculum (pfu/ml)}
\]

The virus was harvested at 48 hr post-infection. Approximately 100-fold amplification of the high titer Cdc45 and TopBP1 P2 baculoviruses were obtained. Using the same method, the highest titer of Cdc45 and TopBP1 P3 baculoviruses were obtained.

**Baculovirus infection**

High Five insect cells were infected individually or co-infected with P3 baculoviruses expressing either Cdc45, TopBP1, DUE-B or ΔCT DUE-B. The Multiplicity of Infection (MOI) of each baculoviruses infection was 5 or 10. After 48 hr, the infected cells were harvested and used for Western blot analysis, co-immunoprecipitation or purification.
Western blot analysis

High Five cells infected with all the baculoviruses described above (Cdc45, TopBP1, DUE-B and ΔCT DUE-B) individually or together were harvested. Cells were lysed in NP-40 containing lysis buffer (1% NP-40, 150 mM NaCl, 300 mM Tris and complete protease inhibitor cocktail (Sigma) and whole cell extracts were resolved on a 10% acrylamide SDS-PAGE gel for 1 hour at 120 volts and 2 hours at 200 volts. Proteins were then transferred to Immobilon-P membranes (Millipore) 1 hour at a constant current of 700 mA, then probed with DUE-B, Cdc45 and TopBP1 antibodies which were used at dilutions of 1:2000 in 5% nonfat dry milk in Tris buffered saline with Tween 20 (TBST). The horseradish peroxidase-conjugated secondary antibodies were used at dilutions of 1:10,000 in 5% nonfat dry milk in TBST as well. DUE-B antibody was raised in rabbits against 6xHis-tagged recombinant human DUE-B expressed in bacteria, which has been described previously (Casper et al., 2005). Cdc45 antibody was purchased from Santa Cruz Biotechnology (sc-20685). TopBP1 antibody was generously provided by Dr. Karnitz (Mayo clinic).

Co-immunoprecipitation

High Five cells cultured in 150 x 25 mm plates were co-infected with two of Cdc45, TopBP1, DUE-B and ΔCT DUE-B baculoviruses, then harvested and lysed in 1 ml of Triton-containing lysis buffer (0.5% Triton X-100, 20 mM HEPES (pH7.4), 150 mM NaCl, 12.5 mM β-glycerophosphate, 1.5 mM MgCl₂, 10 mM NaF, 2 mM dithiothreitol, 1 mM sodiumorthovanadate, 2 mM EGTA, 1 mM phenylmethylsulfonyl fluoride and complete protease inhibitor cocktail (Sigma)). Cell extracts were incubated
overnight with 1 µg of Cdc45, TopBP1 or DUE-B antibody or pre-immune serum (negative control) and 20 µl of protein A-Sepharose beads (prewashed and resuspended in phosphate buffered saline at a 1:1 (v : v) ratio). After incubation, the beads were washed three or four times with lysis buffer and resuspended in 20 µl of lysis buffer. For immunoblotting, the immunoprecipitates were separated by 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting as described above.

**Recombinant Cdc45, TopBP1 and DUE-B protein purification**

High Five cells were washed once with cold PBS, then lysed in lysis buffer containing 300 mM NaCl, 50 mM NaH$_2$PO$_4$ pH 8, 20 mM imidazole and 1% NP-40. 10 µl protease inhibitor cocktail (Sigma) per 1 ml lysate, and 0.5 mM DTT were added to the lysis buffer. 1 ml lysis buffer was added for every 15 million cells. Cells were lysed for 1 hour rotating at 4° C then centrifuged in an Eppendorf microcentrifuge at a maximum speed for 30 minutes at 4° C. The supernatant was then incubated with the nickel nitrilotriacetic acid (Ni-NTA) agarose beads (Qiagen) in a volume of 10 µl beads for ~1.5 ml whole cell extract for 4 hours at 4° C to purify the 6xHis-tagged recombinant Cdc45, TopBP1 and DUE-B proteins. Following three washes with the lysis buffer containing 20 mM imidazole, the purified protein was eluted using 500 µl elution buffer containing 250 mM imidazole. The purified proteins were concentrated using Microcon Centrifugal Filter Devices (Millipore). The proteins whose molecular weights are below 10 kDa were cut off.
Coomassie staining

High Five cells infected with Cdc45 or TopBP1 were harvested and lysed. Whole cell extracts were resolved on a 10% acrylamide SDS-PAGE gel and proteins were then transferred to Immobilon-P membranes (Millipore). The membrane was stained with the 0.1% (w/v) Coomassie blue R250, 20% (v/v) methanol, and 10% (v/v) acetic acid at room temperature for 15 to 20 min with gentle agitation. The Coomassie stain was removed after staining, and the membranes were incubated in the destain solution (50% (v/v) methanol in water with 10% (v/v) acetic acid) and allowed the membranes to destain with gentle agitation until the protein bands were seen without background.

Silver staining

Purified Cdc45, TopBP1 and DUE-B proteins were subjected to SDS-PAGE gel. The gel was placed in several gel volumes of Milli-Q water and shaken for 30 min. The water was replaced by fixing solution (50% ethanol, 10% glacial acetic acid) and shaken for 10 min. Then fixing solution was replaced by rinse solution (50% ethanol) and shaken for 5 min. Rinse solution was replaced by sensitizer (0.02% sodium thiosulphate) and shaken for 2 min. Sensitizing solution was replaced by Milli-Q water and shaken for 2 min. The water was replaced with cold staining solution (0.1% silver nitrate (Sigma Ultrapure) and shaken for 20 min. The gel was washed with water for 1 min (2X). After second wash, developing solution (2% (10 g) sodium carbonate, 200 ul (of 37% v/v) formaldehyde, 20 ml sensitizer) was added and shaken for 1 min. The developing solution was changed and shaken for 5 – 10 min as required or until the expected bands appear. The developing solution was discarded and stop solution (1% acetic acid) was
added and shaken for at least 5 min. The gel was washed with Milli-Q water (3X).

The binding assay of TopBP1, Cdc45 and DUE-B trimeric complex formation

The TopBP1, Cdc45 and DUE-B purified from insect High Five cells were put into 500 µl of 50 mM sodium phosphate buffer and pulled down overnight with 1 µg FLAG antibody and 20 µl of protein G-Sepharose beads (prewashed and resuspended in phosphate buffered saline at a 1:1 ratio). After incubation, the beads were washed three or four times with immunoprecipitate lysis buffer and resuspended in 500 µl of sodium phosphate buffer containing 400 µg/ml Flag peptide (Sigma F3290). After 4 hr incubation, the supernatant was then incubated overnight with 1 µg DUE-B antibody and 20 µl of protein A-Sepharose beads. After incubation, the beads were washed three or four times with immunoprecipitate lysis buffer and boiled with 20 µl sample buffer for 10 min. For immunoblotting, the immunoprecipitates were separated by 10% SDS-PAGE, transferred to Immobilon-P membranes (Millipore), and analyzed by immunoblotting with Cdc45 and DUE-B antibodies described above. A flow chart for this procedure is shown below (Figure 5):
**Figure 5. Flow chart for assay of trimeric complex formation.**

The mixture of purified DUE-B, Cdc45 and TopBP1 proteins was performed experiment according this procedure. Only Cdc45 in the trimeric DUE-B/Cdc45/TopBP1 complex is visualized.
Mass spectrometry analysis sample preparation

2 µg of purified Sf9 DUE-B or HeLa DUE-B, as mentioned above, was dissolved in 100 mM ammonium bicarbonate, pH 8.5. The trypsin (Sigma T6567) was dissolved in 1 mM HCl at a concentration of 1 mg/ml. The trypsin solution was added to the substrate protein solution at a ratio 1:20 (w/w) of enzyme to substrate. The enzyme and protein mixture was incubated 18 hours at 37 °C. Then 2 µl 1% HCl was added to stop reaction. The digested protein samples were characterized by mass spectrometry at the Proteome Analysis Laboratory, Wright State University.
RESULTS

Generation of recombinant Cdc45 and TopBP1 bacmid and baculoviruses

Our laboratory has demonstrated that DUE-B is essential for DNA replication (Casper et al., 2005) and proposed that it is the metazoan homolog of Sld3. Sld3 interacts with Cdc45 and Dpb11 and affects their loading to chromatin at pre-IC formation. To examine whether DUE-B has similar function as Sld3, DUE-B, Cdc45 and TopBP1 recombinant baculoviruses need to be generated. Dr. Michael Kemp previously generated DUE-B and C-terminal truncated DUE-B (ΔCT DUE-B) baculoviruses, therefore Cdc45 and TopBP1 recombinant baculoviruses were generated by using the Bac-to-Bac Baculovirus Expression System (Figure 6).

The pFAStBac™ donor plasmid containing human Cdc45 or TopBP1 cDNA was transformed into DH10Bac competent E. coli. Cdc45 and TopBP1 genes were integrated into parent bacmid with a lacZ-mini-attTn7 fusion to form two kinds of recombinant bacmid. The recombinant Cdc45 and TopBP1 bacmids were isolated by using Mini Plasmid Extraction kit (Omega). PCR analysis and restriction enzyme digestion were performed to identify if Cdc45 and TopBP1 genes were correctly integrated into baculovirus DNA (Figure 7). The M13F and M13R primers were used to identify the recombinant Cdc45 bacmid. Three of four chosen Cdc45 transformed clones had the correct 4.1 kb PCR products but not clone 4 (Figure 7A). The four PCR products of Cdc45 bacmid DNA were digested with the restriction enzyme Pst I. The 4.1 kb
PCR products showed the 2.5 kb, 1.0 kb and 0.5 kb three correct size bands (Figure 7B). The recombinant TopBP1 bacmid DNAs were amplified by PCR using TopBP1F and TopBP1R primers giving the correct 2.5 kb products (Figure 7C). The restriction enzyme BamH I was used to digest the 2.5 kb PCR products to get the correct 2.0 kb and 0.5 kb bands, which confirmed that the PCR products were TopBP1 cDNA (Figure 7D). All these PCR and restriction enzyme results showed human Cdc45 and Xenopus TopBP1 genes successfully integrated into parent bacmid.

Recombinant Cdc45 and TopBP1 bacmid DNA were isolated and transfected into Sf9 insect cells by using Cellfectin (Invitrogen). After 3 days, pure and low-titer recombinant Cdc45 and TopBP1 baculoviruses, called P1 baculoviruses, were obtained. Then the high-titer recombinant Cdc45 and TopBP1 baculoviruses (P2 and P3) were produced by using P1 baculoviruses to infect High Five insect cells.

Optimization of the expression of recombinant Cdc45 and TopBP1 proteins in High Five insect cells

To obtain the high expression of recombinant Cdc45 and TopBP1 proteins in insect cells, the different concentration of recombinant baculovirus used to infect High Five cells and the time course for harvesting infected cells must be performed to determine the point of maximum expression. High-titer P3 recombinant Cdc45 and TopBP1 baculoviruses (MOI (multiplicity of infection) = 5 and 10) were used to infect High Five cells, and the cells were harvested 1 and 2 days after infection. Cells were lysed with lysis buffer containing 1% NP-40 and whole cell extracts were analyzed by western blotting by probing with Cdc45 and TopBP1 antibodies (Figure 8). The western
Figure 6. Generation of recombinant baculovirus and gene expression with the Bac-to-Bac® Expression System.

The gene of interest is cloned into a pFAStBac™ donor plasmid, and the recombinant plasmid is transformed into DH10Bac™ competent cells which contain the bacmid with a mini-attTn7 target site on the bacmid in the presence of transposition proteins provided by the helper plasmid. Colonies containing recombinant bacmids are identified by disruption of the lacZ gene. High molecular weight mini-pre DNA is prepared from selected E.coli clones containing the recombinant bacmid, and this DNA is then used to transfect insect cells.
Figure 7. Generation of Cdc45 and TopBP1 recombinant bacmid.

The pFAStBac™ donor plasmid containing human Cdc45 or Xenopus TopBP1 cDNA was transformed into DH10Bac competent E. coli and formed Cdc45 and TopBP1 expression recombinant bacmids. (A) PCR identification of Cdc45 recombinant bacmid. The bacmid DNA of four Cdc45 clones were isolated and amplified by PCR using M13F and M13R primers. Three of four clones had the correct PCR products. (B) Restriction enzyme digestion of Cdc45 PCR products. The four PCR products of Cdc45 bacmid DNA were digested with the restriction enzyme Pst I and separated using 1% agarose gel electrophoresis. (C) PCR identification of TopBP1 recombinant bacmid. The bacmid DNA of four TopBP1 clones were isolated and amplified by PCR using TopBP1F and TopBP1R primers. All four clones had the correct PCR products. (D) Restriction enzyme digestion of TopBP1 PCR products. The four PCR products of TopBP1 bacmid DNA were digested with the restriction enzyme BamH I and separated using 1% agarose gel electrophoresis (M: marker; +: plasmid as template; -: no template; 1,2,3,4 represent the individual picked clones.)
result suggested that the maximum expression of both Cdc45 and TopBP1 was at 2 days after infection. High expression of Cdc45 and TopBP1 protein was obtained using high-titer P3 recombinant baculovirus (MOI=10) (Lane 9). The results also showed that the proteins expressed in High Five insect cells were the correct Cdc45 and TopBP1 proteins. The molecular weights of Cdc45 and TopBP1 are 62 kDa and 170 kDa respectively. The particular concentration of baculoviruses (MOI=10) and the time course (2 days infection) were used in all the experiments in this thesis.

**Full-length DUE-B interacts with Cdc45 and TopBP1 in High Five insect cell**

Although our laboratory has shown that DUE-B binds to chromatin and is required for Cdc45 and TopBP1 loading on chromatin using the *Xenopus* egg extract system (Chowdhury, unpublished data), and other groups (Schmidt et al., 2007) showed the interaction of human Cdc45 and TopBP1 in HeLa cells, all these experiments cannot eliminate the effects of additional replication factors. In order to test if DUE-B can bind Cdc45 and TopBP1 and minimize the contribution of other proteins, human His-tagged DUE-B, His-tagged Cdc45 and *Xenopus* His-Flag-tagged TopBP1 were overexpressed in High Five insect cells and immunoprecipitation experiments were performed to determine the binding of DUE-B, Cdc45 and TopBP1.

High Five insect cells were co-infected with two of the three recombinant DUE-B, Cdc45 and TopBP1 baculoviruses and harvested 2 days after infection and lysed with lysis buffer. The whole cell extracts were used for co-immunoprecipitation experiments. All the inputs and immunoprecipitated samples were subjected to SDS/PAGE gel and
Figure 8. Optimization of the expression of recombinant Cdc45 and TopBP1 proteins in High Five insect cells.

Cdc45 and TopBP1 recombinant baculoviruses (MOI (multiplicity of infection) = 5 and 10) were used to infect High Five cells, and the cells were harvested 1 and 2 days after infection. The whole cell extracts were used to perform western blotting experiments. Insect cells expressing recombinant Cdc45 and TopBP1 proteins were lysed in buffer containing 1% NP-40 and whole cell lysates were used for western blotting analysis. Lane 1 is lysate from uninfected High Five cells; Lanes 2-5 are the whole lysates of Cdc45 baculovirus infected High Five cells probed with Cdc45 antibody; Lanes 6-9 are the whole lysates of TopBP1 baculovirus infected High Five cells probed with TopBP1 antibody. (Lane 2, 3, 6, 7 insect cells harvested 1 day after infection; Lane 4, 5, 8, 9 insect cells harvested 2 days after infection; Lane 2, 4, 6, 8 insect cell infected with MOI=5 baculoviruses; Lane 3, 5, 7, 9 insect cell infected with MOI=10 baculoviruses; nonspecific bands were used as loading control.)
probed with DUE-B, Cdc45 and TopBP1 antibodies. As shown in Figure 9, when whole cell extracts co-infected with recombinant Cdc45 and DUE-B baculoviruses were immunoprecipitated with Cdc45 antibody and probed with DUE-B and Cdc45 antibodies, the DUE-B protein was pulled down together with Cdc45 (Lane 6). Similar results were obtained when lysates were immunoprecipitated with DUE-B antibody (Lane 7). This result suggested that overexpressed DUE-B binds to Cdc45 in High Five insect cells. The whole cell extracts from cells co-infected with recombinant Cdc45 and TopBP1 baculoviruses were immunoprecipitated with Cdc45 (Lane 8) or Flag-tag antibody (Lane 9), and both results showed that overexpressed Cdc45 also interacts with TopBP1 in insect cells. The interaction of DUE-B and TopBP1 was identified by co-immunoprecipitation experiments with Flag-tag (Lane 10) or DUE-B antibody (Lane 11). These are very exciting results because it is the first time to show that DUE-B interacts with human Cdc45 and *Xenopus* TopBP1, especially since these interactions occur in insect cells, which almost rules out the effects of other replication proteins. After these results were obtained, Shere Myers in our lab also got the similar results in *Xenopus* egg extract and HeLa cells (Myers, unpublished data). All these results suggested that overexpressed human DUE-B, Cdc45 and *Xenopus* TopBP1 interact with each other after minimizing the effects of other human replication proteins. In other words, each pair of these three proteins binds to each other directly. These results also suggested that DUE-B had the similar function as yeast Sld3 protein, which is essential for loading Cdc45 and TopBP1 on chromatin at initiation of DNA replication.
Figure 9. DUE-B interacts with Cdc45 and TopBP1 in High Five insect cells.
High Five insect cells were co-infected with two of the recombinant DUE-B, Cdc45 and TopBP1 baculoviruses and harvested 1 day after infection. The whole cell extracts were used for co-immunoprecipitation experiments. All the input and immunoprecipitated samples were subjected to SDS/PAGE gel and probed with DUE-B, Cdc45 and TopBP1 antibodies. Lanes 1-4: input (Lane 1: uninfected High Five cells; Lane 2: infected with Cdc45 and DUE-B recombinant baculoviruses; Lane 3: infected with Cdc45 and TopBP1 recombinant baculoviruses; Lane 2: infected with TopBP1 and DUE-B recombinant baculoviruses). Lanes 5-11: immunoprecipitated samples (Lane 5: uninfected High Five insect cells, immunoprecipitated with IgG antibody; Lanes 6-7: co-infected with Cdc45 and DUE-B recombinant baculoviruses, Lane 6 immunoprecipitated with Cdc45 antibody and lane 7 immunoprecipitated with DUE-B antibody; Lanes 8-9: infected with Cdc45 and TopBP1 recombinant baculoviruses; Lane 8 immunoprecipitated with Cdc45 antibody and lane 9 immunoprecipitated with Flag-tag antibody; Lane 10-11: infected with TopBP1 and DUE-B recombinant baculoviruses, Lane 10 immunoprecipitated with DUE-B antibody and lane 11 immunoprecipitated with Flag-tag antibody; Black circles indicated antibodies used for immunoprecipitation).
**DUE-B interacts with Cdc45 and TopBP1 through its c-terminal region in High Five insect cells**

Now, we want to know which part of DUE-B is responsible for the interaction with Cdc45 and TopBP1. The crystal structure of DUE-B showed that human DUE-B had an extra C-terminal 59 amino acids compared to bacterial and yeast homologs. DUE-B binds to dsDNA using its C-terminal tail (Kemp et al., 2007). To test whether the C-terminal tail of DUE-B is responsible for the interaction with Cdc45 and TopBP1, the overexpressed C-terminal truncated DUE-B (ΔCT DUE-B) protein was used to check the interaction with Cdc45 or TopBP1 (Figure 10).

High Five insect cells were co-infected with the recombinant ΔCT DUE-B and Cdc45 or ΔCT DUE-B and TopBP1 baculoviruses and harvested 1 day after infection. Cells were lysed in co-immunoprecipitation lysis buffer and the whole cell extracts were used for co-immunoprecipitation experiment. The whole cell extracts co-infected with recombinant Cdc45 and ΔCT DUE-B baculoviruses were immunoprecipitated with Cdc45 and probed with DUE-B and Cdc45 antibodies (Lane 3). The result showed that only Cdc45 protein was pulled down by Cdc45 antibody, but not DUE-B. This result suggested that C-terminal truncated DUE-B cannot interact with Cdc45 in High Five insect cells. The reverse immunoprecipitation experiment, immunoprecipitated with DUE-B antibody, also got similar result (Lane 4). The whole cell extracts overexpressing both ΔCT DUE-B and TopBP1 proteins were performed for immunoprecipitation experiment. Lane 6 was immunoprecipitated with Flag-tag antibody and probed with DUE-B antibody. Only Flag-tagged TopBP1 but not DUE-B showed in the lane. A similar result was given with anti-DUE-B antibody (Lane 7). So ΔCT DUE-B does not bind to TopBP1.
Figure 10. DUE-B interacts with Cdc45 and TopBP1 through its C-terminal region in High Five insect cells.

High Five insect cells were co-infected with the recombinant ΔCT DUE-B, and Cdc45 or ΔCT DUE-B and TopBP1 baculoviruses and harvested 1 day after infection. The whole lysates were used for co-immunoprecipitation experiments. All the input and immunoprecipitated samples were subjected to SDS/PAGE gel and probed with DUE-B, Cdc45 and TopBP1 antibodies. Lane 1: immunoprecipitated with IgG antibody; Lanes 2-4: co-infected with Cdc45 and ΔCT DUE-B recombinant baculoviruses, lane 2: input; Lane 3 immunoprecipitated with Cdc45 antibody and lane 4 immunoprecipitated with DUE-B antibody; Lanes 5-7: co-infected with TopBP1 and ΔCT DUE-B recombinant baculoviruses, lane 5: input; Lane 6 immunoprecipitated with Flag-tag antibody and lane 7 immunoprecipitated with DUE-B antibody. (Black circles indicated antibodies used for immunoprecipitation)
in insect cells either. Together these results indicated that DUE-B suggested with Cdc45 and TopBP1 through its C-terminal region in High Five insect cells.

**Purification of High Five insect cells expressing DUE-B, Cdc45 and TopBP1 proteins**

As mentioned before, full-length DUE-B interacts with Cdc45 and TopBP1 through its C-terminal region in High Five insect cell. Although these experiments were performed in High Five insect cells and minimized the effects of other human replication proteins, such as ORC, MCM2-7, there still exist many insect proteins, which may affect the binding of these three proteins. To definitely eliminate the effect of other proteins, His-tagged DUE-B, His-tagged Cdc45 and His-flag-tagged TopBP1 proteins overexpressed in High Five insect cells were purified and used to check the interaction between these three proteins.

Whole cell extracts from High Five cells expressing the DUE-B, Cdc45 and TopBP1 proteins with His-tag were purified using Ni-NTA agarose separately. Silver staining and western blotting experiments were performed to check purified DUE-B, Cdc45 and TopBP1 proteins. Figure 11A indicated Sf9 His-tagged-DUE-B, His-tagged-Cdc45 and His-Flag-tagged-TopBP1 proteins were obtained. The concentration of small molecular weight protein DUE-B (Lane1), 24kDa, was much higher than Cdc45 (Lane 2) and TopBP1 (Lane 3). Compared with BSA control (Lane 4-6), the His-tagged-DUE-B protein concentration was about 500 ng/µl. The concentration of His-tagged-Cdc45 and His-Flag-tagged-TopBP1 were about 20 ng/µl. The results of western blotting probed with DUE-B, Cdc45 and TopBP1 antibodies confirmed that the purified proteins were the
Figure 11. Purification of High Five insect cells expressed DUE-B, Cdc45 and TopBP1 proteins.

Whole cell extracts from High Five cells expressing the DUE-B, Cdc45 and TopBP1 proteins with His-tag were purified using Ni-NTA agarose separately. (A) Silver staining to check purified DUE-B, Cdc45 and TopBP1 proteins. Three kinds of purified and concentrated proteins were subjected to SDS/PAGE gel and staining with silver nitrate. Lane 1: DUE-B; Lane 2: Cdc45; Lane 3: TopBP1; Lanes 4-6: BSA standard series used for estimate the concentration of all the purified proteins, the concentration of BSA are 200 ng, 50 ng and 500 ng; Lane 7: protein marker. (B) The purified DUE-B, Cdc45 and TopBP1 proteins were subjected to SDS/PAGE gel and probed with DUE-B, Cdc45 and TopBP1 antibodies. Lane 1: DUE-B; Lane 2: Cdc45; Lane 3: TopBP1.
expected Sf9 DUE-B (Lane 1), Cdc45 (Lane 2) and TopBP1 (Lane 3) proteins (Figure 11B).

**DUE-B directly interacts with Cdc45 and TopBP1 in vitro**

After the purified His-DUE-B, His-Cdc45 and Flag-His TopBP1 proteins were obtained from High Five insect cells, interaction experiments between the three proteins were performed *in vitro* to test if DUE-B, Cdc45 and TopBP1 bind to each other directly.

The purified His-DUE-B, His-Cdc45 and Flag-His TopBP1 proteins were mixed together in sodium phosphate buffer and co-immunoprecipitation experiments were performed. All immunoprecipitated samples were subjected to SDS/PAGE gel and probed with DUE-B, Cdc45 and TopBP1 antibodies. As showed as Figure 12, when immunoprecipitated with Cdc45 antibody and probed with TopBP1, DUE-B and Cdc45 antibodies, the TopBP1 and DUE-B protein were pulled down together with Cdc45 (Lane 3). Similar results were obtained when immunoprecipitated with DUE-B and Flag-tag antibody (Lane 4, 5). The three proteins, DUE-B, Cdc45 and TopBP1 were pulled down together. All the data suggest that DUE-B, Cdc45 and TopBP1 interact with each other directly.

**DUE-B, Cdc45 and TopBP1 form one trimeric complex in vitro**

All the results from the insect cells and *in vitro* experiments indicate that DUE-B, Cdc45 and TopBP1 interact with each other directly. However, these experiments cannot prove whether these three proteins form one complex. To check whether the three proteins form one complex, the competitive co-immunoprecipitation experiment was performed (Figure 13).
Figure 12. DUE-B directly interacts with Cdc45 and TopBP1 in vitro.
Three kinds of DUE-B, Cdc45 and TopBP1 proteins purified from High Five insect cells were added to 50 mM Na$_2$PO$_4$ and performed immunoprecipitation experiment. All immunoprecipitated samples were subjected to SDS/PAGE gel and probed with DUE-B, Cdc45 and TopBP1 antibodies. Lane 1: mixture of 100 ng Cdc45, 100 ng TopBP1 and 500 ng DUE-B purified proteins; lane 2 immunoprecipitated with IgG antibody; Lane 3 immunoprecipitated with Cdc45 antibody; lane 4 immunoprecipitated with DUE-B antibody; Lane 5 immunoprecipitated with Flag antibody.
Figure 13. DUE-B, Cdc45 and TopBP1 form one complex in vitro.
The DUE-B, Cdc45 and TopBP1 proteins purified from High Five insect cells were added to 500 µl 50 mM Na$_2$PO$_4$ buffer and pulled down with 1 µg Flag antibody and 20 µl of protein G-Sepharose beads (prewashed and resuspended in phosphate buffered saline at a 1:1 ratio) overnight. After incubation, the beads were washed three or four times with Na$_2$PO$_4$ buffer and resuspended in 500 µl of Na$_2$PO$_4$ buffer containing 400 µg/ml FLAG peptide. After 4 hrs incubation, the supernatant was then incubated with 1 µg DUE-B antibody and 20 µl of protein A-Sepharose beads overnight. After incubation, the beads were washed three or four times with Na$_2$PO$_4$ buffer and boiled with 20 µl sample buffer for 10 mins. All samples were subjected to SDS/PAGE gel and probed with DUE-B and Cdc45 antibodies described above. Lane 1: whole cell extract infected with DUE-B and Cdc45 baculoviruses. Lane 2, the sample including the mixture of purified DUE-B, Cdc45 and TopBP1 proteins. Lane 3: the sample including the mixture of purified Cdc45 and TopBP1 proteins.
The purified His-DUE-B, His-Cdc45 and Flag-His TopBP1 proteins were mixed together in sodium phosphate buffer and pulled down with Flag antibody, which would be expected not to immunoprecipitate dimeric complexes of DUE-B and Cdc45. Excess Flag peptide was added into the product of the pull down and then the supernatant of released proteins were immunoprecipitated with DUE-B antibody. This is expected not to immunoprecipitate dimeric complexes of Cdc45 and TopBP1. The DUE-B immunoprecipitation sample was probed with Cdc45 (Lane 2). As the Lane 2 shows, the Cdc45 protein was pulled down together with DUE-B, which suggested that DUE-B, Cdc45 and TopBP1 proteins interact simultaneously to form one complex in vitro. As a negative control, TopBP1 and Cdc45 were mixed and the same reactions were performed (Lane 3). In the absence of DUE-B, Cdc45 cannot immunoprecipitated with DUE-B antibody.

Mass spectrometry to identify the covalent modification sites of Sf9 DUE-B and HeLa DUE-B

The phosphorylation of DUE-B appears to regulate its function. The previous data from our laboratory showed that Sf9 DUE-B functions as a dominant negative form and inhibited DNA replication in Xenopus egg extracts. But HeLa DUE-B (recombinant DUE-B expressed in HeLa cells) did not inhibit DNA replication and restored DNA replication to DUE-B depleted egg extracts (Casperet al., 2005). The C-terminus region of Sf9 DUE-B and HeLa DUE-B were phosphorylated at different sites (Kemp et al., 2007). CK2 phosphorylated Sf9 DUE-B binds Cdc45 weaker than the unphosphorylated form in Xenopus egg extracts (Chowdhury, unpublished data). According to these data,
we proposed that the covalent modification, i.e. phosphorylation, of DUE-B affects its activity.

To identify the different covalent modification sites of Sf9 DUE-B and HeLa DUE-B, purified DUE-B proteins from High Five cells and HeLa cells were digested by trypsin and then characterized by mass spectrometry (Figure 14). The results of Sf9 DUE-B showed that there is no covalent modification in all peptides detected by mass spectrometry, because the observed values and the expected values of all the peptides are the same. If there are covalent modifications, such as phosphorylation, the peptide mass should be increased 80 Da for each phosphate group. The mass spectrometry analysis of HeLa DUE-B is currently being done, but we expect that there may be covalent modifications in the HeLa DUE-B.
Figure 14. Mass spectrometry analysis of Sf9 DUE-B.
Purified Sf9 DUE-B was digested with trypsin and analyzed by mass spectrometry by the Proteome Analysis Laboratory, Wright State University.
DISCUSSION

The DNA unwinding element binding protein (DUE-B) was identified by our laboratory (Casper et al., 2005; Ghosh et al., 2006) and proven to be required for DNA replication. DUE-B is conserved throughout evolution, from bacteria to humans, but contains a unique 59 amino acid C- terminus present only in vertebrates. In *Xenopus* egg extracts, DUE-B is essential for the replication of sperm chromatin, acting after pre-RC formation and before origin unwinding (Casper et al., 2005; Ghosh et al., 2006). The DUE-B purified from HeLa cells, but not from Sf9 cells, can restore the DNA replication of DUE-B-immunodepleted *Xenopus* egg extracts (Casper et al., 2005). DUE-B co-localizes to origin DUEs with MCM and Cdc45 proteins *in vivo* and it is involved in loading Cdc45 and TopBP1 to chromatin in egg extracts (Chowdhury, unpublished data). DUE-B binds to the origin DNA transiently and it comes off DNA as the origin unwinds during DNA replication (Chowdhury, unpublished data). These results suggest that depletion of DUE-B inhibits DNA replication by preventing the loading of Cdc45 on chromatin.

All the evidence supports the possibility that DUE-B may be the metazoan homolog of yeast Sld3. The co-immunoprecipitation experiments show that DUE-B not only binds to Cdc45 and TopBP1 *in vivo* (Figure 9) and *in vitro* (Figure 12), but also binds to the two proteins directly (Figure 12). The Araki group (Kamimura et al., 2001) reports that in yeast Sld3 (synthetically lethal with dpb11-1) binds to the TopBP1 homolog Dpb11 and loads Cdc45 at the origin unwinding and polymerase-loading steps.
of chromosomal DNA replication. These results indicate DUE-B has the similar property of interacting with Cdc45 and TopBP1. These data also confirm the Hanel group’s data (Schmidt et al., 2007) that human TopBP1 binds to human Cdc45 in vitro and in vivo and is required for Cdc45 loading on chromatin.

The TopBP1 Xenopus homolog Xmus101 and yeast homolog Dpb11 are required for the recruitment of XCdc45 to origins of DNA replication (Van Hatten, et al., 2002; Kamimura et al., 2001). The interaction between Sld3 and Dpb11 is required for the loading of SCdc45 on chromatin. Sld3 binds to the N-terminal BRCT repeats of TopBP1 though its C-terminal region (Araki et al., 1995; Tanaka et al., 2007; Zegerman et al., 2007), and in a similar fashion, DUE-B binds to TopBP1 through its C-terminus (Figure 10). There is no data to show which part of Sld3 binds to SCdc45. Our data show that DUE-B binds to Cdc45 also through its C-terminus, which provides a clue that Sld3 may interact with SCdc45 through its C-terminal region.

The data that DUE-B, Cdc45 and TopBP1 interact with each other (Figure 9 and 12) suggests that these three proteins could form one complex. The data from Figure 13 shows that DUE-B, Cdc45 and TopBP1 form a heterotrimeric complex in vitro. The interaction between Sld3, Cdc45 and Dpb11 has been reported by several groups (Kamimura et al., 2001; Van Hatten, et al., 2002; Tanaka et al., 2007; Zegerman et al., 2007).

The Sf9 DUE-B appears to function as dominant negative DUE-B because its modification is different from functional HeLa DUE-B, which is supported by the mass spectrometry (Figure 14). There is no phosphorylation in the C-terminal region of Sf9 DUE-B, the region that is involved in DNA replication. But C-terminal region of HeLa
DUE-B is phosphorylated at three sites (Kemp et al, 2007). So the phosphorylation of DUE-B is necessary for its replication function. Sld3 is an essential CDK substrate and phosphorylation of Sld3, together with Sld2, is the minimal requirement for CDK-dependent activation of the initiation of DNA replication in budding yeast (Masumoto et al; 2002; Zegerman et al., 2007; Tanaka et al., 2007). Sld3 phosphorylated by CDK is required for binding to the C-terminal BRCT3 region of the TopBP1 yeast homolog Dpb11, which leads to polymerase loading in cooperation with Sld3-SCdc45 complex (Araki et al., 1995; Tanaka et al., 2007; Zegerman et al., 2007). However, the interaction between DUE-B, TopBP1 and Cdc45 is not required DUE-B phosphorylation by S-phase kinases (Figure 9, 12, 13). The DUE-B phosphorylation plays a role in releasing Cdc45 on chromatin (Chowdhury, unpublished data).

The metazoan homolog of Cdc45 and Dpb11 have already been isolated, but not the Sld3 homolog. The main reason is that Sld3, even within fungi, is very poorly conserved. *S. pombe* Sld3 shares only 14% identity and 24% similarity with *S. cerevisiae* Sld3. On the other hands, Cdc45 is well conserved from yeast to higher eukaryotes and the N-terminal BRCT repeats of Dpb11 are conserved in its metazoan homolog TopBP1 (Makiniemi et al., 2001; Yamamoto et al., 2000). All this evidence suggests that Sld3 should, like other important replication proteins such as Cdc45 and TopBP1, have a metazoan homolog.

Although human DUE-B only shares 4% identity and 14% similarity with *S. cerevisiae* Sld3, the C-terminal of DUE-B shares nearly 16% identity and 50% similarity with *S. cerevisiae* Sld3 and *S. pombe* Sld3 (Figure 2). The N-terminus of Sld2, another
Sld family member, shares 20% identity with its metazoan homolog RecQL4 (Sangrithi et al., 2005).

All these evidence supports the hypothesis that the human DNA replication protein DUE-B is the potential functional homolog of yeast Sld3.

To more directly test if DUE-B functions as the Sld3 metazoan homolog, DUE-B will be transformed into Sld3 temperature sensitive fission yeast strains to check whether DUE-B can restore the Sld3 function.
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