

The Role of Protein Kinases in DNA Replication in
Saccharomyces cerevisiae

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I, Stephen Sweet, confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

The initiation of DNA replication at the onset of S phase in eukaryotic cells is a critically important and tightly regulated process. Multiple origins of replication in the genome must be co-ordinately regulated such that duplication of the chromosomes is complete before cell division, whilst also ensuring that no sections of the DNA are over-replicated. In G1 phase of the cell cycle, a large ‘pre-replicative complex’ (pre-RC) forms at origins consisting of a hexameric Origin Recognition Complex (ORC) as well as Cdc6, Cdt1 and another hexameric complex known as the Minichromosome Maintenance (MCM) complex. At the onset of S phase, two cell cycle regulated protein kinases, the Cyclin Dependent Kinase (CDK) and Cdc7, are activated. Phosphorylation of various proteins by these two enzymes triggers formation of large ‘replisome’ complexes, initiation of DNA replication from each origin, and disassembly of the pre-RCs. Pre-RC re-assembly is subsequently inhibited until kinase activity falls again after cell division.

In this study, we have set about identifying substrates of both CDK and Cdc7 involved in DNA replication in the budding yeast *Saccharomyces cerevisiae*. Two techniques are employed, the *in vitro* phosphorylation of arrays of peptides and phosphorylation of pre-RCs assembled in cell-free yeast extracts. Peptide arrays provide a high throughput technique for screening large numbers of potential substrates in a single experiment, whilst pre-RC phosphorylation allows consideration of both tertiary and quaternary structures of the *in vivo* kinase substrate. Several potential novel substrates of both CDK and Cdc7 are revealed. Pre-RC phosphorylation also reveals a previously unreported phosphorylation of Orc1 by a third kinase which has been identified as Casein Kinase II (CKII).

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Chapter 1: Introduction

The eukaryotic cell cycle is divided into four distinct stages; DNA replication, which occurs during the Synthetic (S) phase, cell division, which occurs during the Mitotic (M) phase, and two Gap phases, G1 and G2, which precede S and M phases respectively. The initiation of DNA replication at the onset of S phase is a highly regulated, multi-step process. It is of critical importance to the cell not only to ensure that the entire genome is duplicated before cell division, but also to prevent re-replication of sections of the chromosomes in a single cell cycle. Initiation represents perhaps the most critical point of regulation in the process of DNA replication, and must only be allowed to occur at the correct stage of the cell cycle and when environmental conditions are permissive. It is of no surprise, therefore, that the protein machinery responsible and its regulatory mechanisms are complex.

Our current view of replication initiation derives from the paradigm of the replicon theory, proposed in 1963 by François Jacob, Sydney Brenner and François Cuzin (Jacob et al, 1963). They hypothesised that in order to begin replication a *trans*-acting 'initiator' element would bind to a *cis*-acting 'replicator' in the DNA, which would then trigger downstream replication events. This has proved true for simple prokaryotic and viral systems, also explains aspects of eukaryotic replication. In prokaryotes, the initiator protein DnaA binds to a single sequence specific origin of replication in the chromosome, *oriC*, leading to DNA unwinding and recruitment of replisome components (Messer, 2002). This marks the major regulatory step in the initiation of replication, coupling initiation to cell size and preventing origin re-initiation (Donachie & Blakely, 2003; Kaguni, 2006). In the eukaryotic virus Simian

Virus 40 (SV40), the Large T Antigen protein (TAg) similarly recognises the viral origin and triggers DNA unwinding and replisome formation. In eukaryotes, however, large genome sizes have given rise to the requirement for multiple origins of replication that, with the exception of the budding yeast *Saccharomyces cerevisiae*, remain largely poorly defined and are unlikely to be sequence specific (Cvetic & Walter, 2005). Origins are bound by the six-subunit Origin Recognition Complex (ORC). In G1 phase of the cell cycle a larger pre-replicative complex (pre-RC) forms from which replication can initiate after subsequent activation (Bell & Dutta, 2002). Pre-RC formation and activation is regulated at multiple levels to ensure once-and-only-once replication in each cell cycle (Diffley, 2004).

In both prokaryotic and eukaryotic cells, a replisome must be assembled subsequent to the formation either of the *oriC*-DnaA or pre-RC complexes. Replisomes consist of helicase and polymerase components capable of catalysing the unwinding of the DNA and the coordinated synthesis of both leading and lagging strands (Benkovic et al, 2001). In eukaryotes, formation of the replisome is regulated independently of pre-RC formation, and phosphorylation of several core replisome components by two cell cycle regulated protein kinases, the Cyclin Dependent Kinase (CDK) and Cdc7, is an absolute requirement for replisome formation and activation (Bell & Dutta, 2002). The roles of protein kinases in the positive regulation of replication initiation provide the focus of this study.

1.1 Initiation of DNA replication in *Escherichia coli*

Prokaryotes provide a useful model for understanding both the positive and negative regulation of initiation. *E. coli*, in common with all prokaryotes, contains a single chromosomal origin of replication, *oriC*, which binds the initiator protein, DnaA. DnaA is capable of causing DNA unwinding *in vitro* (Bramhill & Kornberg, 1988).

DNA unwinding by DnaA

E. coli oriC consists of a 245bp region (Oka et al, 1980) containing two elements, an AT rich DNA unwinding element (DUE) containing three tandem repeats of a 13mer sequence (Bramhill & Kornberg, 1988), and a DnaA binding region. The DnaA binding region contains five DnaA boxes R1-5 (Fuller et al, 1984; Matsui et al, 1985) of which three (R1, 2 and 4) bind tightly to DnaA with a K_D of between 3 and 9nM and conform exactly to the DnaA box consensus sequence 5'-TT(A/T)TNCACA-3'. The remaining two (R3 and R5) differ from the consensus by a single base and bind DnaA much more weakly, requiring cooperativity with an adjacent DnaA box (Schaper & Messer, 1995; Speck et al, 1999). In addition to these five sites, *oriC* also contains several other DnaA binding sites, including three I sites which differ from the DnaA box consensus by 3-4 bases (Grimwade et al, 2000) and also several 6-mer sites contained in the 13-mer repeats of the DUE, termed S-M sites (Speck & Messer, 2001). Crucially, binding to these sites is specific to the ATP-bound but not ADP-bound form of DnaA (Ryan et al, 2002; McGarry et al, 2004; Speck & Messer, 2001; Ozaki et al, 2008). Although DnaA binding to double stranded S-M sites is relatively weak, binding to the single-stranded DNA once the DUE is unwound is considerably stronger (Speck & Messer, 2001; Ozaki et al, 2008). DnaA binding to *oriC* was

observed to introduce a 40° kink in the DNA which was initially proposed to be responsible for inducing DNA unwinding by torsional stress (Schaper & Messer, 1995).

DnaA is an oligomeric ATPase of the AAA+ family (Neuwald et al, 1999; Messer et al, 2001) and has four structural domains (Messer et al, 1999). Domain I mediates oligomerisation via a hydrophobic patch on its surface (Abe et al, 2007) as well as binding to several other proteins including the DnaB helicase and DiaA (see below). Domain II appears to be a flexible linker domain and does not show sequence conservation between species (Messer et al, 1999), whilst domain III is the AAA+ domain. In common with other AAA+ proteins, the DnaA AAA+ domain contains an 'arginine finger' (Arg285) which interacts with ATP bound to an adjacent subunit. Domain III therefore contributes to DnaA oligomerisation in the ATP bound but not the ADP bound form of the protein. Arg285 is essential for cooperative binding of DnaA to the ATP-DnaA sensitive I sites (Kawakami et al, 2005). Domain IV is the major DNA binding domain, forming contacts with both the major and minor groove of the DnaA box consensus (Fujikawa et al, 2003). DNA binding also appears to be mediated by two residues in domain III, Val211 and Arg245, which are required for binding to the single but not double stranded S-M sites (Ozaki et al, 2008).

In vitro, DnaA is sufficient to cause unwinding of the DUE, but requires the presence of ATP (Bramhill & Kornberg, 1988). The process is not dependent on ATP hydrolysis however, since ATP γ S can substitute for ATP in open complex formation (Sekimizu et al, 1987). The open complex can be visualised by electron microscopy and contains between 20 and 30 molecules of DnaA (Fuller et al, 1984). The

structure of AMP-PCP bound *A. aeolicus* DnaA reveals a helical filament, formation of which is dependent on the presence of ATP (Erzberger et al, 2006). Helical initiator proteins have been predicted to form a sub-family of AAA+ ATPases, members of which include *E. coli* DnaC and the eukaryotic ORC complex (see below) (Iyer et al., 2003; Erzberger et al., 2006; Mott et al., 2008). The DNA is proposed to wrap around the outside of the DnaA filament, although when modelled to this structure, the Val211 and Arg245 residues of *Thermotoga maritime* DnaA are on the inside of the helix (Ozaki et al, 2008). DnaA binding to *oriC* can be followed by *in vitro* footprinting; DnaA remains bound in its ADP-bound state to DnaA boxes R1, 2 and 4 throughout the cell cycle, forming a larger complex immediately before the onset of replication (Cassler et al, 1995). Together, these findings enable a model of DNA unwinding by DnaA to be proposed. As the ATP-bound form of DnaA builds up in the cell, structural changes in the protein allow the helical filament to form which leads to DNA unwinding, stabilised by interactions between the single-stranded DNA and the DnaA residues Val211 and Arg245.

In addition to DnaA, unwinding of *oriC* is also influenced by several other proteins, including the two histone like proteins IHF and Fis. The sites of interaction of these proteins with *oriC* are known, with Fis binding between DnaA boxes R2 and R3 and IHF adjacent to site R1 (Gille et al, 1991; Filutowicz & Roll, 1990). Fis inhibits DNA unwinding *in vitro*, and prevents DnaA binding to R3 (Hiasa & Marians, 1994). In contrast, IHF stimulates DNA unwinding (Hwang & Kornberg, 1992) and overcomes the inhibition by Fis (Hiasa & Marians, 1994). At the onset of DNA replication, Fis binding to *oriC* is replaced by IHF coincident with the binding of DnaA to site R3 (Cassler et al, 1995). In common with DnaA, binding of IHF to *oriC* introduces a

kink into the DNA (Swinger & Rice, 2004). A third histone like protein, HU, also stimulates DNA unwinding *in vitro*, although its mechanism of action is unclear (Ryan et al, 2002). Finally, DnaA oligomerisation is promoted by binding to a protein called DiaA, a recently discovered homo-tetrameric protein that binds multiple DnaA molecules (Keyamura et al, 2007).

Regulation of replication initiation

As in eukaryotic cells, it is important for prokaryotes to prevent over-replication of the DNA by immediate re-initiation from replication origins which have recently fired. *E. coli* ensures that this is the case by three independent mechanisms; the regulated hydrolysis of ATP by DnaA, sequestration of newly synthesised, hemi-methylated *oriC* by binding to the protein SeqA, and depletion of DnaA by binding to a nearby locus, *datA*.

Regulatory inactivation of DnaA (RIDA)

ATP hydrolysis by DnaA is triggered by the formation of the replisome, being stimulated by the DNA PolIII holoenzyme β clamp (described below) and a second protein named Hda (Kurokawa et al, 1998; Katayama et al, 1998; Kato & Katayama, 2001; Katayama & Crooke, 1995; Su'etsugu et al, 2004). An Hda dimer forms a complex with the β clamp once it is loaded onto the DNA and promotes DnaA ATP hydrolysis by providing an arginine finger to the DnaA ATP binding domain (Xu et al, 2009). Hda has been predicted to trigger ATP hydrolysis in the DnaA unit at the end of the helical filament, thus causing sequential filament disassembly (Erzberger et al., 2006). Hda mutations increase ATP bound DnaA levels from 20 to 70 percent of the total population, resulting in over-initiation (Kato & Katayama, 2001; Riber et al,

2006; Fujimitsu et al, 2008). This mechanism of regulation is known as the Regulatory Inactivation of DnaA, RIDA.

oriC sequestration

oriC contains eleven repeats of the *dam* methyltransferase recognition site GATC, which have been shown to remain hemi-methylated for approximately one third of a cell generation. By contrast, other regions of the genome are re-methylated within minutes of their replication (Cleary et al, 1982; Campbell & Kleckner, 1990). Methylated *oriC* minichromosomes are incapable of transforming *dam*- strains, accumulating as hemi-methylated plasmids which cannot be replicated. Hemi-methylated templates are active for *oriC* mediated replication *in vitro*, however, (Russell & Zinder, 1987; Boye, 1991) so some mechanism must exist *in vivo* to prevent the replication of hemi-methylated *oriC* sites. An experiment to identify mutant strains in which hemi-methylated plasmids were capable of being replicated in a *dam*- background identified the protein SeqA, which was subsequently shown to bind *oriC* during the period for which it remains hemi-methylated. As with Hda, loss of *SeqA* resulted in over-initiation, leading to the conclusion that SeqA binding to hemi-methylated *oriC* inhibits initiation. (Lu et al, 1994; von Freiesleben et al, 1994). In support of this, SeqA has been shown to prevent DnaA binding specifically to sites R5, I2 and I3, although it allows binding to the strong DnaA boxes R1, R2 and R4 (Nievera et al, 2006).

DnaA sequestration

The *datA* locus is immediately adjacent to *oriC* and contains five recognisable DnaA boxes. However, it has been shown to bind approximately five-fold more DnaA

molecules than *oriC* (Kitagawa et al, 1996). The close proximity of *datA* to *oriC* means that it is replicated immediately after initiation and therefore provides a local sink for DnaA molecules immediately after replication. In support of this hypothesis, changing the position of the locus has been shown to have an effect on coordinated chromosome replication, although the locus itself is not essential for viability (Kitagawa et al, 1998). Together, the three processes of RIDA, *oriC* and DnaA sequestration provide overlapping mechanisms whereby *E. coli* prevents over-replication. As in eukaryotes (described below), utilising multiple redundant mechanisms of regulation ensure that disruption of one alone is insufficient to overcome overall control.

Coordination of initiation with cell size

Initiation of replication in *E. coli* occurs at a fixed size, known as the initiation volume, which is insensitive to fluctuations in DnaA levels (Donachie, 1968). This is therefore thought to be regulated by the ratio of ADP-bound to ATP-bound DnaA rather than absolute levels of the protein (Donachie & Blakely, 2003). Inhibition of DNA synthesis during replication leads to an increase in the relative amount of ATP to ADP-bound DnaA from 20 to 80 percent, and resumption of replication leads to multiple synchronous rounds of re-replication and a fall in the amount of ATP bound DnaA (Kurokawa et al, 1999). Recycling of ADP to ATP by DnaA has been shown to be stimulated by acidic phospholipids in the cell membrane (Crooke et al, 1992) (Sekimizu & Kornberg, 1988), and recently by specific sequences in the DNA (Fujimitsu et al, 2009). Newly synthesised DnaA is also expected to bind ATP (Kaguni, 2006; Sekimizu et al., 1987). Thus the levels of ATP bound DnaA fall immediately after initiation, but gradually increase during cell growth until a

threshold is reached at which *oriC* open complex formation is triggered and replication initiates.

Replication initiation in other prokaryotes

DnaA and *oriC* are widely conserved throughout eubacteria, and of those species so far analysed, only *Synechocytis* does not contain a recognisable *oriC* containing clusters of DnaA boxes (Richter et al, 1998). *Bactillus subtilis oriC*, for example, spans 560bp and contains three clusters of DnaA boxes, separated between the second and third clusters by the DnaA gene and with an AT rich region after the third (Ogasawara et al, 1985). This arrangement of DnaA boxes and the DnaA gene is conserved in multiple bacteria, including *Micrococcus luteus*, *Mycoplasma capricolum*, *Spirioplasm citri*, *Mycobacterium*, *Heliobacter pylori* and *Streptomyces*, and has thus been proposed to represent a common primordial *oriC* structure (Ogasawara et al, 1991). Other examples of *oriCs* include *Streptomyces lividans*, which has a 600bp *oriC* containing 19 DnaA boxes (Jakimowicz et al, 1998), and *Thermus thermophilus*, which contains thirteen DnaA boxes in two opposingly orientated clusters of six with one central box (Schaper et al, 2000).

DnaA structures are also adapted to the species in which they are found. For example, both *Streptomyces* and *Thermophilus* have GC rich DNA, and both have origins rich in DnaA boxes. However, whilst *Streptomyces* has a weaker DnaA box consensus containing a G or C at position 3, it contains a high affinity DnaA protein (Majka et al, 1999). *Thermophilus*, on the other hand, has a weaker binding DnaA protein but maintains the strong DnaA box consensus (Schaper et al, 2000).

Events downstream of DNA unwinding

In prokaryotes, DNA unwinding at the origin by DnaA is sufficient to trigger replisome formation and initiation of replication. Components of the *E. coli* replisome were initially identified by mapping of temperature-sensitive mutants defective in DNA replication (Carl, 1970) (Wechsler & Gross, 1971) and include the PolIII holoenzyme (described below), the hexameric DnaB helicase, the hexameric helicase loader DnaC, the primase DnaG and single-stranded binding protein (SSB). A double hexameric complex of DnaB₆DnaC₆ forms in an ATP dependent manner in solution (Wahle et al, 1989) and is recruited to the open complex via interactions between DnaA and both DnaB and DnaC, and DnaC interactions with single-stranded DNA (Learn et al, 1997; Marszalek & Kaguni, 1994; Sutton et al, 1998; Mott et al, 2008). DnaC is a structural paralog of DnaA, and is proposed to interact with the AAA+ domain at the end of the DnaA spiral (Mott et al, 2008). Recruitment of the DnaB-DnaC leads to the formation of a 'pre-priming complex', observed by EM to contain DnaA and DnaB but not DnaC (Funnell et al, 1987).

The pre-priming complex can be stabilised with a DnaB mutant defective in helicase activity. Analysis of complexes formed with this mutant shows that two DnaB hexamers are loaded in a head to head fashion, increasing the extent of unwinding in the open complex from 23 to 65 base pairs in the absence of helicase activity (Fang et al, 1999). DnaC is responsible for the loading of the DnaB hexamer via a 'ring-breaker' mechanism (Davey & O'Donnell, 2003). When bound in the presence of ATP, DnaC holds the ring shaped DnaB hexamer open, allowing entry of the DNA into the central cavity. This triggers ATP hydrolysis, release and closure of DnaB (Davey et al, 2002). The DnaB hexamers each encircle single-stranded DNA, and

migrate along the DNA with 5'-3' polarity (Kaplan, 2000; LeBowitz & McMacken, 1986). After loading of the DnaB hexamers, DnaC leaves the complex (Fang et al, 1999). The two active DnaB helicases slide past each other, and after sufficient unwinding the primase DnaG is recruited through interactions with DnaB (Fang et al, 1999). Recruitment of the polymerase holoenzyme to the primed site through interactions with DnaB and DnaG completes replisome formation.

The PolIII holoenzyme is a complex structure consisting of two PolIII core enzymes, one for the leading and one for the lagging strand, a β clamp processivity factor for each polymerase and a heptameric clamp loading complex. Each polymerase consists of a trimer of α , ϵ and θ subunits, of which α contains polymerase and ϵ exonuclease activity (Maki et al, 1985; Maki & Kornberg, 1985; McHenry & Crow, 1979; Studwell-Vaughan & O'Donnell, 1993). Both PolIII core enzymes are held onto the DNA by a dimeric β clamp, each monomer of which forms a crescent and which therefore together encircle the DNA and hold the polymerase in place, increasing its processivity from only 10 nucleotides per binding event to more than 50kb (Kong et al, 1992; Kuwabara & Uchida, 1981; LaDuca et al, 1986; Stukenberg et al, 1991).

The β clamp is itself loaded onto the DNA by the clamp loader complex which coordinates both PolIII core enzymes as well as the DnaB helicase and DnaG primase, and which therefore forms the heart of the replisome.

The clamp loader consists of γ , τ , δ , δ' , χ , ϕ subunits in a stoichiometry of $\gamma_1 \tau_2 \delta_1 \delta'_1 \chi_1 \phi_1$ (Glover & McHenry, 2000; Onrust et al, 1995; Pritchard et al, 2000). The γ and τ proteins are products of the same gene (*dnaX*), with τ being a larger product with two extra domains responsible for binding to PolIII α and DnaB (Gao & McHenry,

2001a; Gao & McHenry, 2001b). A pentamer of $\delta\delta'\gamma_3$ is a minimal enzyme capable of loading the β clamp, and forms a circular complex of structurally related subunits (Jeruzalmi et al, 2001a). δ binds to β , holding the dimer open by reducing the curvature of each of the crescent monomers (Jeruzalmi et al, 2001b). β binding by δ is stimulated by ATP binding by γ_3 , which are AAA+ family ATPases (Hingorani & O'Donnell, 1998; Naktinis et al, 1995). Binding to DNA then stimulates ATP hydrolysis, resulting in release of β around the DNA (Bloom et al, 1996; Turner et al, 1999). PolIII and the clamp loader compete for binding to β , so release of the clamp allows binding to the polymerase (Lopez de Saro et al, 2003; Naktinis et al, 1996).

The extra subunits of the complete clamp loader complex serve to coordinate the replisome machinery. Binding of PolIII and DnaB by the two extra domains of the τ subunits couple the leading and lagging strand polymerases to the helicase, and allows recycling of the lagging strand polymerase at each Okazaki fragment. The χ subunit binds to DnaG and to single-stranded DNA (Glover & McHenry, 1998; Kelman et al, 1998), and is therefore responsible both for the recruitment of the entire assembly to the primed origin, and also recycling of DnaG.

1.2 Eukaryotic replication initiation

Eukaryotic origins of replication

Unlike prokaryotes, the large size of eukaryotic genomes necessitates the coordinate activity of multiple origins of replication which must be regulated such that the genome is replicated in a timely fashion, whilst also ensuring that no origin fires twice in a single round of replication. Regulation is therefore more complex, since initiation cannot be universally inhibited following firing from a single origin.

The initial identification of a eukaryotic origin came from the observation that a specific DNA sequence, termed the Autonomously Replicating Sequence (ARS1) was capable of allowing the extrachromosomal maintenance of plasmids in *Saccharomyces cerevisiae* (Stinchcomb et al, 1979). The *S. cerevisiae* genome is estimated to contain approximately 200 to 400 origins (Rivin & Fangman, 1980) and approaches to identify them have included plasmid maintenance assays (Shirahige et al, 1993), two dimensional gel electrophoresis (Friedman et al, 1997), microarrays (Raghuraman et al, 2001; Wyrick et al, 2001; Yabuki et al, 2002) and mapping single-stranded DNA in hydroxyurea treated cells (Feng et al, 2006).

S. cerevisiae ARS elements are between 100 and 200bp in length and contain an essential A element containing the ARS Consensus Sequence (ACS) 5'- (A/T)TTTA(T/C)(A/G)TTT(A/T)-3' (Van Houten & Newlon, 1990) as well as B elements, three of which are found in ARS1 and any two of which are sufficient to maintain its origin function (Marahrens & Stillman, 1992). Together, the A and B1 elements in ARS1 function as a binding site for the eukaryotic initiator, the origin

recognition complex (ORC, described below) (Rao & Stillman, 1995) (Rowley et al, 1995). The B2 element is AT rich and was proposed to act as the site of DNA unwinding, since experiments showed that it could be functionally substituted with other easily unwound sequences (Huang & Kowalski, 1993) (Huang & Kowalski, 1996). However, comparison of different DNA sequences able to give B2 function at ARS1 did not show a correlation between the stability of the element and functionality (Wilmes & Bell, 2002). Instead, they were required to contain a second, imperfect match to the ARS consensus sequence, which may act by binding ORC or another pre-RC component (Wilmes & Bell, 2002) (Marahrens & Stillman, 1992) (Bell & Stillman, 1992). The B3 element contains a binding site for the transcription factor Abf1, which can be functionally replaced with binding sites for other transcriptional activators (Diffley & Stillman, 1988) (Diffley & Stillman, 1989) (Marahrens & Stillman, 1992). Similar analysis of ARS307 revealed the presence of two B elements, although there is very little conservation of this region between origins (Theis & Newlon, 1994). Abf1 also acts as an enhancer at other origins, for example ARS121, although most origins are found in non-transcribed regions of the genome (Walker et al, 1990). The start site for replication in ARS1 has been mapped to a single base pair between the B1 and B2 elements (Bielinsky & Gerbi, 1999).

Origins of replication in the fission yeast *Schizosaccharomyces pombe* were similarly identified by plasmid transformation, and have also been shown to be active in their chromosomal loci (Clyne & Kelly, 1995). However, *S. pombe* ARS elements are much larger than those of *S. cerevisiae*, stretching from 0.5 to 1 kb, and do not appear to contain any sequence specific binding consensus for ORC. Instead, *S. pombe* origins appear to be composed of stretches of AT rich sequences and are therefore

helically unstable. Deletion of small patches abrogates origin function, but patterns of AT rich elements show no conservation between origins and do not share sequence identity. Further, AT rich patches can be replaced with alternative, random stretches of AT rich DNA (Clyne & Kelly, 1995) (Okuno et al, 1999). Genome wide analysis has identified 384 'A+T rich islands', and of 20 randomly tested for ARS activity, 18 were active origins (Segurado et al, 2003). *S. pombe* Orc4 has been shown to contain repeats of an AT hook DNA binding motif, which is unique to fission yeast ORC and which partially explains origin specification in this species (Chuang & Kelly, 1999). Microarray mapping of origin binding proteins as well as BrdU incorporation into early firing origins in hydroxyurea treated cells identified 460 sites of pre-RC formation, of which 218 overlapped with the predicted AT rich island origins (Hayashi et al, 2007).

In metazoans, origins of replication move even further away from the replicon model of sequence defined replicator elements in the DNA. In both *Xenopus* and *Drosophila* embryonic systems, initiation appears to initiate at random throughout the genome (Hyrien & Mechali, 1992) (Mahbubani et al, 1992) (Shinomiya & Ina, 1991), although specificity is introduced during development, (Hyrien & Mechali, 1993) (Hyrien et al, 1995) (Sasaki et al, 1999). The random nature of initiation events in *Xenopus* and *Drosophila* embryos gives rise to a problem known as the 'random completion problem', since replication in these systems must complete within a finite time (Hyrien et al, 2003). The solution to this conundrum is still unclear, but may involve either structural definition of origins at fixed distances or the formation of an excess of complexes capable of acting as origins of replication which are then

regulated by a system of lateral inhibition. An increase in the rate of origin firing through S phase has also been proposed (Goldar et al, 2008).

Some evidence for sequence specific recruitment of ORC to origins of replication in *Drosophila* has been found from studies of chorion gene locus amplification in follicle cells. Replication of one of these loci was found to depend on two regions, ACE3 and ori β , of which ACE3 stimulates ORC binding to ori β and which together can direct amplification of an extragenic locus (Lu et al, 2001). *In vivo* evidence indicates that ORC binding to these regions is sequence dependent (Austin et al, 1999) requiring multiple elements in both ACE3 and ori β (Zhang & Tower, 2004). However, *in vitro* the affinity of dmORC for origin and non-origin DNA is similar (Remus et al, 2004). Any plasmid transfected into *Drosophila* Schneider cells will undergo autonomous replication, regardless of sequence (Smith & Calos, 1995).

Mammalian cells show a similar lack of sequence specificity, and plasmid replication assays have shown that any piece of human DNA of sufficient length will allow plasmid propagation in human kidney 293S cells (although bacterial DNA sequences are less efficient), initiating from random sites as in *Drosophila* (Heinzel et al, 1991) (Krysan et al, 1993). However, a handful of approximately 20-30 origins have been identified, which fall into two categories (Todorovic et al, 1999); ‘zones of initiation’, containing multiple potential sites of initiation, and origins where initiation begins at a single specific site in each cell cycle. Zones of initiation include the human rDNA locus and the Chinese hamster Rhodopsin and DHFR loci (Coffman et al, 2006; Vaughn et al, 1990). Site specific origins include the human lamin B2 gene, a 500bp region in which the site of initiation has been defined to a single nucleotide. The

lamin B2 origin can direct ectopic initiation, and is thus a true replicator sequence (Altman & Fanning, 2004). A further example is the human β -globin locus, which can similarly drive ectopic replication (Aladjem et al, 1998). However, initiation from this locus is also sensitive to deletions in a region 50kb upstream (Kitsberg et al, 1993).

No sequence identity is found between different mapped mammalian origins, and biochemical studies of human ORC indicate that beyond a slight preference for AT rich DNA, DNA binding is completely sequence independent (Vashee et al, 2003). Mechanisms of origin selection other than sequence specificity of ORC binding may include transcriptional regulation, DNA methylation, histone modification or recruitment of ORC via another sequence specific binding protein. Transcription may regulate origin selection either positively or negatively, and is likely to exert its effects through changes in chromatin structure. Almost all origins in *S. cerevisiae* are found in intergenic regions, and the transition from random to specific initiation in *Xenopus* occurs as transcription begins at the mid-blastula transition (Hyrien et al, 1995). However, transcriptional activators are required for the function of some origins, such as Abf1 at ARS1 in *S. cerevisiae*. DNA methylation at CpG sites appears to inhibit initiation, and under-methylated CpG islands are often associated with origins of replication (Delgado et al, 1998). Conversely, histone acetylation seems to promote initiation, and tethering histone deacetylases to the DNA decreases origin activity (Aggarwal & Calvi, 2004). Evidence for ORC recruitment by interaction with other chromatin bound factors comes from both *Drosophila* and human cells (Beall et al, 2002; Norseen et al, 2008; Tatsumi et al, 2008) (Atanasiu et al, 2006).

Origin recognition by the Origin Recognition Complex (ORC)

The first factor to be identified as specifically binding to the sequence specific origins of *S. cerevisiae* was Abf1, identified by its ability to cause an ARS specific DNA gel shift when purified from a yeast extract (Diffley & Stillman, 1988; Diffley & Stillman, 1989). Similar experiments employing a footprinting technique identified a six subunit complex termed the Origin Recognition Complex (ORC) (Bell & Stillman, 1992). ORC and Abf1 bind to ARS1 constitutively through the cell cycle and form a footprint on ARS1 *in vitro* resembling that seen *in vivo* in cells arrested in G2/M (Diffley et al, 1994), with ORC protecting the A and B1 elements and Abf1 binding to element B3 (Marahrens & Stillman, 1992). Temperature sensitive mutations subsequently confirmed the requirement of ORC for DNA replication; various *S. cerevisiae* Orc2 temperature-sensitive mutants arrest at the non-permissive temperature with unreplicated DNA, and show a defect in plasmid maintenance (Bell et al, 1993; Foss et al, 1993; Micklem et al, 1993). Both *orc2-1* and *orc5-1* temperature-sensitive alleles show defects in initiation from ARS1 when grown at the permissive temperature, measured by bubble arc formation in two-dimensional gel electrophoresis (Loo et al, 1995). ORC analogues have been identified in a diverse range of eukaryotic organisms, indicating that they represent a conserved method of origin recognition, even in organisms in which the origin is not sequence specific (Duncker et al, 2009).

The ORC complex is a heterohexamer made up of subunits Orc1-6, named in order of size (Bell et al, 1995), and binds to the DNA in an ATP dependent manner requiring both the A and B1 element of ARS1 (Bell & Stillman, 1992) (Rao & Stillman, 1995)

(Rowley et al, 1995). Sequence specific DNA binding of the *S. cerevisiae* complex is dependent on all subunits of the complex except Orc6, and Orc1, 2 and 4 all appear to bind to the major groove of the ACS. In addition, Orc5 crosslinks to both A and B1 elements (Lee & Bell, 1997). Although in *S. cerevisiae* Orc6 is not required for DNA binding, in *Drosophila* it is, and in both organisms it is an essential subunit of the complex (Li & Herskowitz, 1993; Balasov et al, 2007) It has been shown to be required for the recruitment of downstream components (see below) (Chen et al, 2007). The exact mechanism of DNA binding by the complex remains unclear however; the AT hook of *S. pombe* Orc4 is the only definitive DNA binding motif and is not conserved in other organisms.

Recent structural studies have provided information about the mechanism of DNA binding in archaeal ORC homologues (Dueber et al, 2007; Gaudier et al, 2007; Liu et al, 2000). Archaea appear to have origins reminiscent of those in prokaryotes, containing repeats of an 'origin recognition box' (ORB) in proximity to an AT rich region, but initiator proteins resembling those of eukaryotes (Grabowski & Kelman, 2003). Specifically, most species contain one or a few copies of an Orc1/Cdc6 homologue which is responsible for recognising the ORB. Orc1/Cdc6 from both *Aeropyrum pernix* and *Solfolobus solfataricus* bind the DNA through both a canonical winged helix DNA binding motif and their AAA+ domain. DNA binding introduces a 35° kink in the DNA and causes DNA unwinding (Gaudier et al, 2007)(Gaudier et al., 2007). Although the exact correlation between DNA binding mechanisms in archaeal and eukaryotic ORC proteins remains unknown, the winged helix domain may be conserved in eukaryotic Orc1 (Liu et al, 2000), and the eukaryotic ORC AAA+ domain shares with its archaeal counterpart an extended DNA binding loop.

The DNA interaction via both domains may therefore be a structural feature common to both eukaryotic and archaeal proteins, although ORC binding to the DNA in eukaryotes does not induce DNA unwinding (Gaudier et al, 2007).

ORC has ATPase activity, and Orc1-5 have all been identified as members of the AAA+ ATPase family (Klemm et al, 1997; Speck et al, 2005). Of these, Orc1 and Orc5 have been shown to bind and hydrolyse ATP, although it is the Orc1 ATPase activity, inhibited by double stranded DNA, that is important for the function of the complex, discussed below (Bowers et al, 2004; Klemm et al, 1997). ATPase activity in this subunit requires an ‘arginine finger’ from the adjacent Orc4 subunit, and mutation of this residue results in a non-functional complex (Bowers et al, 2004). An EM structure of the *Drosophila* ORC complex shows a filament structure similar to that observed for DnaA (Clarey et al, 2008; Clarey et al, 2006; Erzberger et al, 2006), and binding of ATP to the complex induces tightening of the filament which could explain ATP specific binding of ORC to ARS1. EM structures and subunit tagging experiments have assigned the positions of individual ORC subunits within the filament (Chen et al, 2008).

Although ORC appears to be constitutively associated with origins in *S. cerevisiae* (Diffley et al, 1994), it may be regulated in a cell specific manner in other organisms. In *Xenopus*, ORC seems to be released from the chromatin after initiation (Rowles et al, 1999; Sun et al, 2002). Mammalian Orc1 also seems to be reduced on the chromatin during mitosis (Kreitz et al, 2001; Natale et al, 2000). In support of this,

mammalian G1 nuclei can replicate in a *Xenopus* extract depleted of ORC, whilst G2 nuclei cannot (Yu et al, 1998).

Pre-replicative complex formation

The solution to the problem of coordinate regulation of multiple origins of replication has been shown to be a system of origin 'licensing' in which individual origins are licensed for initiation during G1 phase of the cell cycle, de-licensed at the onset of replication, and re-licensing inhibited until the subsequent G1 phase. Cell fusion studies showed that addition of a G1 phase nucleus to an S phase cell would induce replication in the G1 phase nucleus, but the same was not true of a G2 phase nucleus (Rao & Johnson, 1970). Experiments in *Xenopus* showed that DNA added to an egg extract could undergo formation of a nuclear envelope and a single round of replication (Blow & Laskey, 1986). G2 nuclei added to a G1 extract did not replicate, however, unless the nucleus was permeabilised (Blow & Laskey, 1988). This led to the 'licensing factor' theory, which hypothesised that DNA is bound by a licensing factor in G1 phase which is inactivated or destroyed on entry into S phase, and which is prevented from relicensing replicated chromatin until breakdown of the nuclear envelope at metaphase (Blow & Laskey, 1988). In fact, regulation of origin licensing is through the activity of the cyclin dependent kinase, as will be described.

Following the pattern of footprinting of ARS1 *in vivo* during the cell cycle reveals the existence of two complexes, a minimal complex present in G2-M phases consisting of ORC and Abf1, and a more extensive complex present in G1 phase. These were termed the pre and post replicative complexes (Diffley et al, 1994). In this respect, eukaryotic origins are similar to their prokaryotic counterparts, in which two distinct

complexes can also be resolved by footprinting during the cell cycle (Cassler et al, 1995). Formation of the pre-RC is considered responsible for the licensing of G1 phase nuclei.

The components of the pre-RC were defined in the latter half of the 1990s, and comprise Cdc6, Cdt1 and the hexameric MiniChromosome Maintenance (MCM) complex. Origin licensing can be reconstituted *in vitro* using these minimal purified components (Gillespie et al, 2001; Kawasaki et al, 2006). The end point of licensing is thought to be loading of the MCM complex in a salt stable manner onto the DNA, and ORC, Cdc6 and Cdt1 are all dispensable for replication in a *Xenopus* extract after this point (Rowles et al, 1999). Early evidence indicated that the MCM complex may function as the replicative helicase, and there is now convincing data that this is indeed the case (described below) (Koonin, 1993; Lee & Hurwitz, 2000; Moyer et al, 2006; Pacek et al, 2006).

Cdc6 was first identified as a gene required for the initiation of DNA replication in *S. cerevisiae* in a screen for cell division mutants (Hartwell et al, 1973). Temperature sensitive *cdc6* mutants were shown to result in a plasmid loss phenotype which could be complemented by increasing the number of ARS elements on the DNA (Hogan & Koshland, 1992), a phenotype similar to that later seen with ORC subunits (Loo et al, 1995). Over-expression of Cdc6 could complement the temperature-sensitive phenotype of *orc5-1* (Liang et al, 1995) and it was shown to be required for pre-RC formation but not binding of ORC to the origin (Cocker et al, 1996; Santocanale & Diffley, 1996). Like ORC subunits 1-5, Cdc6 is a member of the AAA+ ATPase family, and is highly related to Orc1. As with Orc1, mutation of the ATP binding or

hydrolysis motifs in Cdc6 results in a non-functional protein (Perkins & Diffley, 1998; Weinreich et al, 1999). Homologues of Cdc6 have been identified in other eukaryotes by sequence similarity to the yeast protein (Coleman et al, 1996; Kelly et al, 1993; Williams et al, 1997), and in *Xenopus*, immunodepletion of Cdc6 inhibits the replication of double but not single-stranded DNA, which can be restored by the addition of recombinant Cdc6 (Coleman et al, 1996).

The MCM proteins were identified as *S. cerevisiae* mutants defective either in plasmid maintenance (mini-chromosome maintenance) or cell cycle progression (Maine et al, 1984; Moir et al, 1982). Six proteins, Mcm2-7, are highly related, sharing a central 240 amino acid domain termed the MCM box, and appear to function together as a complex (Koonin, 1993; Madine et al, 1995; Labib et al, 2001). Although the sequence outside of the central conserved domain is highly divergent between proteins, it is conserved between species, arguing for a specific role of each protein. In support of this, deletion of any individual protein is lethal (for example (Gibson et al, 1990)).

Evidence for the role of the MCM proteins in the formation of the pre-RC came first from experiments in *Xenopus* egg extracts, in which the search for a positive licensing factor led to the identification of Mcm3, which had previously been suggested to act as a G1 phase specific licensing factor in yeast and *Xenopus* due to its behaviour cycling in and out of the cell nucleus (Kubota et al, 1995; Yan et al, 1993; Hennessy et al, 1990). A complex of MCM proteins was shown to bind to the chromatin during G1 and be displaced during S and G2 phases (Donovan et al, 1997; Madine et al, 1995). Cdc6 was required for this recruitment of the MCM complex to chromatin

(Aparicio et al, 1997; Donovan et al, 1997; Tanaka et al, 1997), but neither ORC nor Cdc6 was required for its maintenance. The MCM complex was shown to be required for formation of the G1 phase footprint on ARS305 (Labib et al, 2001) and was shown to be stable on the chromatin at salt concentrations up to 300mM NaCl (Donovan et al, 1997). Both ORC and Cdc6 can be removed from the chromatin after MCM association without affecting the ability of the licensed DNA to replicate in a *Xenopus* extract. The essential function of ORC/Cdc6 was therefore inferred to be the loading of a salt stable MCM complex onto the chromatin (Rowles et al, 1999). Interestingly, in both *Xenopus* and yeast it appears that MCM complexes are loaded in more than ten-fold excess of the number of active origins, the reason for which remains unclear (Lei et al, 1996).

Cdt1 was discovered first in *S. pombe* (Hofmann & Beach, 1994), and homologues were later identified in *Xenopus*, *Drosophila*, human cells and *S. cerevisiae* (Devault et al, 2002; Maiorano et al, 2000; Tanaka & Diffley, 2002; Whittaker et al, 2000). It was shown to behave in a manner similar to Cdc6, and is similarly required for the loading of the MCM complex both *in vivo* and *in vitro* (Devault et al, 2002; Maiorano et al, 2000; Nishitani et al, 2000). In *S. cerevisiae*, Cdt1 binds constitutively to Mcm2-7 (Tanaka & Diffley, 2002), and the Mcm2-7 binding C terminal domain of mouse Cdt1 has recently been shown to form a winged helix fold (Khayrutdinov et al, 2009).

Although the mechanism of Mcm2-7 loading by ORC, Cdc6 and Cdt1 is still unclear, recent biochemical studies have provided some details. Cdc6 and Cdt1 interactions with the chromatin are independent, and the Cdt1 interaction is transient, being

stabilised by the presence of the non-hydrolysable ATP analogue ATP γ S (Gillespie et al, 2001; Maiorano et al, 2000; Randell et al, 2006). As well as binding constitutively to Mcm2-7, Cdt1 in *S. cerevisiae* also interacts with the C terminus of Orc6. A fusion of Orc6 with Cdt1 is capable of driving a single round of Mcm2-7 complex loading, although reiterative loading is prevented (Chen et al, 2007). Blocking ATP hydrolysis by Orc1 by introducing a mutation into the arginine finger of Orc4 blocked the reiterative loading of Mcm2-7, but a single round of loading was permitted (Bowers et al, 2004). However, blocking ATP hydrolysis entirely by both ORC and Cdc6 completely abolished salt stable loading of the complex (Randell et al, 2006). A model for Mcm2-7 complex loading by ORC, Cdc6 and Cdt1 has therefore emerged. Orc6 acts to recruit and Mcm2-7-Cdt1 complex, whilst ATP hydrolysis by Cdc6 and Orc1 act sequentially to catalyse the loading of the complex and its release from the pre-RC in order to allow reiteration of the process.

Interestingly, EM structures of *S. cerevisiae* ORC either alone or in complex with Cdc6 show that Cdc6 contributes to the structure to form an asymmetric ring with a diameter similar to the Mcm2-7 complex. The six AAA+ domains of Orc1-5 and Cdc6 could therefore form a ring loader machine with function comparable to the RFC (below) or *E. coli* clamp loader complexes (Perkins & Diffley, 1998; Speck et al, 2005).

Mcm2-7 complex structure and function

The initial suggestion that an MCM complex acts as the replicative helicase came from the observation that the central AAA+ domain shares common motifs with a family of DNA and RNA helicases (Koonin, 1993), coupled with the fact that unlike ORC and Cdc6 the complex appears to migrate with the replisome after initiation (Aparicio et al, 1997). Degradation of Mcm2-7 during S phase was shown to prevent replication fork progression (Labib et al, 2000). As well as the complete hexamer, various subcomplexes can also be readily purified, including Mcm4,6,7, Mcm2,4,6,7 and Mcm3,5 (Lee & Hurwitz, 2000). Of these, helicase activity was observed in the Mcm4,6,7 complex, but puzzlingly not in the complete hexamer (Ishimi, 1997; Lee & Hurwitz, 2000). EM reconstruction of the structure of the Mcm4,6,7 complex revealed a hexameric ring (Sato et al, 2000), a structure not only analogous to *E. coli* DnaB, but also to the replicative helicases from a variety of other systems, including SV40, Papillomavirus and the bacteriophages T4 and T7 (Dong et al, 1995; Egelman et al, 1995; Li et al, 2003a; Sedman & Stenlund, 1996; VanLoock et al, 2002; Yang et al, 2002). Further evidence that the complex is likely to act as the replicative helicase came from archaea, which all contain at least one homologue of the eukaryotic Mcm2-7 proteins. Archaeal MCM proteins from several species have been shown to form oligomeric complexes with a robust helicase activity (Chong et al, 2000; Gomez-Llorente et al, 2005; Grainge et al, 2003; Kelman et al, 1999; Pape et al, 2003; Shechter et al, 2000). The most recent experiments with the eukaryotic complex have demonstrated both a weak helicase activity in the complete Mcm2-7 hexamer (Bochman & Schwacha, 2008) and a robust helicase activity when combined with two accessory factors, Cdc45 and GINS (described below) (Moyer et al, 2006; Pacek et al, 2006).

Despite the availability of several structures of the archaeal proteins, however (Bae et al, 2009; Brewster et al, 2008; Fletcher et al, 2003; Liu et al, 2008), reviewed in (Sakakibara et al, 2009), the mechanism of action of the complex remains unclear. Several models have been suggested, including a steric exclusion model analogous to *E. coli* DnaB, in which the complex migrates along single-stranded DNA and forces the duplex apart by excluding the other strand from the central channel (Kaplan et al, 2003; Lee & Hurwitz, 2001), a rotary pump model, in which hexamers are located at distant sites and pump DNA towards the replisome (Laskey & Madine, 2003), a model based on the SV40 virus large T antigen, which operates as a double hexamer and extrudes single-stranded DNA at the hexamer interface (Wessel et al, 1992), and a ploughshare model in which a structural unit, which may be part of the MCM complex or be formed by another protein, forces the DNA apart as it leaves the central channel (Takahashi et al, 2005). It is interesting to speculate that Cdc45 and GINS may in part be required to provide the ploughshare that this model predicts.

Activation of the pre-RC

Unlike in prokaryotes, where formation of the large ATP-DnaA complex is sufficient to cause DNA unwinding and replisome formation, pre-RCs in eukaryotes are not immediately active for replication fork formation. There is no evidence for unwinding of the DNA on pre-RC formation, despite loading of a central part of the helicase, and indeed, the temporal gap between pre-RC formation in G1 and firing of the origin at the start of S phase would make this impractical. Activation of the replisome is via the activity of two cell cycle regulated protein kinases, the cyclin dependent kinase (CDK) and Cdc7.

CDK

CDK is the principal driver of the eukaryotic cell cycle, and its activity is regulated by the presence of a variety of regulatory subunits, termed 'cyclins' due to oscillation of their expression and stability during the cell cycle. In yeast only one CDK exists, Cdc28 in *S. cerevisiae* and Cdc2 in *S. pombe* (Nurse & Bissett, 1981). These regulate different stages of the cell cycle by association with different sets of cyclins, which can be categorised according to their expression pattern as acting in G1 phase, S phase or M phase of the cell cycle. In *S. pombe* there are four cyclins, the G1 phase cyclin Puc1 (Forsburg & Nurse, 1991; Martin-Castellanos et al, 2000), the S phase cyclins Cig1 and Cig2, and the M phase cyclin Cdc13 (Mondesert et al, 1996; Moreno et al, 1989). In *S. cerevisiae* there are nine, Cln1-3 and Clb1-6 (Nasmyth, 1993). Cln1-3 are G1 cyclins, expressed in late G1 phase in response to nutrient availability in order to trigger commitment to the cell cycle by activating the expression of the B type cyclins (Levine et al, 1995). The S phase cyclins Clb5 and Clb6 act initially to trigger DNA replication (Schwob & Nasmyth, 1993; Donaldson et al, 1998; Epstein & Cross, 1992) and subsequently the M phase cyclins Clb1-4 drive mitosis (Fitch et al, 1992). Degradation of cyclins at the end of mitosis occurs by a mechanism common to all eukaryotic cells and is triggered by activation of the anaphase promoting complex/cyclosome (APC/C), an E3 ubiquitin ligase which targets the cyclins for destruction via the proteasome (reviewed in (Zachariae & Nasmyth, 1999)). APC activity is regulated by association with two regulatory subunits, Cdc20 and Cdh1, of which APC^{Cdc20} is activated first, by phosphorylation of various APC subunits by M phase CDK activity leading to Cdc20 association (Fang et al, 1998; Shteinberg et al, 1999). This targets the M phase cyclins for destruction, leading to dephosphorylation of the APC and dissociation of Cdc20. Cdh1 binding, which is in

contrast inhibited by CDK phosphorylation of Cdh1, is now allowed and maintains APC activity. APC^{Cdh1} targets Cdc20 for degradation, and its activity remains high until Cdh1 is re-phosphorylated in late G1 phase (Zachariae et al, 1998).

In higher eukaryotes, CDK activities are slightly more complex. Four CDKs regulate the cell cycle, CDK1, 2, 4 and 6, and a host of other CDKs control other cellular processes, such as transcription (Morgan, 1997). CDKs 4 and 6 associate with cyclin D in response to growth factors in order to trigger commitment to division by degrading the protein pRb (Bartek et al, 1996; Sherr, 1995). pRb binds to and suppresses the activity of E2F family members, which are transcriptional activators (van den Heuvel & Dyson, 2008). Activation of E2F leads to transcription of downstream cyclins, therefore leading to cell cycle progression. CDK2 interacts with cyclin E to trigger the initiation of DNA replication, and subsequently binds cyclin A throughout S phase. Mitosis is triggered subsequent to S phase by the association of CDK1 with cyclin B (Nigg, 1995).

In both yeast and higher eukaryotes, CDK activity is also dependent on phosphorylation at a conserved threonine (Thr160 in human CDK2) by the CDK Activating Kinase (CAK). In *S. cerevisiae* this is the 44-kDa protein Cak1 (Espinoza et al, 1996; Kaldis et al, 1996; Thuret et al, 1996), whilst in higher eukaryotes, the CAK activity is itself a CDK, Cdk7-Cyclin H (Nigg, 1996). Cdk7-cyclin H is also involved in transcription, phosphorylating the C terminal domain of RNA polII (Fisher, 2005). Although CDK phosphorylation by CAK is necessary for function, it does not seem to be rate-limiting or regulated during the cell cycle (Espinoza et al, 1996). CDK activity is also regulated by the presence of inhibitory subunits (CKIs)

such as *S. cerevisiae* Far1 and Sic1, *S. pombe* Rum1, and the higher eukaryotic Cip/Kip and Ink4 families (Morgan, 1997). In *S. cerevisiae*, *SIC1* transcription is triggered in late mitosis by the nuclear import of the transcription factor Swi5, and Sic1 is degraded at the end of G1 following phosphorylation by the Cdc28-Cln complex (Schwob et al, 1994; Verma et al, 1997). It therefore provides a mechanism of inhibiting CDK activity throughout G1 phase of the cell cycle.

Finally, CDK activity is also regulated by inhibitory phosphorylation of the conserved tyrosine Tyr15, which provides a mechanism of regulation of CDKs in an all-or-nothing manner due to positive feedback loops. The classic model of Tyr15 regulation is that of phosphorylation of Cdc2 by Wee1 and dephosphorylation by Cdc25 in *S. pombe* (Coleman & Dunphy, 1994). CDK dependent Wee1 phosphorylation during mitosis inhibits activity, whilst similar phosphorylation of Cdc25 increases its activity, hence triggering CDK1 dephosphorylation at the beginning of mitosis. Other kinases, such as polo-like kinase, also contribute to the Wee1 and Cdc25 phosphorylation during G2 phase (Coleman & Dunphy, 1994). Considerable structural information is available regarding the mechanism of activation of CDK activity by cyclins and CAK activity, as well as Tyr15 phosphorylation and binding by CKIs, and is reviewed in (Morgan, 1997).

The earliest evidence that CDK activity is required for activation of origins of replication came from studying replication *in vitro* of the viral SV40 system, in which the viral origin recognition protein (large T antigen, TAg) required CDK dependent phosphorylation to initiate replication (McVey et al, 1989). A more direct role for activation of chromosomal replication was demonstrated in *Xenopus*, where depletion

of CDK prevented egg extracts from initiating replication, although they remained capable of formation of intact nuclei and of elongating replication forks formed in undepleted extracts (Blow & Nurse, 1990). Treatment of extracts with a protein kinase inhibitor also inhibited initiation (Blow, 1993). In *S. cerevisiae*, a *cln1 cln2 clb5 clb6* deletion mutant arrested with unreplicated DNA (Schwob & Nasmyth, 1993).

The exact substrates involved in triggering DNA replication have until recently remained elusive however. CDK requires a minimal consensus sequence of S/T-P for substrate phosphorylation, and a basic residue at the +3 position strengthens this consensus, S/T-P-x-K/R (Songyang et al, 1994). Recruitment via an 'RxL' motif is also known to aid substrate specificity, thought to be mediated by binding to the cyclin subunit (Adams et al, 1996; Wilmes et al, 2004). Screening for *in vitro* phosphorylation of GST Tagged proteins from budding yeast identified 360 targets of Cdc28, including Orc1, Orc2, Orc6, Cdc6 and Mcm3 (Ubersax et al, 2003). Although most of these substrates did not show specificity to either Clb2 or Clb5 bound Cdc28, several of the replication proteins were phosphorylated tenfold more rapidly by Clb5-Cdc28 than by Clb2-Cdc28 (Loog & Morgan, 2005). The essential substrates however have been shown to be the two downstream factors Sld2 and Sld3, phosphorylation of each of which stimulates binding to a third factor, Dpb11 (Tanaka et al, 2007; Zegerman & Diffley, 2007; Tak et al, 2006). The roles of each of these proteins will be described below. CDK also plays a negative role in regulating pre-RC formation, which will also be described in a subsequent section.

Cdc7

The Cdc7 kinase is regulated similarly to CDK; it is expressed constitutively but its activity is regulated by association with a cyclically expressed regulatory subunit, Dbf4. It has therefore been termed the Dbf Dependent Kinase, DDK. Cdc7 was discovered in Hartwell's cell division mutant screen (Hartwell et al, 1973) and Dbf4 in a screen for mutants defective in the initiation of DNA replication (Johnston & Thomas, 1982a; Johnston & Thomas, 1982b). Cdc7 was shown to be a serine-threonine kinase (Patterson et al, 1986; Yoon & Campbell, 1991) and its activity varied with the cell cycle, despite protein levels remaining constant (Yoon et al, 1993). Dbf4 and Cdc7 were shown to interact both genetically and physically (Dowell et al, 1994; Kitada et al, 1992), and Dbf4 levels to fluctuate during the cell cycle, driven both by changes in expression (Chapman & Johnston, 1989) and in protein stability, regulated like the cyclins by the anaphase promoting complex (Ferreira et al, 2000; Oshiro et al, 1999; Weinreich & Stillman, 1999). *S. cerevisiae* Cdc7-Dbf4 was shown through one-hybrid assays to interact with replication origins in a manner dependent on the ORC binding ACS sequence (Dowell et al, 1994) and is required for the firing of individual origins, rather than as a global S phase trigger (Bousset & Diffley, 1998; Donaldson et al, 1998). Origin interaction is via Dbf4, since the Cdc7 and Dbf4 interacting domains of Dbf4 could be functionally separated, locating to the C and N termini of the protein respectively (Dowell et al, 1994). Cdt1 has recently been proposed to play a role in recruiting Cdc7-Dbf4 to origins in human cells through an interaction with the catalytic subunit (Ballabeni et al, 2009).

Cdc7 and Dbf4 both have homologues in higher eukaryotes. Hsk1 was cloned from *S. pombe* as a kinase with 65% similarity to Cdc7, and was required for DNA

replication (Masai et al, 1995). Homologues were subsequently found in a variety of higher eukaryotes (Jiang & Hunter, 1997; Kim et al, 1998). The *S. pombe* Dbf4 homologue Dfp1 was identified as an activating subunit which purified with Hsk1 (Brown & Kelly, 1998), and independently through two-hybrid screening (Takeda et al, 1999). Human Dbf4, named 'Activator for S-phase Kinase' (ASK) was also identified by two-hybrid screening (Kumagai et al, 1999). Expression of both Dfp1 and ASK is regulated like Dbf4 in a cell cycle dependent manner (Brown & Kelly, 1998; Kumagai et al, 1999; Takeda et al, 1999) and are responsible for the activation of Hsk1 and mammalian Cdc7 respectively. Depletion of Cdc7 from *Xenopus* egg extracts resulted in a complete loss of replication activity (Jares & Blow, 2000). Sequence comparison of Dbf4 homologues has identified three conserved motifs at the N and C termini and in the central portion of the protein, termed motifs N, M and C. Motifs M and C are both required for binding and activation of Cdc7, whilst motif N is required for chromatin association (Ogino et al, 2001). As well as its role in replication, Cdc7 also has separate functions in several processes including meiosis, the DNA damage checkpoint and control of mitotic exit (Marston, 2009; Ogi et al, 2008; Miller et al, 2009) .

Origin activation by Cdc7

A steadily growing body of evidence suggests that the substrates of Cdc7-Dbf4 include subunits of the Mcm2-7 complex. A Cdc7 bypass mutant exists (*bob1*) which contains a proline to leucine point mutation in Mcm5 (Hardy et al, 1997), although Mcm5 does not itself appear to be a Cdc7 substrate. Instead, other members of the complex are efficiently phosphorylated by the kinase. Purified Cdc7-Dbf4 from *S. cerevisiae* phosphorylates Mcm2, 3, 4, 6 and 7 *in vitro*, as well as undergoing

autophosphorylation on both subunits (Weinreich & Stillman, 1999). Mcm2-7 complex subunit phosphorylation by Cdc7 was also reported in *S. pombe* and higher eukaryotes, with Mcm2 reported as the primary substrate, both alone and in the context of an Mcm2-4-6-7 complex (Brown & Kelly, 1998; Jiang et al, 1999; Kihara et al, 2000; Kumagai et al, 1999; Lei et al, 1997; Masai et al, 2000; Sato et al, 1997; Takeda et al, 1999). Phosphorylation of the Mcm2-7 complex has been shown to be stimulated by loading onto the DNA (Francis et al, 2009). Phosphorylation of human Mcm2 and Mcm4 by Cdc7 is within the N terminus, and mapping of phosphorylation sites has shown a requirement for an acidic or phosphorylated residue in the +1 position (Cho et al, 2006; Ishimi et al, 2001; Masai et al, 2006; Montagnoli et al, 2006). Multiple Cdc7 phosphorylation sites in *S. cerevisiae* Mcm4 were also shown to be located in the N terminus of the protein, and efficient phosphorylation required a binding site immediately C terminal to the region of phosphorylation (Sheu & Stillman, 2006). Phosphorylation sites in the N terminus were required for S phase progression, but the entire region could be functionally substituted with the N terminus of Mcm2, which has also been shown to be phosphorylated in two residues, S164 and S170, of which S170 is essential for growth but can be bypassed with *cdc7-bob1* (Bruck & Kaplan, 2009). Cdc7-Dbf4 seems to be recruited to Mcm2 by a similar mechanism as to Mcm4. It thus appears that Mcm2 and Mcm4 represent essential Cdc7 substrates, although whether they form a minimal set is unknown. A previous report has suggested that phosphorylation of the N termini of Mcm2 and Mcm4 is redundant (Masai et al, 2006).

One possible explanation for the bypass of kinase function by the modification of a protein which is not itself a substrate is that phosphorylation of adjacent subunits in

the complex induces a structural change which is mimicked by the Mcm5 point mutation (Hoang et al, 2007). In support of this, Mcm5 has been mapped to a position adjacent to Mcm2 in the complex (Davey et al, 2003). Structural changes are induced in the *M. thermoautotrophicus* protein when an equivalent mutation is introduced (Fletcher et al, 2003). However, phosphorylation of neither the Mcm2-4-6-7 complex nor the complete Mcm2-7 hexamer activates its helicase activity, so phosphorylation may provide a binding site for other downstream factors. In support of this phosphorylation of the N terminus of Mcm4 has been shown to facilitate its interaction with the downstream factor Cdc45 on the chromatin (Masai et al, 2006). However, Cdc45 is also an *in vitro* target of Cdc7 (Nougarede et al, 2000), as is Pol α (Weinreich & Stillman, 1999), so it remains unclear whether or not the Mcm2-7 complex subunits do in fact represent the only important substrates of Cdc7 *in vivo*. There is as yet no phospho-site specific bypass of the kinase.

Interaction of CDK and Cdc7 activities

The ability of a phosphorylated residues in the +1 position to stimulate substrate phosphorylation by Cdc7 (Cho et al, 2006) implies that the two kinases may function together in the phosphorylation of Cdc7 substrates. Indeed, multiple SSP motifs are found in the N termini of Mcm2, Mcm4 and Mcm6. However, the temporal order of CDK and Cdc7 function has been a matter of some debate. Reports have shown that CDK activity is required upstream of Cdc7 function in *S. cerevisiae* (Nougarede et al, 2000), but the reverse was demonstrated in *Xenopus* (Walter, 2000). Facilitation of Mcm2 phosphorylation by Cdc7 by prior phosphorylation by CDK has been confirmed *in vitro* with the human proteins, but no evidence exists *in vivo* (Cho et al, 2006; Masai et al, 2000; Masai et al, 2006). It could be, however, that CDK functions

at two points in triggering replication initiation, first by priming substrates for phosphorylation by Cdc7, and second by triggering the binding of Sld2 and Sld3 to Dpb11. CDK has been shown to prime the phosphorylation of at least one other substrate of Cdc7, involved in double strand break formation in meiosis (Wan et al, 2008).

Prevention of re-replication

As well as its positive role in stimulating DNA replication at the onset of S-phase, CDK activity also plays a well-documented negative role in inhibiting reformation of the pre-RC, thus acting as a master-regulator of the oscillatory nature of pre-RC formation and DNA replication. During the period in G1 when CDK activity is low, origin licensing is allowed but licensed origins cannot fire. At the onset of S phase, CDK activity rises and origin firing from pre-RCs occurs, but re-licensing is inhibited until CDK activity falls again at the onset of anaphase (Diffley, 2004). Insulatory mechanisms also exist to ensure that re-initiation does not occur during periods when CDK activity is at intermediate levels. Thus, for example, in *S. cerevisiae* the G1 cyclins Cln1-3 are capable of preventing pre-RC formation but are poor at triggering replication initiation (Drury et al, 2000; Labib et al, 1999; Schwob & Nasmyth, 1993; Tanaka & Diffley, 2002). Pre-RC reformation is therefore inhibited before forks are allowed to fire. Similarly, at the end of mitosis Clb5 is destroyed by APC^{Cdc20} before Clb2, which is destroyed by APC^{Cdh1}. Clb2, like Cln1-3 is poor at stimulating initiation but is sufficient to inhibit pre-RC formation (Donaldson, 2000). Origin licensing and firing are therefore clearly isolated from each other at both the beginning and end of G1 phase.

Although CDK inhibition of re-replication was first demonstrated in *S. pombe*, in which deletion of the mitotic cyclin Cdc13, overexpression of the CKI Rum1 or transient inhibition of a temperature-sensitive Cdc2 mutant all resulted in re-initiation (Broek et al, 1991; Hayles et al, 1994; Moreno & Nurse, 1994), the regulatory mechanisms involved are best understood in budding yeast. Inactivation of CDK in G2 phase in *S. cerevisiae* induces reformation of the pre-RC, and subsequent reactivation causes re-replication (Dahmann et al, 1995). Conversely, activation of CDK in G1 phase prevents pre-RC formation (Detweiler & Li, 1998). All of the components of pre-RC formation are regulated, and these mechanisms act together to produce a complete block to re-initiation. Cdc6 is periodically expressed and degraded (Drury et al, 1997; Piatti et al, 1995), due both to inhibition of nuclear import of its transcriptional activator Swi5 by CDK phosphorylation (Moll et al, 1991) and targeting of the protein for degradation by CDK phosphorylation dependent ubiquitination by the E3 ligase SCF^{Cdc4} (Drury et al, 1997; Drury et al, 2000). Later in mitosis, Cdc6 is partially stabilised by association with Clb2, allowing protein levels to build up although its licensing activity remains inhibited (Drury et al, 2000; Mimura et al, 2004). The Mcm2-7 complex is regulated by periodic accumulation in the nucleus, and this regulation is not dependent on Cdc6 and is therefore independent of pre-RC formation (Labib et al, 1999; Nguyen et al, 2000). Inactivation of CDK activity in mitosis leads to nuclear accumulation of the complex, and the inverse is true during G1 (Labib et al, 1999; Nguyen et al, 2000). Cdt1 regulation seems intimately related to this; Cdt1 and Mcm2-7 remain constitutively associated with each other, and in the absence of either, neither one shows nuclear accumulation. Furthermore, addition of a nuclear localisation signal to Mcm7 causes constitutive nuclear localisation of both Mcm2-7 and Cdt1 (Tanaka & Diffley, 2002). Nuclear

localisation of the Mcm2-7-Cdt1 complex appears to be regulated by a composite nuclear localisation signal (NLS) in Mcm2 and Mcm3, and an adjacent nuclear export signal (NES) in Mcm3 (Liku et al, 2005). Phosphorylation of multiple CDK phosphorylation motifs clustering around the NLS/NES site in Mcm3 causes nuclear export of the complex, whilst the introduction of alanine mutations in the phosphorylated residues cause nuclear retention (Liku et al, 2005).

Finally, phosphorylation of Orc2 and Orc6 by CDK also inhibits their function, although it does not seem to affect their origin association (Nguyen et al, 2001). In addition, Clb5 binds to an RXL motif in Orc6 and this also appears to be inhibitory to origin function (Wilmes et al, 2004). Deregulation of all three of the regulatory mechanisms, Cdc6, Cdt1/Mcm2-7 and ORC, is required to induce significant re-replication by FACS analysis (Nguyen et al, 2001), although some re-replication may also occur after deregulating ORC and Cdc6 alone (Green et al, 2006; Tanny et al, 2006). The partial redundancy of the mechanisms involved means that inactivation of one alone is insufficient to cause re-replication, and underlines the importance of regulation of this step in DNA replication.

In *S. pombe*, it initially appeared that inhibition of pre-RC reformation was simpler than in *S. cerevisiae*, since stabilisation of the Cdc6 homologue Cdc18, the expression and degradation of which is regulated like *S. cerevisiae* Cdc6 in a CDK dependent manner, was sufficient to induce re-replication (Jallepalli et al, 1997; Jallepalli et al, 1998; Lopez-Girona et al, 1998; Muzi Falconi et al, 1996; Nishitani & Nurse, 1995). However, physiological levels of stable Cdc18 do not deregulate replication, so at least one other mechanism must also exist (Muzi Falconi et al, 1996; Nishitani &

Nurse, 1995). It has since been shown that Cdt1 and ORC are also regulated, although unlike in *S. cerevisiae*, Cdt1 is regulated by degradation rather than nuclear import (Gopalakrishnan et al, 2001; Hu & Xiong, 2006; Nishitani et al, 2000). The M phase CDK Cdc2-Cdc13 also appears to bind to *S. pombe* Orc2, sterically inhibiting pre-RC formation (Vas et al, 2001; Wuarin et al, 2002). Cdt1 overexpression combined with a stable copy of Cdc18 is sufficient to cause re-replication (Gopalakrishnan et al, 2001; Nishitani et al, 2000; Yanow et al, 2001).

In higher eukaryotes, prevention of re-licensing is in addition controlled by the presence of another protein factor termed Geminin, discovered in *Xenopus* as an APC substrate and found to bind and inhibit the activity of Cdt1 (McGarry & Kirschner, 1998; Tada et al, 2001; Wohlschlegel et al, 2000). Geminin binding to Cdt1 inhibits its binding to the Mcm2-7 complex, and therefore pre-RC formation (Li & Blow, 2005; Saxena et al, 2004). Not all Geminin is degraded at anaphase, about half of the total population remains stable. It therefore appears that ubiquitination alone in the absence of degradation is sufficient to inhibit its Cdt1 binding activity (Li & Blow, 2004). A further mechanism of regulation which seems unique to higher eukaryotic cells is the CDK independent degradation of Cdt1 by ubiquitination triggered by its interaction with PCNA on the chromatin (Arias & Walter, 2005; Arias & Walter, 2006). Thus, Cdt1 is degraded specifically in S phase, when PCNA is present on the chromatin in the replisome (see below). Cdt1 binding to PCNA may create a binding site for the E3 ubiquitin ligase Cul4-Ddb1^{Cdt2} (Arias & Walter, 2005; Jin et al, 2006; Arias and Walter, 2006).

In combination with degradation of Geminin, stabilisation of Cdt1 causes re-replication, although deregulation of either protein alone is insufficient (Arias & Walter, 2006; Li & Blow, 2005; Yoshida et al, 2005). In mammalian cells, there is also evidence that Cdt1 degradation is triggered by CDK phosphorylation, leading to ubiquitination via SCF^{Skp2} (Li et al, 2003b; Liu et al, 2004; Sugimoto et al, 2004; Takeda et al, 2005). Cdt1 is protected from degradation by this pathway by binding to Geminin, thus allowing Cdt1 levels to build up during mitosis (Ballabeni et al, 2004). In this way Geminin plays a positive as well as negative role in licensing, acting analogously to Clb2 in *S. cerevisiae*, protecting Cdc6 from degradation prior to G1 phase. Although with the exception of SCF mediated degradation of Cdt1 these mechanisms are only indirectly dependent on CDK activity via regulation of the cell cycle, there is also evidence that CDK phosphorylation of ORC regulates its activity, potentially by Orc1 proteolysis and regulation of chromatin binding of the complex (Mendez et al, 2002; Ohta et al, 2003; Tatsumi et al, 2003).

Events downstream of origin activation

The SV40 replisome

Many of the factors involved at the eukaryotic replication fork were identified from *in vitro* reconstitution of the replication of plasmids containing an SV40 origin of replication. Replication in this system requires multiple cellular components, as well as the viral protein Large T Antigen (TAg) (Li & Kelly, 1984; Stillman et al, 1985). TAg is a hexameric protein that acts as both the SV40 origin recognition complex and the replicative helicase, hence considerably simplifying the system of eukaryotic replication, removing the requirement for any of the pre-RC components and the

cellular replicative helicase, as well as overcoming cell cycle regulatory mechanisms (Fanning & Zhao, 2009). Analysis of the remaining required cellular components led to the identification of the requirement for two replicative polymerases, Pol α and Pol δ , the trimeric single-stranded DNA binding protein RPA, the clamp loader complex Replication Factor C (RFC) and the trimeric clamp complex Proliferating Cell Nuclear Antigen (PCNA) (Melendy & Stillman, 1991; Prelich et al, 1987b; Wobbe et al, 1987; Prelich et al, 1987a; Podust et al, 1992; Fairman et al, 1989; Tsurimoto et al, 1989; Waga et al, 1994; Waga & Stillman, 1994).

Pol α was initially thought to be the only replicative polymerase and is the only enzyme capable of initiating synthesis *de novo*, associating with a primase as well as a polymerase activity (Conaway & Lehman, 1982). It consists of four subunits, of which polymerase activity is contained in the largest and primase activity in the smallest (Kaguni et al, 1983a; Kaguni et al, 1983b). In fact, Pol α has only low processivity, and is replaced after primer synthesis by another, high processivity polymerase. Pol α is therefore thought to act as the eukaryotic primase. Pol δ is one such high processivity polymerase, and is stabilised by interactions with the PCNA clamp analogously to the *E. coli* PolIII - β complex. Pol δ consists of four subunits in *S. pombe* and higher eukaryotic cells, and three in *S. cerevisiae* (Burgers & Gerik, 1998; Podust et al, 2002; Zuo et al, 2000). In addition to Pol α and Pol δ , a third polymerase, Pol ϵ , is required for replication *in vivo* but is dispensable for SV40 replication *in vitro* (Morrison et al, 1990; Waga & Stillman, 1994). ChIP studies have localised Pol ϵ to origins prior to S phase and shown that it moves away from the origin with the replication fork after the onset of S phase (Aparicio et al, 1997; Feng et al, 2003). It is a tetrameric complex, with polymerase activity in the largest subunit

(Chilkova et al, 2003). Intriguingly, however, the catalytic domain of the protein could be deleted without affecting viability (Kesti et al, 1999). It may therefore be that the only essential role of the protein is at the initiation step of DNA replication (Feng et al, 2003). It is now believed that in wild-type cells, Pol ϵ replicates the leading strand, whilst Pol δ replicates the lagging strand (Pursell et al, 2007; Nick McElhinny et al, 2008).

The RFC clamp loader is a pentameric complex responsible for loading of the PCNA clamp at a primed site. The five subunits (Rfc1-5) are similar to each other, containing a common RFC box, as well as to the δ and γ subunits of the *E. coli* clamp loader. The pentameric structure implies a similarity to the minimal *E. coli* $\delta\delta'\gamma_3$ clamp loader complex, and a crystal structure in complex with PCNA and ATP γ S has been solved (Bowman et al, 2004). Rfc1 provides the primary interaction with PCNA, and has been proposed to act analogously to *E. coli* δ , whilst Rfc2-4 are thought to be γ -like, forming a trimeric ATPase subassembly (Cai et al, 1997). The entire complex forms a helical filament which could thread the DNA (Bowman et al, 2004). PCNA itself is highly structurally analogous to β , except that it is composed of a trimer of proteins with two domains each, rather than a dimer of proteins each with three domains. In each case, the product is a six-domain ring encircling the DNA (Krishna et al, 1994). A model for PCNA loading by RFC is emerging in which ATP binding by the RFC complex promotes both PCNA and DNA interactions, holding PCNA open in a spiral conformation. ATP hydrolysis causes closure of the ring and release of the complex around the DNA (Gomes et al, 2001; Chen et al, 2009; Johnson et al, 2006). RFC competes with the polymerase for binding to the clamp, so release of PCNA allows recruitment of Pol δ or Pol ϵ (Mossi et al, 1997;

Oku et al, 1998). Recognition of the primer-template junction by RFC has been suggested to be due to the structure of the filament, which could not accommodate a rigid double stranded B helix (Bowman et al, 2004).

The model for replisome formation during SV40 replication is therefore as follows. Origin recognition by TAg causes unwinding which is stabilised by RPA, which binds and recruits Pol α (Dornreiter et al, 1992). Pol α synthesises the leading strand primer and perhaps some downstream DNA, then disengages due to its intrinsically low processivity. The primer-template junction is now recognised by the RFC-PCNA complex, leading to PCNA loading. Release of PCNA after loading leads to Pol δ or Pol ϵ recruitment through interaction with PCNA. As the fork is extended, Pol α primes each Okazaki fragment.

Maturation of Okazaki fragments requires in addition the flap endonuclease Fen1, which removes the RNA primer, and DNA ligase I (Barker & Johnston, 1983; Johnston, 1983). Other proteins, such as RNase HI and the yeast helicase Dna2 could also be involved (Bae & Seo, 2000; Budd & Campbell, 1997; Turchi et al, 1994). Fen1 interacts with and is stimulated by PCNA, hence RNA primer removal is stimulated by completion of the upstream Okazaki fragment (Li et al, 1995; Vijayakumar et al, 2007). The eukaryotic replisome has been extensively reviewed in (Waga & Stillman, 1998) and (Johnson & O'Donnell, 2005).

Other factors involved in replisome formation

Several other proteins are required for replisome formation and function *in vivo* which are not required for SV40 replication *in vitro* and which do not form part of the pre-replication complex. Principal amongst these are Cdc45 and the GINS tetramer (Sld5, Psf1, Psf2 and Psf3) which as mentioned, function together with Mcm2-7 to form the replicative helicase, therefore functionally replacing SV40 TAg at the replication fork. Mcm10 is another factor which is required for replication fork formation and elongation *in vivo*, whilst Dpb11, Sld2 and Sld3, the essential CDK substrates, are required for replisome formation but are not directly required for elongation.

Cdc45 has long been known to be involved in the replisome (Moir et al, 1982; Hopwood & Dalton, 1996). It is recruited to origins in a CDK and Cdc7 dependent manner (Zou & Stillman, 2000), travels with the replication fork after initiation (Aparicio et al, 1997) and is required for replisome progression (Tercero et al, 2000). Experiments in both *Xenopus* and *S. cerevisiae* have shown that it is essential for recruitment of the DNA polymerases α and ϵ to the chromatin, and origin binding of Cdc45 and Pol α appears to be sequential, with recruitment of the polymerase but not Cdc45 blocked in the absence of DNA unwinding (Aparicio et al, 1999; Mimura & Takisawa, 1998; Walter & Newport, 2000; Zou & Stillman, 2000). Various reports have also demonstrated interaction with ORC, RPA, the Mcm2-7 complex, Pol α and Sld3 (Kamimura et al, 2001; Kukimoto et al, 1999; Mimura et al, 2000; Saha et al, 1998; Uchiyama et al, 2001; Walter & Newport, 2000; Zou & Stillman, 2000).

The GINS complex, by contrast, is a relative newcomer to the set of replication proteins. The Sld5 subunit was identified as a protein synthetically lethal with Dpb11, and the other subunits, Psf1, Psf2 and Psf3, by genetic and physical interactions with Dpb11 and with each other. The four proteins were found to form a stable complex which can be purified from cell extracts (Takayama et al, 2003). The complex was also identified in a screen for temperature-sensitive DNA replication factors (Kanemaki et al, 2003). Mutants in Sld5 and Psf1 are defective in DNA replication, showing similar phenotypes to mutants in components of the pre-RC, Cdc45, Sld2, Sld3 and Dpb11. GINS associates with origins at the point of their activation and travels with the replication fork (Takayama et al, 2003). The association of the complex with the chromatin requires Sld3, and Cdc45 interaction with the chromatin requires GINS, suggesting a sequential pathway of recruitment (Yabuuchi et al, 2006). GINS has been suggested to bind and stimulate both Pol α and Pol ϵ , and the archaeal homologue has also been found to interact with the archaeal primase (De Falco et al, 2007; Marinsek et al, 2006; Seki et al, 2006). Disruption of GINS complex during G1 phase in *S. pombe* results in the loss of both Pol ϵ and Cdc45 recruitment to the chromatin, but not of Pol α , which must be recruited by a different pathway (Pai et al, 2009). In agreement with the finding described below that GINS forms an integral part of the eukaryotic replicative helicase, the archaeal homologue was suggested to couple polymerase and helicase components of the fork, a function which may well be shared with its eukaryotic counterpart (Marinsek et al, 2006).

Identification of the Mcm2-7, Cdc45, GINS complex as the complete replicative helicase came from the observation that the three complexes are enriched at the site of

DNA unwinding when helicase and polymerase activities at the fork are uncoupled (Pacek et al, 2006). Mcm2-7, Cdc45 and GINS were also observed to associate after purification of a larger 'replisome progression complex', enriched for helicase components at the replication fork (Gambus et al, 2006). Purification of Cdc45 from *Drosophila* led to the enrichment of Mcm2-7 and GINS, which were therefore inferred to form a stable complex termed the CMG complex. This was shown to have helicase activity, the first demonstration of helicase activity in the complete Mcm2-7 hexamer (Moyer et al, 2006). Both EM and crystal structures of the human GINS complex are available, revealing multiple inter-subunit interactions to form a stable complex, and suggesting that the C terminal domain of Psf1 is of critical importance for the function of the complex, possibly by mediating the interaction with Cdc45 or Mcm2-7 (Boskovic et al, 2007; Chang et al, 2007; Choi et al, 2007; Kamada et al, 2007). Interestingly, GINS was also shown to bind single-stranded DNA, possibly stabilising DNA unwinding by the Mcm2-7 complex (Boskovic et al, 2007).

The roles of the remaining proteins in replisome formation and activity, however, are much less clear. Mcm10 was discovered in the minichromosome maintenance screen and also in a screen for mutants defective in DNA synthesis during mitosis (Maine et al, 1984; Solomon et al, 1992). It is required both for both initiation and elongation stages of DNA replication (Kawasaki et al, 2000; Merchant et al, 1997). Mcm10 localises to origins and shows a weak interaction with ORC, although it can associate with chromatin independently (Kawasaki et al, 2000). Mcm10 shows genetic interactions with various replication fork components, including DNA Pol ϵ and δ subunits, Mcm7 and Cdc45, and a physical interaction between Mcm10 and Mcm7 is important for its function since the *mcm7-1* allele suppresses the phenotype of

mcm10-1 by restoring the binding between the two mutant proteins (Homesley et al, 2000; Kawasaki et al, 2000). Initial experiments in *S. cerevisiae* suggested that the protein is constitutively chromatin bound and is required for Mcm2-7 but not ORC association with the chromatin (Homesley et al, 2000), but this was later disputed (Sawyer et al, 2004). Experiments in *Xenopus*, *S. pombe* and *S. cerevisiae* all suggest that it functions downstream of pre-RC assembly but upstream of Cdc45 recruitment and DNA unwinding, but although *Xenopus* Mcm10 chromatin association was shown to require Mcm2-7, *S. pombe* Mcm10 was also shown to bind chromatin constitutively (Gregan et al, 2003; Sawyer et al, 2004; Wohlschlegel et al, 2002). Suggested roles for the protein include the recruitment and activation of Cdc7 (Lee et al, 2003), stabilization and recruitment of DNA Pol α (Chattopadhyay & Bielinsky, 2007; Ricke & Bielinsky, 2004; Yang et al, 2005; Zhu et al, 2007), recruitment of Cdc45 (Gregan et al, 2003; Sawyer et al, 2004), interaction with PCNA (Das-Bradoo et al, 2006) and primase activity (Fien & Hurwitz, 2006). However, the significance of these activities and how they are coordinated is unclear. The protein also binds both single and double stranded DNA and has been reported to form hexameric oligomers (Okorokov et al, 2007; Robertson et al, 2008; Warren et al, 2008). Binding to single-stranded DNA and to Pol α have been shown to be competitive (Warren et al., 2009)

Dpb11 was first identified as a gene required for S phase progression which suppresses mutations in Pol ϵ (Araki et al, 1995). The two proteins interact physically and are recruited to origins of replication in a mutually dependent manner (Masumoto et al, 2000). *S. cerevisiae* Dpb11 is homologous to *S. pombe* Cut5 and the higher eukaryotic TopBP1. These proteins all contain multiple phosphopeptide binding

BRCT repeats, four of which are found in the *S. cerevisiae* protein (Araki et al, 1995; Bork et al, 1997; Makiniemi et al, 2001; McFarlane et al, 1997). Finally, Sld2 and Sld3 were both identified in a screen for genes which are synthetically lethal at the permissive temperature with the temperature-sensitive *dpb11-1* mutant (Kamimura et al, 1998; Kamimura et al, 2001).

Sld2 and Sld3 have both been shown to be substrates of CDK involved in promoting DNA replication initiation (Tak et al, 2006; Tanaka et al, 2007; Zegerman & Diffley, 2007). The essential site of phosphorylation of Sld2 is at residue T84, and phosphorylation has been shown to promote binding to Dpb11 via BRCT repeats three and four. Phosphorylation of Sld2 T84 can be bypassed with an aspartate point mutation (Tak et al, 2006). The essential sites of phosphorylation of Sld3 have been shown to be T600 and S622, and phosphorylation similarly promotes binding to Dpb11, via BRCT repeats 1 and 2. Phosphorylation of Sld3 can be bypassed by creating a fusion with Dpb11 (Zegerman & Diffley, 2007). When combined, the two phosphomimicking mutants allow replication in S phase in the absence of CDK activity, or in G1 phase when combined with the *cdc7-bob1* Cdc7 bypass (Zegerman & Diffley, 2007). In addition Sld3 has also been demonstrated to bind Cdc45, and recruitment of the two proteins to the chromatin is mutually dependent (Kamimura et al, 2001). A mutation in Cdc45 also bypasses the requirement for Sld3 phosphorylation, and similarly allows DNA replication in the absence of CDK activity when combined with eth Sld2 T84D mutation (Tanaka et al, 2007). Despite the apparent importance of Sld2 and Sld3 in *S. cerevisiae*, however, no definite higher eukaryotic homologues have been reported. ReqQ4 has been suggested to be a higher

eukaryotic homologue of Sld2, but as yet no homologue of Sld3 is known (Matsuno et al, 2006; Sangrithi et al, 2005).

Thus, even with these minimal components the eukaryotic replisome is already a large, interconnected complex. *In vivo*, the situation is further complicated by the presence of many other accessory components also involved in the process of elongation. A replisome progression complex (RPC) consisting of proteins associated with the helicase component of the replisome was isolated by tandem purification of GINS and Mcm2-7, and contained in addition to the factors outlined above Mrc1, Tof1, Ctf4, Csm3, Spt16, Pob3 and Top1 (Gambus et al, 2006). Spt16 and Pob3 make up the FACT histone chaperone thought to be important for transcription and replication through chromatin (Okuhara et al, 1999), Tof1 and Csm3 are involved in fork pausing at barriers to replication (Calzada et al, 2005; Mohanty et al, 2006; Tourriere et al, 2005), Mrc1 is involved in damage signalling to Rad53 and stabilisation of paused replication forks (Alcasabas et al, 2001; Katou et al, 2003; Osborn & Elledge, 2003), Ctf4 interacts with Pol α and may be important for sister chromatid cohesion (Hanna et al, 2001; Kouprina et al, 1992; Miles & Formosa, 1992) and Top1 (topoisomerase I) is important for reducing DNA supercoiling (Kim & Wang, 1989). Mrc1 has also been shown to be important for stabilising the Pol ϵ interaction with the replisome, interacting directly with the catalytic subunit (Lou et al, 2008) and thus playing a role coordinating the polymerase and helicase components. Given its interaction with Pol α , GINS may also share this role, as could Ctf4 (Gambus et al, 2009; Tanaka et al, 2009). The structure of the complete replisome and the manner of coordination of leading and lagging strand synthesis with each other and with the helicase remains to be elucidated.

1.3 Conclusions

Much progress has been made in the field of DNA replication since James Watson and Francis Crick first described its structure in 1953 (Watson & Crick, 1953). Many areas of the field are now in a relatively advanced state. Replication has been particularly well characterised in *E. coli*, but the process is also well understood in eukaryotes. The essential factors have mostly been identified and positioned within the processes of initiation and elongation. A clear picture of the pre-replicative complex has emerged, and the process of its cell cycle regulation has been extensively studied. The essential substrates of at least one of the two required protein kinases are known, and a simplified viral replication fork has been reconstituted *in vitro*.

However, as the field progresses many questions of detail emerge, and the complexities of the complete eukaryotic replisome remain perplexing. The structures of many of the components remain to be solved, and their interactions elucidated.

How does ORC interact with the DNA, and what is the mechanism of Mcm2-7 complex loading? What causes DNA unwinding and how is it coordinated with replication fork formation? What is the function of Cdc7 and do the Mcm2-7 complex subunits represent its only physiological substrates? If so, what is the functional consequence of their phosphorylation? What is the significance of Sld2 and Sld3 phosphorylation and their interaction with Dpb11? How do the factors of the replisome fit together to form a coordinated replication fork? Clearly, there is much scope for future study.

This thesis will focus on the roles protein kinases in the initiation of DNA replication initiation, and aims to analyse the substrates and sites of phosphorylation of CDK and

Cdc7 in *S. cerevisiae*. Phosphorylation of Orc1 by casein kinase II (CKII) is also revealed. Ultimately, the field of eukaryotic replication initiation will be greatly aided by complete reconstitution of the process *in vitro*, and purification and characterisation of the regulatory kinases is an important step in this process.

Chapter 2: Materials and Methods

2.1 General DNA techniques

Oligonucleotide preparation

Oligonucleotides were obtained from Sigma-Aldrich. Lyophilized DNA was pelleted by brief centrifugation, dissolved in the required volume of sterile water to give a final concentration of 100 μ M and stored at -20°C.

PCR amplification from genomic DNA or plasmid templates

General PCR reactions were carried out using a Peltier Thermal Cycler PTC-200 (MJ Research) using conditions as follows: 50 μ l reaction volumes containing 100ng template DNA (yeast genomic DNA or purified plasmid DNA), 20pmoles each primer, 1x High Fidelity Phusion PCR buffer (Finnzymes), 0.2mM each dATP, dCTP, dGTP and dTTP (TaKaRa) and 1 unit Phusion DNA polymerase (Finnzymes). Standard cycling conditions were 30 cycles of 95°C (2 min), 95°C (30 sec), 50°C (30 sec), 72°C (2 min), 72°C (2 min) where the first and last steps were included only in the first and last cycles respectively. Modifications were made where necessary. For colony PCR from yeast cultures, please see Yeast Manipulation. PCR products were purified after amplification using a High Pure PCR kit (Roche) according to the manufacturer's instructions.

Agarose gel electrophoresis

0.8% agarose gels were prepared in TAE (40mM tris-acetate, 1mM EDTA, pH 8.0) containing 0.5µg ml⁻¹ ethidium bromide. DNA was loaded in 1x DNA loading buffer (1.5% Ficoll, 10mM Tris HCl pH 7.5, 1mM EDTA, 0.1% Orange G, 0.1% Xylene Cyanol) and gels run at 70V in TAE until the desired separation was reached. For analysis of PCR reactions, 2µl samples were run and visualised using a UV transilluminator (BioRad). For cloning reactions, 200ng cleaved backbone or insert DNA was run and visualised using a Dark Reader (Clare Chemical Research), followed by bands excision and DNA purification into a final volume of 50µl using a High Pure PCR kit (Roche). For colony PCR, 10µl samples of each reaction were run and visualised using a UV transilluminator (BioRad).

Enzymatic reactions

Restriction endonucleases and T4 DNA ligase were obtained from NEB and used with buffers and protocols supplied with the enzymes. Restriction digests were generally carried out for 1 hour at the specified optimal temperature. DNA ligations were carried out for 2 hours at room temperature or overnight at 16°C. For cloning, 200ng backbone or insert DNA were cleaved with the relevant restriction endonucleases and purified by agarose gel electrophoresis as described above. 2µl backbone and 6µl insert DNA were ligated in a final reaction volume of 20µl and half of the total DNA transformed into *E. coli* strain DH5α.

Sequencing

Sequencing reactions were performed using a BIG DYE ABI PRISM Terminator Cycle Sequencing Ready Reaction Kit according to the manufacturers instructions, using appropriate oligos of 20bp length.

2.2 *E. Coli* manipulation

Cell growth

Cells were grown in suspension in LB (0.5% bacto-tryptone, 0.25% bacto-yeast extract, 170mM NaCl, pH7.0) at 37°C, or 24°C for protein expression. For solid phase growth, LB was supplemented with 2% agar. Medium was obtained from the Cancer Research UK cell production facility. For selective growth, medium was supplemented with ampicillin (100µg ml⁻¹), kanamycin (50µg ml⁻¹) or chloramphenicol (25µg ml⁻¹). 1000x antibiotic stocks were stored at -20°C.

Transformation

Competent cells were prepared as follows. *E. coli* strain DH5α was freshly streaked from frozen stocks and 100ml cultures grown to OD₅₅₀ 0.4 in ψ broth (0.5% yeast extract, 2% tryptone, 10mM KCl, 20mM MgSO₄). Cells were pelleted at 3000rpm, 4°C for 10 minutes and re-suspended in 33ml ice-cold solution RF1 (100mM RbCl, 50mM MnCl₂, 30mM potassium acetate pH 7.5, 15mM CaCl₂, 15% w/v glycerol, pH

5.8 with acetic acid, filtered). Cells were incubated on ice for 10 minutes before re-pelleting and resuspension in 8ml ice-cold solution RF2 (10mM MOPS pH 6.8, 10mM RbCl, 10mM KCl, 100mM CaCl₂, 15% w/v glycerol, pH 6.8 with NaOH, autoclaved). Cells were incubated on ice for a further 10 minutes before aliquoting in 200µl fractions and freezing at -80°C. Competent *E. coli* of strain BL21 RIL were a gift of D. Booze. Cells were transformed by thawing 70µl aliquots of competent cells on ice and incubating with DNA (10µl ligation reaction or 100ng purified plasmid DNA) on ice for 30 minutes. Cells were heat shocked at 42°C for 90 seconds, cooled on ice and 1ml LB medium added. Cells were grown at 37°C for 1 hour before pelleting and plating on selective LB.

Plasmid DNA preparation

Plasmid DNA was amplified by the growth of 1.5 ml cultures of cells overnight in selective LB. Cells were pelleted by centrifugation at 3000g for 3 minutes and plasmids purified using a Mini-Prep kit (QIAGEN) according to the manufacturer's instructions.

Protein expression

Protein expression vectors were transformed into *E. coli* strain BL21 RIL. Cultures were grown to a density of approximately OD₅₉₅=0.5 and protein expression induced by the addition of 1mM IPTG to the medium. For verification of protein expression, 500µl samples were taken from a 10ml culture growing at 37°C at 1 hour time points for 5 hours after induction. Cells were pelleted at 13,000rpm for 1 minute and resuspended in 200µl Laemmli buffer. Samples were boiled for 10 minutes, 10µl aliquots separated by SDS-PAGE and proteins visualised by Coomassie staining. For

batch purification, 1 litre cultures were grown at 24°C and protein expression induced for 16 hours overnight.

Extract production

Cells were pelleted by centrifugation at 4000rpm for 10 minutes and resuspended in 25ml lysis buffer (50mM Tris HCl, pH 7.5, 1M NaCl, 0.05% NP-40, 1mM EDTA, 2mM β mercapto-ethanol (β ME), 10% w/v glycerol) per 1 litre of cell culture. One Complete Protease Inhibitor tablet (Roche) was dissolved in 500 μ l sterile water and added to the cell suspension before lysing the cells by sonication for three 30sec intervals, incubating for 1 minute on ice between each cycle. Lysed cells were centrifuged in a Beckman ultracentrifuge using a Ti70 rotor at 50,000g for one hour. Soluble cell extract was decanted from pelleted cell debris and used for subsequent protein purification steps.

2.3 Yeast manipulation

Cell Growth

Cells were grown in suspension in YP (1% yeast extract, 2% bacto-peptone) supplemented with 2% glucose, raffinose or galactose. Temperatures were 30°C for wild type, or 24°C for temperature-sensitive strains. For solid phase growth, medium was supplemented with 2% agar. For selective growth, drop-in medium (2% agar, 1x yeast nitrogen base, 2% glucose) was supplemented with the appropriate amino acids; adenine, 5mg ml⁻¹, uracil 2mg ml⁻¹, leucine, 10mg ml⁻¹, tryptophan 2mg ml⁻¹, histidine 10mg ml⁻¹. Cell cycle arrest was achieved in exponentially growing cultures

using α factor (G1 arrest) at $5\mu\text{g ml}^{-1}$, or 100ng ml^{-1} for ΔBar1 strains, or Nocodazole (mitotic arrest) (Sigma Aldrich) at $5\mu\text{g ml}^{-1}$. α factor was obtained from the Cancer Research UK peptide synthesis facility.

Transformation

10ml cultures of cells were grown to a density of 1×10^7 cells ml^{-1} in YP supplemented with 2% glucose. Cells were pelleted by centrifugation at 3000rpm for 2 minutes at room temperature and washed in 10mls of sterile water. Cells were re-pelleted, resuspended in 1ml sterile water, transferred to an Eppendorf tube and pelleted once more by brief centrifugation in a benchtop microfuge. Cells were washed in 1ml sterile Li/TE (0.1M lithium acetate pH 7.5, 10mM Tris HCl pH7.5, 1mM EDTA) prepared fresh from 10x stocks, re-pelleted and finally re-suspended at a density of 2×10^9 cells ml^{-1} in 50 μl Li/TE. 5 μl herring sperm DNA (10mg ml^{-1} in sterile water, phenol chloroform extracted and sonicated to shear into approximately 2kb fragments) and 1-2 μg plasmid or PCR product DNA were added to the cells, followed by mixing and addition of 300 μl Li/PEG (0.1M lithium acetate pH7.5, 10mM Tris HCl pH7.5, 1mM EDTA, 40% w/v polyethylene glycol (PEG) 3550), prepared fresh from stock solutions. Cells were mixed vigorously and incubated at 30°C with agitation for 30mins. DMSO was added to a final concentration of 10% and heat shock was carried out at 42°C for 15mins. Cells mixed once more, cooled on ice and pelleted by brief centrifugation in a microfuge. Transformed cells were resuspended in 40 μl TE and plated on appropriate selective medium. For transformation with pSS1 and pSS2, plasmids were first linearised in the *ura3* region with NcoI restriction endonuclease for 1.5 hours.

Colony PCR

For verification of DNA integration into the genome, PCR amplification of the transformed locus was carried out directly from selections of yeast colonies growing on selective plates. Small quantities of cells were transferred using a Gilson pipette tip to 20µl reaction vessels containing 1x ExTaq buffer (TaKaRa), 20pmoles each primer and 0.2mM each dATP, dGTP, dCTP and dATP. 2 units of ExTaq (TaKaRa) were added and PCR carried out using a Peltier Thermal Cycler PTC-200 (MJ Research) for 34 repeats of the following cycling conditions: 94°C (2 min), 94°C (2 min), 55°C (2 min), 72°C (2 min), 72°C (2 min). The first and last steps were included in only the first and last cycles, respectively. 10µl samples of each reaction were analysed directly by agarose gel electrophoresis.

TCA protein extraction

Whole cell protein extractions were carried out by TCA precipitation (Foiani *et al.*, 1994). Samples of approximately 1×10^8 cells were collected and pelleted by centrifugation at 3000rpm for 2 minutes. The cell pellet was washed in 1ml sterile water, transferred to an Eppendorf tube and re-pelleted by brief centrifugation in a benchtop microfuge. Cells were finally resuspended in 200µl 20% tri-chloroacetic acid (TCA). An equal volume of 0.2mm diameter sterilized glass beads were added and cells broken open by vortexing for 1 minute. Supernatant was recovered and beads washed twice in 5% TCA, adding each wash to the previous supernatant. Precipitated proteins were pelleted by centrifugation at 3000rpm for 10 minutes, supernatant discarded and proteins resuspended in 200µl 1x Laemmli buffer. pH was neutralised with 5-10µl 2M Tris base. Samples were boiled for 10 minutes and cell

debris pelleted by centrifugation at 3000 rpm for 10 minutes. The supernatant was transferred to a fresh Eppendorf tube and 10µl samples analysed by SDS-PAGE and Western blotting.

Genomic DNA preparation

For preparation of genomic DNA, a 10ml culture was grown to a density of 2×10^7 cells ml^{-1} , cells pelleted by centrifugation at 3000rpm for 2 minutes, transferred to an Eppendorf tube and re-pelleted by brief centrifugation in a benchtop microfuge. Cells were resuspended in 200µl lysis buffer (100mM NaCl, 10mM Tris HCl pH 8.0, 1mM EDTA) and 200µl 25:24:1 phenol:chloroform:isoamyl-alcohol added. Cells were broken open by vortexing for 30 seconds in the presence of a 200µl volume of 0.2mm diameter sterile glass beads. 200µl TE was added (100mM Tris HCl pH 8.0, 1mM EDTA) and mixed by vortexing again for 5 seconds. The mixture was centrifuged for 2 mins at 13,000 rpm and the aqueous phase transferred to a fresh Eppendorf tube. DNA was ethanol precipitated by the addition of two volumes of 100% ethanol and re-centrifuging for 2 minutes at 13,000 rpm. The DNA pellet was washed in 70% ethanol, allowed to dry at room temperature and resuspended in 50µl TE with 50µg ml^{-1} RNaseA. RNA was degraded by incubation at 37°C for 1 hour and DNA stored at -20°C.

Protein expression

For induction of Clb5 and Dbf4 expression in strains ySS4, ySS5 and ySS9, asynchronous cultures were grown to a density of approximately 2×10^7 cells ml^{-1} YP supplemented with 2% raffinose, and 2% galactose added for 2.5 hours before extract

production as described below. For ORC and Cdc6 overexpression in a G1 arrest of strain ySC17, cells were grown to 2×10^7 cells ml^{-1} in YP supplemented with 2% glucose, arrested in G1 phase with α factor for 4 hours, pelleted by centrifugation at 4000rpm for 20 minutes and medium replaced with fresh YP containing α factor and 2% galactose. Protein expression was allowed to continue overnight at 24°C.

Extract preparation

For protein purification, extracts were prepared as follows. Cells were pelleted by centrifugation at 4000rpm for 20 minutes, washed twice in 500ml wash buffer (25mM Hepes KOH pH7.5, 0.8M sorbitol) followed by resuspension in 50ml of lysis buffer (25mM Hepes KOH pH7.5, 100mM NaCl, 2mM CaCl_2 , 0.02% NP-40, 10% w/v glycerol). Cells were re-pelleted by centrifugation at 3500 rpm for 5 minutes and supernatant discarded. Lysis buffer was added containing a mixture of 5x protease inhibitors and 10mM β ME, to a total of one-quarter of the volume of the cell pellet. 1x protease inhibitors consisted of 1 mM 4-(2-aminoethyl)-benzenesulfonyl fluoride hydrochloride (AEBSF), 2 $\mu\text{g}/\text{ml}$ aprotinin, 1 mM benzamidine hydrochloride, 10 $\mu\text{g}/\text{ml}$ leupeptin, and 1 $\mu\text{g}/\text{ml}$ pepstatin A. Cells were flash frozen by dripping the cell suspension into liquid nitrogen using a Gilson pipette. Frozen cells were broken open using a Freezer Mill (model 6850, Glen Creston) and ground cell powder thawed on ice. An equal volume of lysis buffer supplemented with 1x protease inhibitors and 2mM β ME was added and salt concentration raised to 300mM NaCl to extract chromatin bound proteins. Extract was rotated at 4°C for 30 minutes before clarification by ultracentrifugation at 50,000rpm for 1 hour at 4°C (Optima L-100 XP Ultracentrifuge, Beckman Coulter). Aqueous phase extract was separated from cell

debris and the lipid phases and used immediately for subsequent purification steps. For ySC17 extract preparation for the loading assay, the protocol followed was as above, with the following modifications. Lysis buffer was (100mM Hepes KOH pH 7.5, 0.8M sorbitol, 50mM potassium glutamate, 10mM MgCl₂ and 2mM EDTA), and after thawing powdered cell extract, the twofold dilution and chromatin extraction steps were omitted. Extract was aliquoted into 100µl fractions, flash frozen in liquid nitrogen and stored at -80°C.

2.4 Insect cell manipulation and baculovirus production

Cell growth

Sf9 and Hi5 cells were grown at 24°C in Grace's medium (Invitrogen) supplemented with 10% Fetal Bovine Serum (FBS). Cells at appropriate densities on plates or in suspension were obtained from the Cancer Research UK cell production facility.

Baculovirus production

Virus production utilised the Invitrogen Bac-to-Bac system (Invitrogen) and was carried out according to manufacturers' instructions. Briefly, Cdc7 was cloned into pFastBacI between EcoRI and StuI restriction sites, and Dbf4 into pFastBacHTA between BamHI and SalI restriction sites to create a 6x-His tagged construct.

Plasmids were transformed into DH10Bac cells (Invitrogen) which were then plated on solid L-agar containing tetracycline (10µg ml⁻¹), kanamycin (50µg ml⁻¹), gentomycin (7 µg ml⁻¹), XGal (100µg ml⁻¹) and IPTG (40µg ml⁻¹). White colonies

were selected and Bacmids purified according to the specified protocol.

Recombinants were screened for correct recombination by PCR amplification of the bacmid template using M13 forward and reverse primers. Bacmid DNA was used to transfect Sf9 cells using Cellfectin Reagent (Invitrogen) according to the following protocol. 1 µg bacmid DNA was combined with 6 µl cellfectin in a total volume of 200 µl Grace's medium, unsupplemented (Invitrogen). After incubation for 15 min at room temperature, transformation mixture was added to Sf9 cells in 6 well plates containing 1×10^6 cells per well, freshly washed into 800 µl unsupplemented Grace's medium. DNA was incubated with the cells for 5 hours at 24°C, after which time medium was removed and replaced with 2 ml Grace's medium supplemented with 10% FBS. Transfected cells were allowed to grow for 96 hours, after which time the medium was removed and stored in the dark at 4°C as viral stock P1. P2 amplified viral stock was created by infection of a 30 ml suspension culture of Sf9 cells at 1×10^6 cells ml⁻¹ with 1 ml P1 stock for 72 hours at 24°C. P3 amplified viral stock was created by infection of a 300 ml suspension culture of Sf9 cells at 1×10^6 cells ml⁻¹ for 72 hours at 24°C. Cells were pelleted and medium stored in the dark at 4°C as final viral stock P3.

Protein expression

Protein expression was carried out in Hi5 cells, and optimal conditions were determined empirically. Titrations of 1, 2, 5, 10 and 50 µl of viral stock were incubated with Hi5 cells in 24 well plates containing 6×10^5 cells per well for 24, 48, 72 or 96 hours at 24°C. After the required incubation, medium was removed and cells resuspended in 200 µl Laemmli buffer. 10 µl samples were separated by SDS-

PAGE and proteins visualised by Coomassie staining. Final batch purifications were performed in sets of twenty 150mm plates containing Hi5 cells at approximately 60% confluence. 300µl Dbf4 virus and 1200µl Cdc7 virus were mixed and incubated with the cells for 96 hours.

Extract production

Proteins were purified from a nuclear extract prepared as follows. Cells were washed from the plates into ice-cold PBS (137mM NaCl, 2.7mM KCl, 10mM Na₂PO₄, 1.7mM KH₂PO₄, pH 7.4) containing in addition 10mM MgCl₂. Cells were centrifuged at 3000rpm for 10 minutes, washed in ice-cold PBS containing 10mM MgCl₂, repelleted and resuspended in 10 volumes of hypotonic buffer (25mM Hepes KOH pH 7.5, 15mM NaCl, 2mM MgCl₂, 0.1mM EDTA, 2mM β-ME, 0.2mM PMSF and 1x Complete Protease Inhibitor cocktail (Roche)). Cells were incubated on ice for 15 minutes and broken open using an ice cold dounce homogenizer using a B pestle. Supernatant was transferred to a 50ml falcon tube and the salt concentration adjusted to 100mM NaCl. Nuclei were pelleted by centrifugation at 3,500rpm for 15 minutes, supernatant discarded and nuclei resuspended in (25mM Hepes KOH pH 7.5, 100mM NaCl, 2mM MgCl₂, 0.1mM EDTA, 2mM β-ME, 0.2mM PMSF and 1x Complete Protease Inhibitor cocktail (Roche)). One tenth volume of a cold saturated solution of ammonium sulphate was added and rotated for 30 minutes at 4°C. Nuclear extract was spun at 50,000 rpm for one hour in an ultracentrifuge (Optima L-100 XP, Beckman Coulter). The pellet was discarded and proteins in the supernatant precipitated with 0.3g ml⁻¹ solid ammonium sulphate. Precipitate was pelleted by centrifugation at 12,000rpm for 15 minutes, supernatant discarded and proteins resuspended in purification buffer (25mM Tris HCl pH 7.5, 500mM NaCl, 0.05% NP-40, 5mM imidazole, 10% glycerol, 2mM βME, 1x Protease Inhibitor Cocktail

(Roche)). If necessary, precipitated proteins were stored overnight at -80°C before resuspension.

2.5 Protein analysis

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

10% polyacrylamide gels were prepared according to (Sambrook & Russell, 2000) using a Mini-Protean II gel system (BioRad) or Anachem MV2-DC gel system (Anachem). An appropriate volume of concentrated Laemmli buffer (Sambrook & Russell, 2000) was added to samples to make a final concentration of 1x (50mM Tris HCL pH 6.8, 2% SDS, 0.1% bromophenol blue, 10% glycerol) before boiling for 10 minutes. Proteins were loaded onto gels polyacrylamide and run at 200V until the desired separation had been achieved. Benchmark markers (Invitrogen) were used to determine protein sizes by silver or Coomassie staining (0.5 or 5µl respectively), or Benchmark Pre-stained markers (10µl) (Invitrogen) for Western blotting.

Western blotting

Following SDS-PAGE, gels were equilibrated in transfer buffer (48mM Tris base, 39mM glycine, 0.0375% SDS, 20% methanol) and transferred to Hybond ECL nitrocellulose membrane (Amersham) using a semi-dry blotter (Owl) at 0.5A for 30 minutes. Where necessary, protein was visualised on the membrane by staining with PonceauS (2% w/v PonceauS, 3% w/v tri-chloroacetic acid), incubating in stain

solution for 1 minute followed by destaining in water until protein bands were visible. Membranes were blocked in TBS-T (25mM Tris HCl pH 8, 140mM NaCl, 2.5mM KCl, 0.1% Tween-20, 0.2% NP-40) containing 5% dried milk (Marvel) for 30 minutes at room temperature followed by incubation with the appropriate primary antibody in TBS-T containing 5% milk for 1 hour at room temperature or overnight at 4°C overnight. Membranes were washed for 10 minutes in TBS-T and incubated with horseradish peroxidase (HRP) conjugated secondary antibody in TBS-T containing 5% milk for one hour at room temperature, unless the primary antibody was already coupled to HRP. Membranes were given three 10 minute washes in TBS-T followed by visualisation HRP-conjugated antibodies with Enhanced Chemiluminescence (ECL) reagents (Amersham) according to the manufacturer's instructions.

Protein staining

Proteins were visualised after SDS-PAGE by silver staining or Coomassie staining according to the following protocols. For silver staining, gels were washed sequentially for 15 minutes each in 50% methanol, 5% methanol and 32µM DTT. Gels were then rinsed in a solution of 1mg ml⁻¹ silver nitrate followed by a further 15 minute incubation in the same. Gels were rinsed three times in sterile H₂O followed by a solution of 300µM sodium carbonate supplemented with 0.02% formaldehyde. Gels were then incubated in the same until protein bands were visible. Staining was quenched by the addition of crystalline citric acid and gels washed thoroughly in H₂O. For Coomassie staining, proteins were first fixed for 20 minutes in a solution of 50% methanol and 10% acetic acid, followed by rinsing and incubation in H₂O for 10 minutes. Gels were then incubated in GelCode Blue Stain Reagent (Thermo Scientific) for 30 minutes at room temperature, followed by washing in H₂O.

³²P visualisation

Radiolabel incorporation into dried gels or peptide array membranes were visualised as specified either by autoradiography or using a phosphorimager. For autoradiography, gels were exposed for the appropriate time to X-ray film (Amersham Hyperfilm MP, GE Healthcare) in a film cassette at -80°C. For phosphorimager analysis, gels were exposed to a phosphorscreen (Molecular Dynamics) for varying times between 30 minutes to 16 hours before analysis using a STORM 840 phosphorimager (Molecular Dynamics). Where necessary, phosphorimager results were further quantified using ImageQuant software (Molecular Dynamics).

Mass spectrometry

For analysis of purified ORC and pre-replicative complexes, proteins were separated on a NuPAGE 3-8% Tris-Acetate Gel (Invitrogen) run in 1x NuPAGE Tris-acetate SDS running buffer (Invitrogen) using X-SureLock gel apparatus (Invitrogen). Gels were stained using GelCode Blue Stain Reagent (Thermo Scientific) without prior fixing of proteins. Sample containing lanes were each cut into 30 equally sized slices which were stored in 500µl sterile H₂O and analysed by electrospray ionization mass spectrometry by the Cancer Research UK Protein Analysis facility.

2.6 Protein purification methods

TAP tag purification

Yeast extracts for purification of TAP tagged proteins were prepared as described above. A 200 μ l bed volume of calmodulin affinity resin (Stratagene), preequilibrated in binding buffer (25mM Hepes KOH pH 7.5, 100mM NaCl, 2mM CaCl₂, 0.02% NP-40, 10% w/v glycerol), was added per 10ml of extract. Extract was rotated at 4°C for 3 hours, after which time beads were collected in a gravity flow column (BioRad, Poly-Prep) and washed twice with 10ml binding buffer. Proteins were eluted in five 200 μ l aliquots of elution buffer (25mM Hepes KOH pH 7.5, 100mM NaCl, 0.02% NP-40, 10% w/v glycerol, 1mM EDTA, 1mM EGTA). Elution fractions were combined in a non-stick Eppendorf tube and a 50 μ l bed volume of IgG sepharose beads (GE Healthcare), preequilibrated in elution buffer, was added. Beads were rotated overnight at 4°C, then collected in a gravity flow column and washed twice with 3ml calmodulin affinity resin elution buffer. Beads were then resuspended in a final volume of 500 μ l calmodulin affinity resin elution buffer in a non-stick Eppendorf tube and 6x-His tagged TEV protease (Cancer Research UK peptide synthesis facility) added at a concentration of 0.1mg ml⁻¹. Cleavage was allowed to proceed for 4 hours at 4°C. Beads were collected in a gravity flow column and flow through containing eluted proteins collected. Beads were rinsed with a further 200 μ l buffer. TEV was removed by application of the eluate to a 200 μ l bed volume Ni-NTA agarose (Qiagen). Samples were taken at each step of the purification and diluted twofold in Laemmli buffer for analysis by SDS-PAGE, silver staining and Western blotting.

HisTrap purification

Extracts containing 6xHis tagged proteins were applied to a 1ml HisTrap column (GE Healthcare) using an AKTA-Prime FPLC machine (GE Healthcare). The column was washed extensively in H₂O followed by binding buffer (25mM TrisHCl pH 7.5, 500mM NaCl, 5mM imidazole, 0.05% NP-40, 10% glycerol, 2mM βME, 0.2mM PMSF). Extract was applied at a flow rate of 0.5ml min⁻¹, followed by manual washing with wash buffer (25mM TrisHCl pH 7.5, 500mM NaCl, 40mM imidazole, 0.05% NP-40, 10% glycerol, 2mM βME, 0.2mM PMSF) until weakly binding contaminants were observed by UV absorbance to be washed away. Bound proteins were then eluted over a 20 ml gradient from 40mM to 1M imidazole, continuously collecting 500μl fractions. Peak protein elution fractions were determined by UV absorbance, samples diluted twofold in 2x Laemmli buffer and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing the protein of interest were pooled and dialysed where necessary into an appropriate buffer for subsequent purification steps. Columns were subsequently washed back into H₂O and stored at 4°C.

GSTrap purification

Extracts containing GST tagged proteins were applied to a 1ml GSTrap column (GE Healthcare) using an AKTA Prime FPLC machine (GE Healthcare). The column was washed extensively in H₂O followed by binding buffer (25mM Hepes KOH pH 7.5, 500mM NaCl, 0.05% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM βME, 0.2mM PMSF) before application of cell extract at a flow rate of 0.5ml min⁻¹. The column was washed in a further 10ml of binding buffer before stepping into an elution buffer (25mM Hepes KOH pH 8, 500mM NaCl, 0.05%, 20mM reduced glutathione, NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM βME, 0.2mM

PMSF). Fresh elution buffer was made before each purification, and pH adjusted where necessary. Ten 500 μ l fractions were collected and samples diluted twofold in 2x Laemmli buffer. Proteins were analysed by SDS-PAGE followed by Coomassie staining, and peak fractions containing the protein of interest were pooled and dialysed where necessary into an appropriate buffer for subsequent purification steps. Columns were subsequently washed extensively with elution buffer followed by H₂O, and stored at 4°C.

Gel filtration

Gel filtration purification steps were carried out using a Superdex 200 HR 10/30 (GE Healthcare) using an AKTA Prime FPLC machine (GE Healthcare). Proteins were first concentrated into 500 μ l using a Microcon Ultracel YM-10 (Millipore) filter device in a benchtop microfuge. The gel filtration column was washed into elution buffer (25mM Hepes KOH pH 7.5, 500mM NaCl, 0.05% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM β ME, 0.2mM PMSF), sample applied and proteins collected in 500 μ l fractions over 1 column volume (24ml). Peak protein elution fractions were determined by UV absorbance, samples diluted twofold in 2x Laemmli buffer and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing the protein of interest were dialysed into a buffer containing (25mM Hepes KOH pH 7.5, 100mM NaCl, 0.05% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM β ME, 0.2mM PMSF), flash frozen in liquid nitrogen and stored at -80°C.

Ion exchange chromatography

For purification by ion exchange chromatography, proteins were dialysed into buffer containing 25mM Hepes KOH pH 7.5, 100mM NaCl, 0.05% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM β ME, 0.2mM PMSF and applied to a 1ml Q sepharose column (Amersham Pharmacia) using an AKTA Prime FPLC machine (GE Healthcare) according to the manufacturers' instructions. The column was washed extensively in H₂O followed by binding buffer, and protein applied at a flow rate of 0.5 ml min⁻¹ followed by washing with 10ml of binding buffer. Bound protein was eluted over a 10ml gradient from 100mM to 1M NaCl, collecting 500 μ l fractions. Peak protein elution fractions were determined by UV absorbance, samples diluted twofold in 2x Laemmli buffer and analysed by SDS-PAGE followed by Coomassie staining. Fractions containing the protein of interest were dialysed into a buffer containing 25mM Hepes KOH pH 7.5, 100mM NaCl, 0.05% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 2mM β ME, 0.2mM PMSF, flash frozen in liquid nitrogen and stored at -80°C. For purification of λ phosphatase treated Cdc7-Dbf4, samples were applied to a 100 μ l MonoQ column using a SMART FPLC machine (Pharmacia). Protein was eluted over a 2ml gradient between 100mM and 1M NaCl, collecting 50 μ l fractions which were analysed as above.

2.7 Peptide array methods

Membrane synthesis and background detection

Membranes were designed to specification and synthesised by the Cancer Research UK peptide synthesis facility using an Intavis Multiprep peptide synthesiser (Intavis Bioanalytical Instruments AG, Cologne, Germany) on PEG derivatised cellulose membranes (Intavis). For detection of background binding of ^{32}P γATP , peptides were dissolved in the Cdk2-CyclinA reaction buffer (25mM HEPES KOH pH 7.5, 100mM potassium acetate, 0.05% NP-40, 1mM EGTA, 1mM EDTA, 10% glycerol) for 2-3 hours. When all peptides were seen to have dissolved, buffer was removed and replaced with 2.5ml (a sufficient volume to cover the membrane surface) fresh buffer per membrane, containing in addition 5mM MgCl_2 , 10 μM ATP and 10 μCi $^{32}\text{P}\gamma\text{ATP}$ per membrane. Membranes were covered to prevent evaporation and incubated for 1 hour at room temperature. Membranes were subsequently rinsed in an excess of reaction buffer, then incubated overnight at room temperature in 0.5% phosphoric acid in order to compete away non-specifically bound ATP. Membranes were then given three further washes in 0.5% phosphoric acid, rinsed in 96% ethanol and allowed to dry at room temperature. Radiolabel was visualised using a phosphorimager (STORM 840, Molecular Dynamics) after exposure overnight to a phosphor screen (Molecular Dynamics).

Cdk2-CyclinA phosphorylation

Peptides were dissolved again in reaction buffer as described above. Buffer was removed and replaced with 2.5ml per membrane fresh buffer containing in addition 0.5 μM Cdk2-cyclinA, 5mM MgCl_2 , 10 μM ATP and 10 μCi $^{32}\text{P}\gamma\text{ATP}$ per membrane. Vessels were covered and reactions allowed to proceed at room temperature for 1 hour. The reaction was stopped by the addition of an excess of 1% SDS and heating

to 65°C for 1 hour to denature and remove membrane bound CDK, before incubating overnight in 0.5% phosphoric acid. Membranes were then subjected to three further washes in 0.5% phosphoric acid, then sequential washes in methanol at 50%, 25%, 12.5% and 6.25% to remove SDS from peptide bonds. Membranes were rinsed in 96% ethanol and allowed to dry at room temperature before visualisation of radiolabel as above. Radiolabel incorporation into each spot on the array was quantified using ImageQuant software (Molecular Dynamics).

λ phosphatase treatment and data analysis

Phosphorylated peptides were re-dissolved in λ phosphatase buffer (50mM Tris HCl pH 7.5, 100mM NaCl, 0.1mM EGTA, 2mM DTT) followed by incubation in 2.5ml per membrane of the same containing in addition 2mM MnCl₂ and 30,000U per membrane λ phosphatase (NEB). Membranes were washed in 1% SDS for 1 hour at 65°C, followed by multiple washes in sterile H₂O and sequential methanol washes as described above. Membranes were rinsed in 96% ethanol, dried and radiolabel visualised and quantitated as above. Signal at each spot following phosphatase treatment was subtracted from signal prior to phosphatase treatment. Final background normalisation was achieved by subtraction of average levels of background seen at sites containing no peptide. The final data set was plotted as heat maps using software designed by the Cancer Research UK bioinformatics group.

Cdc28-Clb5 and Cdc7-Dbf4 phosphorylation

For Cdc28-Clb5 phosphorylation, radiolabel remaining on the arrays after phosphorylation with Cdk2-CyclinA was allowed to decay to negligible levels over a six month period. Phosphorylation reactions, phosphatase treatment and data analysis was carried out as described for Cdk2-CyclinA with a slight variation in buffer conditions (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100μM ATP, 1mM EGTA, 1mM EDTA, 0.05% NP-40, 10% glycerol, 10μCi ³²P γATP per membrane). 200ng purified Cdc28-Clb5 was used per membrane. Reactions were allowed to proceed for 1 hour at 30°C. For phosphorylation with Cdc7-Dbf4, fresh synthesised membranes were used. Peptides were dissolved in the reaction buffer described for Cdc28-Clb5, and approximately 33μg Cdc7-Dbf4 used per membrane. Reactions were allowed to proceed for 1 hour at 30°C. ATP was added to each 2.5ml aliquot of buffer immediately prior to addition to the membrane in order to prevent Dbf4 autophosphorylation before peptide phosphorylation. In each case, membrane washing procedures, λ phosphatase treatment and data analysis were as described above.

Cdk2-cyclinA stimulation of Cdc7-Dbf4 phosphorylation

Arrays were phosphorylated with Cdk2-cyclinA as described above, but in the absence of ³²P γATP. Membranes were subsequently washed in 1% SDS at 65°C for 1 hour followed by an overnight incubation in 0.5% phosphoric acid and sequential washes in methanol as described above. Buffer was then changed to that described for Cdc7-Dbf4 phosphorylation, and phosphorylation carried out as described in the presence of ³²P γATP.

Cdc7-Dbf4 binding assays

Peptides were solubilised in PBS-T containing 1% BSA (137mM NaCl, 2.7mM KCl, 10mM Na₂PO₄, 1.7mM KH₂PO₄, pH 7.4 with HCl, 0.5% NP-40, 0.1% Tween-20, 1% BSA), then incubated for 1 hour at room temperature in 2.5ml per membrane of the same containing in addition 10µg cdc7-Dbf4. Membranes were subsequently washed 3 times for 15 minutes per wash in PBS-T followed by a further incubation overnight at 4°C in 10ml per membrane PBS-T 1% BSA and anti-Dbf4 antibody (CR-UK) or HRP-conjugated anti-His antibody (AbCam) at a 1:1000 dilution. Membranes were washed briefly in PBS-T, and for detection of Dbf4 antibody, incubated for 1 hour at room temperature in 10ml per membrane PBS-T containing HRP-conjugated ProteinA. Membranes were washed three times for 15 minutes per wash in PBS-T, and HRP-conjugated antibodies detected using ECL reagents (Amersham) according to the manufacturers instructions.

2.8 Loading assays

DNA production and binding to streptavidin beads

Biotinylated DNA for loading assays was prepared by amplification of 1kb fragments of DNA containing wild type or A box linker substitution DNA from plasmids

pars/WTA and pARS/858-865 (Marahrens and Stillman, 1992) respectively using primers RSP23 (AGCGGATAACAATTTTCACACAGG) and ARS13'bio (biotin-CTGTTTTGTCTTGGAAAAAAGCACTACC), where ARS13'bio was biotinylated at the 5' end. 8 independent 50µl PCR reactions were carried out with each template, products purified into a final volume of 200µl TE (10mM Tris HCl pH 7.5, 1mM EDTA). 600µl suspended volume of magnetic streptavidin beads (Dynabeads M-280 Streptavidin, Invitrogen) were washed in a buffer containing 10mM Tris HCl pH 7.5, 1mM EDTA and 2M NaCl, then resuspended in 200µl of the same. A magnetic rack was used to isolate the beads between wash steps. DNA was added and beads rotated for 2 hours at room temperature. DNA bound beads were subsequently washed three times in a buffer containing 10mM Hepes KOH pH 7.5, 1mM EDTA and 1M potassium acetate, then given three further washes in a buffer containing 10mM Hepes KOH pH 7.5 and 1mM EDTA. Beads were finally resuspended in 200µl of the same. 10µl samples of PCR products and DNA bound to beads (0.5% of the total) were analysed side by side by agarose gel electrophoresis to ensure efficient binding of DNA to the beads, after first denaturing the biotin-streptavidin interaction by resuspending the bead sample in 1% SDS and boiling for 10 minutes.

Loading assays

Loading assays were performed using ySC17 extract prepared as described above. 10µl aliquots of DNA beads were taken per reaction and 7.5µl of buffer removed. 17.5µl of 2x assay buffer added to make a final concentration of 50mM Hepes KOH pH 7.5, 20mM magnesium acetate, 0.2mM EDTA, 0.1mM ZnCl₂, 1.6mg ml⁻¹

poly(dIdC) (Sigma-Aldrich), 40 μ M creatine phosphate, creatine kinase, 20 μ M DTT, 6mM ATP and a 2x protease inhibitor mix (1x protease inhibitors were as specified for ySC17 extract production). 20 μ l extract was added and incubated with agitation at 24°C for 20 minutes. Beads were then washed three times in a buffer containing 50mM Hepes KOH pH 7.5, 300mM potassium glutamate, 10% glycerol, 5mM magnesium acetate, 1mM EDTA and 1mM EGTA. Beads were subsequently resuspended in 40 μ l Laemmli buffer, boiled for 10 minutes and 10 μ l aliquots analysed by SDS-PAGE followed by silver staining and Western blotting. For analysis of supernatants by Western blotting, a sample equivalent to 0.5 μ l of extract was run.

Loading assay phosphorylation

Loaded DNA was washed as described above, then resuspended in an appropriate volume of buffer to give a final volume of 18 μ l after kinase addition. Buffer conditions were (25mM Hepes KOH pH 7.5, 100mM NaCl, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA). For phosphorylation with Cdc7-Dbf4, 5 μ l endogenously purified enzyme was used. For phosphorylation with Cdc28-Clb5, 1 μ l endogenously purified enzyme was used. For phosphorylation in the absence of exogenously added kinase, beads were resuspended directly into a final volume of 18 μ l. 2 μ l of a 10x concentration ATP, MgCl₂ mix was added to give final concentrations of 100 μ M ATP, 5mM MgCl₂ and 5 μ Ci ³²P γ ATP per reaction. Phosphorylation was allowed to proceed for 1 hour at 30°C. Beads were

subsequently washed three times in reaction buffer and resuspended in a final volume of 40µl Laemmli buffer.

2.9 Other experimental methods

Kinase assays

In general, kinase assays were performed in 20µl reactions, with buffer conditions, reaction times, substrate and enzyme concentrations as specified below. In almost all cases, control reactions were carried out in the absence of kinase. Reactions were begun by the addition of a 2µl of a 10x concentration solution of ATP and MgCl₂, and stopped by the addition of an equal volume of 2x Laemmli buffer and boiling for 10 minutes. ***Histone H1 phosphorylation by Cdc28-Clb5:*** 20µg histone H1, 5µl (100ng) endogenous Cdc28-Clb5, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 0.1mg ml⁻¹ BSA, 5µCi ³²P γATP per reaction), 20 minutes at 30°C. ***MCM subunit phosphorylation by Cdc7-Dbf4:*** 2µg each Mcm2-7 subunit/Cdt1, 2µl (40ng) endogenous Cdc7-Dbf4, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction) for 30 minutes at 30°C. ***Mcm2-N phosphorylation by***

phosphatase and mock treated baculovirus Cdc7-Dbf4: 0.5µg Mcm2-N with 100ng phosphatase and mock treated enzyme respectively, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 30 minutes at 30°C. ***Timecourse of Mcm2-N phosphorylation by baculovirus Cdc7-Dbf4:*** 5µg Mcm2-N with 0.8µg Cdc7-Dbf4, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), varying time periods at 30°C. ***Mcm2-N phosphorylation by baculovirus Cdc7-Dbf4 after Cdc7-Dbf4 pre-phosphorylation:*** 5µg Mcm2-N with 0.4µg de-phosphorylated Cdc7-Dbf4, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 10 minutes at 30°C. Pre-phosphorylation of Cdc7-Dbf4 was achieved by adding Mcm2-N substrate to each reaction after the appropriate time period. ***Mcm10 phosphorylation by baculovirus Cdc7-Dbf4:*** 10µg Mcm10 with 1µg Cdc7-Dbf4, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 30 minutes at 30°C. ***Sld2, Sld3 and Dpb11 phosphorylation by Cdc7-Dbf4:*** 4µg Sld2, Sld3 or Dpb11 with 5µl (100ng) endogenous Cdc7-Dbf4, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 30 minutes at 30°C. ***Orc1 phosphorylation by Myc tagged kinases:*** 2µl purified Orc1 with the indicated volume of normalised ProteinA beads containing the relevant kinase, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 1 hour at

30°C. **ORC phosphorylation:** 400ng each complex, (25mM Hepes KOH pH 7.5, 100mM NaCl, 5mM MgCl₂, 100µM ATP, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA, 5µCi ³²P γATP per reaction), 1 hour at 30°C.

λ phosphatase treatments

For λ phosphatase treatment of Cdc28-Clb5, 10µl (200ng) of enzyme purified from ySS8 was diluted twofold into a final buffer containing 25mM Hepes KOH pH7.5, 200mM NaCl, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA and 2mM MnCl₂. 40U of λ phosphatase (NEB) was added and reactions allowed to proceed for 30 minutes at 30°C. Reactions were stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. One quarter of the total reaction was separated by SDS-PAGE and analysed by silver staining. For Cdc7-Dbf4, essentially the same procedure was followed but with 2µg of baculovirus purified Cdc7-Dbf4. To purify phosphatase and mock treated Cdc7-Dbf4, 200µl aliquots were dialysed into reaction buffer as above, 4000U λ phosphatase added to the appropriate sample. Reactions were allowed to proceed for 30 minutes at 30°C. Cdc7-Dbf4 was subsequently purified away from λ phosphatase on a 100µl MonoQ column using a SMART FPLC machine (Pharmacia) as described above.

Antibody crosslinking to proteinA agarose

To crosslink monoclonal 9E11 antibody to ProteinA agarose (Pierce), a 200 μ l bed volume of beads was washed in 0.2M sodium borate (0.2M Boric acid pH adjusted to 9.0 with NaOH) and incubated with 1mg 9E11 (Cancer Research UK antibody production facility) for 1 hour at room temperature, with rotation. Beads were subsequently washed in 0.2M sodium borate pH 9 and resuspended in a final volume of 1.5ml. 0.8mg of DMP (Sigma-Aldrich) was added and pH readjusted to 9.0 with NaOH. Crosslinking was allowed to proceed for 1 hour at room temperature, after which time beads were washed into 0.2M ethanolamine (pH 8.0 with HCl) and incubation continued for 2 hours at room temperature. Beads were washed three times with PBS (137 mM NaCl, 2.7 mM KCl, 10 mM Sodium Phosphate dibasic, 2 mM Potassium Phosphate monobasic, pH 7.4) and resuspended in a final volume of 1.5ml. Antibody crosslinked beads were stored at 4°C.

Protein immunoprecipitation

To pull down 9xMyc tagged kinases from yeast extracts, 20 μ l of 9E11 crosslinked ProteinA beads per reaction, or an equivalent volume of beads with no antibody, were washed into (25mM Hepes KOH pH 7.5, 100mM NaCl, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA) and added to 100 μ l aliquots of extract from the relevant strain. Beads were incubated with rotation for 1 hour at 4°C, after which time the beads were washed twice in (25mM Hepes KOH pH 7.5, 500mM NaCl, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA) and once in (25mM Hepes KOH pH 7.5, 100mM NaCl, 0.02% NP-40, 10% glycerol, 1mM EGTA, 1mM EDTA) before

finally resuspending in a final volume of 50 μ l. For Western blotting, 0.5 μ l samples of input and flow through extract, and 5 μ l samples of bead bound proteins were made up to 10 μ l in Laemmli buffer and separated by SDS-PAGE. To normalise the concentrations of each of the kinases after purification on ProteinA beads, samples were titrated and signal strengths compared by Western blotting. Beads were diluted in the required volume of buffer to equalise protein concentrations.

Ypk1 depletion

Ypk1 was partially depleted from extract of strain ySS14 by the following procedure. 100 μ l of 9E11 crosslinked ProteinA beads or an equivalent volume of beads containing no antibody were pelleted by centrifugation at 3000rpm for 30 seconds. Buffer was removed and replaced with 100 μ l aliquots of cell extract. Beads were incubated with rotation at 4 $^{\circ}$ C for 1 hour. Beads were subsequently removed from the extract by centrifugation of the suspension into a 1.5ml Eppendorf tube through a 200 μ l Gilson pipette tip with the end cut off, containing a single 0.5mm diameter sterile glass bead. Beads were retained in the pipette tip whilst extract passed through. The procedure was twice repeated to give a final depleted extract. For Western blot analysis, 0.5 μ l samples of extract were made up to 10 μ l in Laemmli buffer and separated by SDS-PAGE.

Fluorescence Activated Cell Sorting (FACS) analysis

For analysis of rates of S phase progression, 100ml cultures were grown to 1×10^7 cells ml^{-1} and arrested in G1 with α factor. Cells were subsequently pelleted by centrifugation at 3000rpm for 10 minutes and resuspended in fresh buffer containing $5 \mu\text{g ml}^{-1}$ nocodazole. For each sample to be analysed, 1×10^7 cells were taken and fixed in 1ml 70% ethanol and stored overnight at 4°C . Fixed cells were then washed with 1ml 50mM Tris HCl pH 7.6, and resuspended in 1ml Tris HCl pH 7.6 containing 0.2mg ml^{-1} RNaseA. Cells were incubated for 4 hours at room temperature with rotation. Cells were then pelleted and resuspended in 0.5ml 5mg ml^{-1} pepsin (Sigma) dissolved in 55mM HCl. Cells were now incubated at 37°C for 30 minutes, then washed once with 1ml FACS buffer (180mM Tris HCl pH7.6, 190mM NaCl, 70mM MgCl_2). Cells were resuspended in 0.5ml FACS buffer containing $50 \mu\text{g ml}^{-1}$ propidium iodide (Sigma). Cells were then sonicated briefly and $50 \mu\text{l}$ samples diluted in 1ml 50mM Tris HCl pH 7.6 before analysis on a FACS Scan machine (Becton Dickenson).

2.10 Yeast strains

Strain name	Genotype	Use
W303	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100</i>	Background strain
ySC17	<i>MATa ade2-1 ura3-1 his3-11,1 can1-100 bar1::HisG trp1::p404-GAL1-10-ORC3,4 lys2::plys2-GAL1-10-ORC2,5 his3::p403-GAL1-10-ORC1,6 pep4::KanMX ura3::pSF322CDC6</i>	Loading assay (Bowers et al., 2004)
ySC4	<i>MATa ade2-1 ura3-1 his3-11,15 trp1-1 leu2-3,112 can1-100 cdc15-2 omns bar1::kanMX pep4::HIS3</i>	Background strain for protein purification
ySS4	(ySC4) <i>ura3-1::pSS1 GAL1-10-DBF4-TAP^{TCP}-URA3</i>	Cdc7-Dbf4 purification (this study)
ySS5	(ySC4) <i>ura3-1::pSS2 GAL1-10-CLB5-TAP^{TCP}-URA3</i>	Cdc28-Clb5 purification (this study)
ySS9	(ySC4) <i>ura3-1::pSS2 GAL1-10-CLB5-TAP^{TCP}-URA3 Sic1::TRP1</i>	Cdc28-Clb5 purification (this study)
yDR11	<i>MATa ade2-1 ura3-1 his3-11,15 can1-100 bar1::HisG trp1::p404-GAL1-10-ORC3,4 lys2::plys2-GAL1-10-ORC2,5 his3::p403-GAL1-10-ORC1,6 pep4::KanMX GAL1-10-ORC1::GAL1-10-ORC1-TAP^{TCP}-URA3</i>	ORC purification (D. Remus)
yDR14	(ySC4) <i>MCM4::MCM4-TAP^{TCP}-URA3</i>	Mcm2-TAP ^{TCP} (D. Remus)
yDR15	(ySC4) <i>MCM2::MCM2-TAP^{TCP}-URA3</i>	Mcm2-TAP (D. Remus)

ySS9	(ySC17) <i>CKA1::CKA1-9MYC-hphNT1</i>	Cka1 Myc tag (this study)
ySS10	(ySC17) <i>YPK1::YPK1-9MYC- hphNT1</i>	Ypk1 Myc tag (this study)
ySS11	(ySC17) <i>GIN4::GIN4-9MYC- hphNT1</i>	Gin4 Myc tag (this study)
ySS12	(ySC17) <i>CMK1::CMK1-9MYC- hphNT1</i>	Cka1 Myc tag (this study)
ySS13	(ySC17) <i>CKA1::CKA1-9MYC- hphNT1</i> <i>CKA2::natNT2</i>	Cka1 Myc tag, ΔCka2 (this study)
ySS14	(ySC17) <i>YPK1::YPK1-9MYC- hphNT1</i> <i>YPK2:: natNT2</i>	Ypk1 Myc tag, ΔYpk2 (this study)
ySS15	(ySC17) <i>GIN4:: hphNT1</i>	ΔGin4 (this study)
ySS16	(ySC17) <i>CMK1:: hphNT1</i> <i>CMK2:: natNT2</i>	ΔCmk1, ΔCmk2 (this study)

2.11 Antibodies

Primary	Dilution	Secondary	Dilution
PAP (Sigma)	1:1000	-	-
Cdc28 (CR-UK)	1:10,000	HRP-anti Mouse IgG	1:10,000
9E10 (CR-UK)	1:1000	HRP-anti Mouse IgG	1:10,000
9E11 (CR-UK)	1:1000	HRP-anti Mouse IgG	1:10,000
Orc6 (CR-UK)	1:1000	HRP-anti Mouse IgG	1:10,000
Cdc6 (CR-UK)	1:1000	HRP-anti Mouse IgG	1:10,000
Mcm2 (yN-19, Santa-Cruz)	1:2000	HRP-anti Goat IgG	1:10,000
Abf1 (CR-UK)	1:10,000	HRP-ProteinA	1:10,000

Chapter 3: Proteomic approaches to the identification of protein kinase substrates

3.1 Introduction

The onset of DNA replication is triggered in eukaryotes by the activity of two cell cycle regulated kinases, the cyclin dependent kinase (CDK, Cdc28 in *S. cerevisiae*) and the Dbf4 dependent kinase Cdc7 (DDK). Of these, CDK is the better understood, with an established consensus motif for substrate phosphorylation (Songyang et al, 1994) and, although not known at the beginning of this study, just two essential S phase promoting substrates; Sld2 and Sld3 (Tak et al, 2006; Tanaka et al, 2007; Zegerman & Diffley, 2007). Phosphorylation of each of these proteins stimulates binding to a third protein, Dpb11, and the combination of a fusion of Dpb11 to Sld3 with a phosphomimicking mutant of Sld2 in *S. cerevisiae* results in a bypass of the requirement for CDK in S phase (Zegerman & Diffley, 2007). However, the functional significance of Sld2 and Sld3 binding to Dpb11 is unknown and it remains possible that other non-essential substrates also contribute to ensuring timely and complete replication.

In contrast, the complete set of essential substrates of Cdc7 has not yet been determined, although a considerable body of evidence indicates that they include subunits of the MCM complex, in particular Mcm2 and Mcm4 (Bruck & Kaplan, 2009; Sheu & Stillman, 2006; Weinreich & Stillman, 1999). A mutant in which Cdc7

function in *S. cerevisiae* is bypassed does exist, and contains a point mutation in Mcm5 (Hardy et al, 1997). However, Mcm5 is not itself a target of the enzyme (Weinreich & Stillman, 1999). Phosphorylation sites in the N termini of both Mcm2 and Mcm4 have been mapped in both metazoans and *S. cerevisiae* (Cho et al, 2006; Ishimi et al, 2001; Masai et al, 2006; Montagnoli et al, 2006; Sheu & Stillman, 2006; Bruck & Kaplan, 2009). Several other proteins are also phosphorylated by Cdc7 *in vitro*, including Cdc45 and the catalytic subunit of Pol α (Nougarede et al, 2000; Weinreich & Stillman, 1999).

At the start of this study we were therefore interested in identifying substrates of both CDK and Cdc7 involved in promoting replication. We began by using a peptide array phosphorylation approach. Endogenous Cdc7-Dbf4 and Cdc28-Clb5 were both purified from *S. cerevisiae*, yielding active kinases although in limited quantities. Cdc7-Dbf4 was also purified after over-expression in insect cells using a baculovirus protein expression system. Peptide arrays were synthesised covering a complete set of *S. cerevisiae* replication proteins. Array phosphorylation experiments were then performed, first with human Cdk2-cyclinA then with purified *S. cerevisiae* enzymes.

A peptide array technique has previously been successfully used to identify individual phosphorylated residues in substrates of the kinase Rad53, which have been validated *in vivo* (Zegerman and Diffley, submitted). The technique has several advantages as an approach to kinase substrate identification. Large numbers of proteins can be screened in a single experiment, and phosphorylated residues identified easily. However, it also suffers the disadvantage that potential substrates are presented to kinases outside of the context of full-length proteins, without consideration of

secondary or tertiary protein structures or the possible requirement for distant kinase binding sites for substrate recognition.

This chapter will begin by presenting the principle of design of the peptide arrays, followed by the purification of each kinase and the results of array phosphorylation by each in turn.

3.2 Construction of peptide arrays

The robotic synthesis of arrays of peptides directly onto cellulose membranes has been well described in a recent review (Hilpert et al, 2007). Peptides are synthesised by the sequential oligomerisation of amino acids in grids onto a cellulose sheet pre-activated with a poly-ethylene glycol (PEG) linker. Previous reports have described the use of such arrays both for the identification of protein kinase substrates (for example (Loog et al, 2000)) and the identification of protein binding sites (Thorslund et al, 2007).

We therefore set out to use peptide arrays to identify possible substrates of Cdc28 and Cdc7 from a complete set of *S. cerevisiae* replication proteins. Six membranes were synthesised containing a semi-exhaustive set of replication proteins each split into sets of 26 amino acid peptides. Each peptide was designed to overlap the previous one by 13 amino acids such that each residue was represented on the array twice, with the exception of the first and last 13 amino acids of each protein. Each membrane in the set contained 600 peptides in a 20x30 grid, where each spot was on average 4mm in

diameter and contained approximately 50nmol of peptide. Each membrane was 150x200mm in size. Array synthesis was carried out by the Cancer Research UK peptide synthesis facility using an Intavis Multi pep peptide synthesiser (Intavis Bioanalytical Instruments AG, Cologne, Germany) and PEG derivatised membranes (Intavis). Figure 1 illustrates the principle of the array technique, showing the overlap between the first four peptides of Sld3, the first protein on membrane 1, along with a diagram of how the peptides are arranged on each membrane and a Ponceau S stain of membrane 1. The uneven staining of peptides appears to be sequence specific, and is likely to be due to the differing hydrophobicities of the peptides. The position of each peptide in the array is given by the coordinates (A,1) (A,2)...(T,30), with *x* and *y* coordinates given by letters and numbers respectively.

Table 1 lists the proteins present on the membranes, along with the sites of their respective peptides. The entire set of pre-RC components is present; Orc1-6, Cdc6, Cdt1 and Mcm2-7, as are the downstream components Cdc45, the four GINS subunits (Sld5, Psf1-3), Dpb11, Sld2, Sld3 and Mcm10. Also included are the remaining essential components of the replisome; the three polymerases Pol α (Pol11), δ (Pol3) and ϵ (Pol2) along with their accessory subunits (Pol12, Pri1 and Pri2 for Pol α ; Pol31 and Pol32 for Pol δ ; Dpb3 and Dpb4 for Pol ϵ), the five RFC components Rfc1-5, PCNA (Pol30), and the three subunits of RPA (Rfa1-3). The Okazaki fragment maturation factors Fen1, Dna2 and Cdc9 are present, as are the three topoisomerases Top1, 2 and 3. Finally, several factors involved in regulating chromosome stability are also included; Mrc1 and Tof1 which are involved in stabilising paused replication forks and signalling to Rad53 (Katou et al, 2003; Osborn & Elledge, 2003), Sgs1 and Srs2, which are helicases involved in the suppression of inappropriate recombination

(Pfander et al, 2005; Versini et al, 2003), Elg1 which is also involved in the suppression of recombination and forms an alternative RFC complex with Rfc2-5 (Ben-Aroya et al, 2003), Ctf4 and Ctf18, which are required for sister chromatid cohesion (Hanna et al, 2001; Mayer et al, 2001), and Smc5, Smc6, Nse1 and Nse3, which form a complex involved in the DNA damage response (Zhao & Blobel, 2005). Proteins are grouped on the arrays in such a way as to give the most logical peptide distribution between membranes.

The sequence of individual peptides over the complete set of membranes is provided in Appendix 1 at the end of the thesis.

Figure 1. Peptide array synthesis.

A. An illustration of protein division into 26mer amino acid peptides. Each peptide overlaps the previous one by 13 amino acids such that each residue is represented on the array twice, with the exception of the first and last 13 residues of each protein.

The first four peptides of Sld3 are shown, the first protein on membrane 1.

B. The layout of peptides on each membrane. Six columns and the first six rows are shown for representational purposes, although the actual membranes each contain 20 columns and 30 rows.

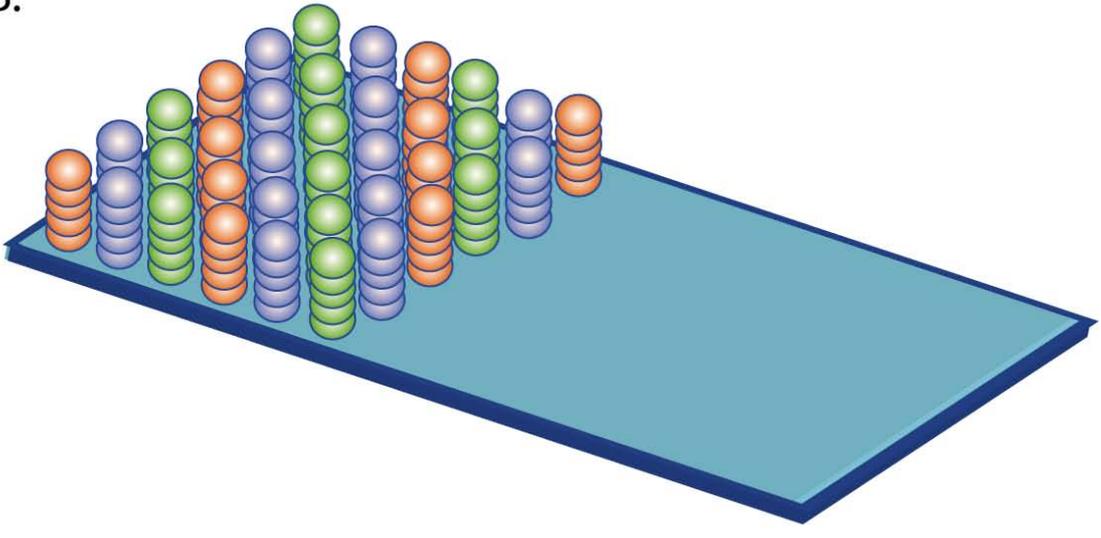
C. A Ponceau S stain of membrane 1. The uneven staining of peptides is explained by the differing hydrophobicities of each peptide.

A. Sld3

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A1 METWEVIASVKEATKGLDLSLDHPLI
A2          TKGLDLSLDHPLIIKSEDVPSNILQL
A3                    IKSEDVPSNILQLLQKKNRRQLKHIC
A4                                LQKKNRRQLKHICMKS.....
  ⋮
  
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B.



C.

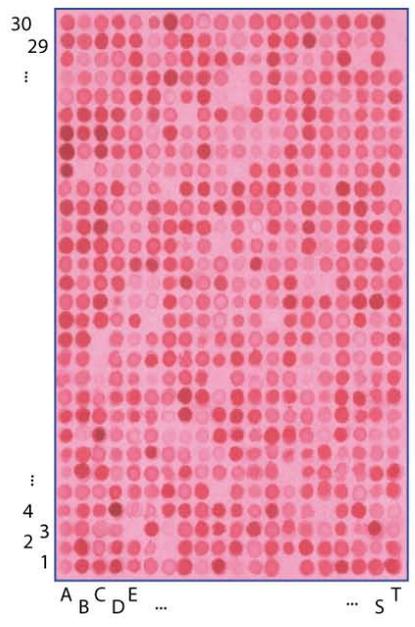


Table 1. Protein arrangement on the six membranes. Proteins present on each of the six membranes in the array are listed, along with the locations of their peptides on each membrane. Proteins are arranged in such a way as to give the most logical peptide distribution across the membranes. Individual peptide sequences are provided in Appendix 1 at the end of the thesis.

Protein	Peptides	Array	Protein	Peptides	Array
Sld3	A1-B21	1	Orc2	A1-B17	5
Orc1	B23-E2		Orc3	B19-D5	
Orc4	E4-F13		Cdc7	D7-E14	
Orc5	F15-G20		Clb6	E16-F14	
Orc6	G22-H24		Cdc28	F16-G7	
Cdc6	H26-J4		Sld2	G9-H12	
Cdt1	J6-K21		Cdc45	H14-J2	
Mcm2	K23-M30		Dpb11	J4-L1	
Mcm3	N2-P15		Sld5	L3-L24	
Mcm4	P17-R27		Psf1	L26-M10	
Mcm5	R29-T27		Psf2	M12-M27	
Mcm6	A1-C18		Psf3	M29-N12	
Mcm7	C20-G15		Pol12	N14-P7	
Mcm10	G17-H29	Pri1	P9-Q9		
Dbf4	I1-J24	Pri2	Q11-R21		
Pol1	J26-N17	Pol31	R23-S29		
Pol2	N19-S25	Pol30	T1-T19		
Clb5	S27-T30	Rfa3	T21-T29		
Dna2	A1-D27	Pol3	A1-C24	6	
Mrc1	D29-G22	Rfa1	C26-E12		
Tof1	G24-J28	Rfa2	E14-F3		
Top1	J30-L28	Pol32	F5-F30		
Top2	L30-P18	Rfc1	G2-I7		
Top3	P20-R9	Rfc2	I9-J5		
Dpb2	R11-T3	Rfc4	J7-J30		
Rfc3	T5-T30	Rfc5	K2-K28		
Sgs1	A1-D21	Fen1	K30-L28		
Srs2	D23-G22	Csm3	L30-M23		
Smc5	G24-J17	Cdc9	M25-O22		
Smc6	J19-M13	Nse1	O24-P18		
Ctf4	M15-O25	Nse3	P20-Q12		
Ctf18	O27-Q22				
Elg1	Q24-S23				
Dpb3	S25-T9				
Dpb4	T11-T25				

Prior to phosphorylation of the arrays with either CDK or Cdc7, it was determined whether any of the peptides bound ATP independently of enzyme activity. The peptides were first dissolved in the buffer that would subsequently be used for CDK phosphorylation, which was then removed and replaced with a small volume (2.5ml per membrane) of the same buffer containing in addition 10 μ M ATP and 10 μ Ci 32 P γ ATP per membrane. Buffer was dripped onto the membranes using a Gilson pipette in order to ensure that coverage was complete, and vessels covered during incubation to prevent evaporation. Membranes were incubated for 1 hour at room temperature, after which time non-specifically bound phosphate was competed away by incubation overnight in 0.5% phosphoric acid. Membranes were then washed three more times in 0.5% phosphoric acid, rinsed in 96% ethanol and allowed to dry at room temperature. Radiolabel on the dried membranes was visualised using a phosphorimager (STORM 840, Molecular Dynamics) after overnight exposure to a phosphor screen (Molecular Dynamics). No background 32 P binding to any of the peptides was observed after this treatment, indicating that radiolabel incorporation after incubation with protein kinase was likely to be due to peptide phosphorylation.

3.3 Cdk2-cyclinA phosphorylation of peptide arrays

The project was begun with the purification of endogenous Cdc28-Clb5 and Cdc7-Dbf4 from *S. cerevisiae*, which will be described later. However, quantities of these enzymes was limiting, and the initial array phosphorylation experiment was therefore carried out with human cyclinA-CDK2. This enzyme can be readily purified in an active form after co-expression with the CDK activating kinase (CAK) in *E. coli*, and

was expected to give a measure of specificity of peptide phosphorylation *in vitro*, since CDK is known to require an S/T-P motif for substrate phosphorylation (Songyang et al, 1994). The human enzyme may also show similar substrate specificity to its yeast homologue. Purified enzyme was a gift from D. Booze.

Array phosphorylation was carried out as described in Materials and Methods. After the control experiment in the absence of kinase, peptides were re-dissolved in reaction buffer which was then replaced as before with 2.5ml per membrane of the same buffer containing in addition 10 μ M ATP and 5mM MgCl₂, as well as Cdk2-cyclinA at a concentration of 0.5 μ M and 10 μ Ci ³²P γ ATP per membrane. Phosphorylation was carried out for 1 hour at room temperature, after which time the reaction was stopped by the addition of an excess of 1% SDS and heating to 65°C for 1 hour. The SDS incubation step was designed to denature and remove bound CDK from the membranes. Non-specifically bound phosphate was then competed away as before with 0.5% phosphoric acid overnight, followed by three further washes in 0.5% phosphoric acid and four sequential washes in 50%, 25%, 12.5% and 6.25% methanol to remove SDS from peptide bonds. Membranes were then rinsed in 96% ethanol and allowed to dry at room temperature, followed by visualisation of radiolabel using a phosphorimager. Radiolabel incorporated into each spot was quantified using ImageQuant software (Molecular Dynamics).

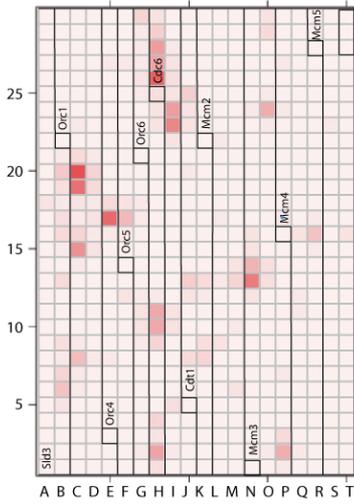
A promising pattern of radiolabel incorporation into the arrays was observed at this stage (Figure 3). In order to verify that radiolabel incorporation was due to peptide phosphorylation, the membranes were treated with λ phosphatase (Cohen & Cohen, 1989; Zhuo et al, 1993). Peptides were re-dissolved in λ phosphatase buffer and

incubated with λ phosphatase (NEB, 30,000U per membrane) for 1 hour at 30°C. Membranes were once again washed in 1% SDS and methanol, followed by drying, visualisation and quantification of the radiolabel. A general reduction in specific but not background signal of approximately 50% was observed, varying slightly between peptides. The signal in each spot of the array after phosphatase treatment was then subtracted from the signal prior to phosphatase treatment to give a specific peptide phosphorylation signal. Since some background noise was still present at this stage, manifested in apparent phosphate incorporation into sites that did not contain peptide, average background signal at these sites was further deducted from the general data set.

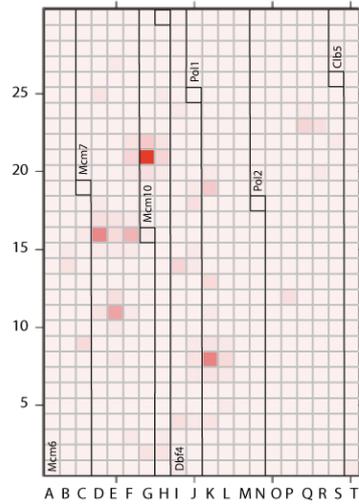
The data was then plotted as a series of 'heat maps', using software designed by the Cancer Research UK bioinformatics group. The results are shown in Figure 2. Heat maps are laid out as grids arranged in an identical manner to the membranes, with darker red spots indicating strong phosphorylation and white spots indicating an absence of phosphorylation. Phosphorylation is measured in arbitrary units and the scale is kept constant between membranes, indicated on the right hand side. Also marked on the grids are the positions of individual proteins, with protein names given at the N terminus of each. For comparison, an image of ^{32}P incorporation into membrane 1 prior to λ phosphatase treatment is also shown, in Figure 3.

Figure 2. Cdk2-cyclinA phosphorylation of peptide arrays. Membranes containing arrays of peptide spots were phosphorylated by human cyclinA-Cdk2 in the presence of ^{32}P γATP . Radiolabelled spots were quantified, membranes treated with λ phosphatase and radiolabel re-quantified in order to ensure that ^{32}P incorporation was due to peptide phosphorylation. Post phosphatase radiolabel levels were subtracted from the pre-phosphatase levels, as were background levels observed at sites containing no peptide. Results are plotted as a series of heat maps, with darker red spots indicating stronger phosphorylation. The number of each membrane is indicated alongside the relevant plot, and the positions of each protein on the array indicated. The scale is kept constant between membranes to allow comparison and is indicated on the right hand side.

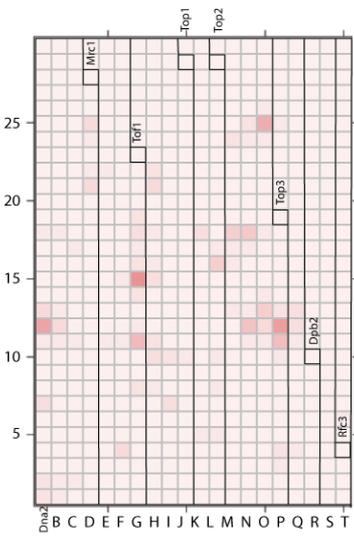
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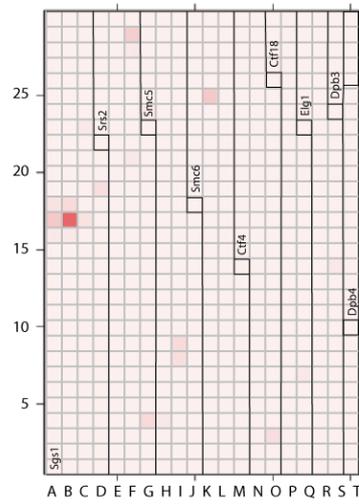
2.



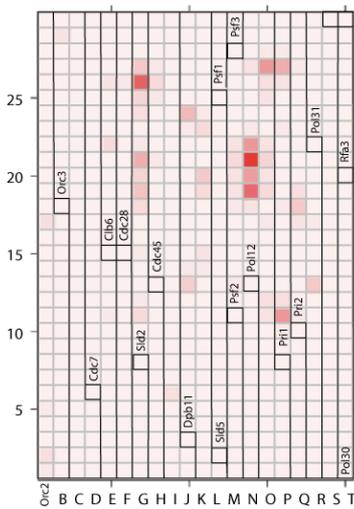
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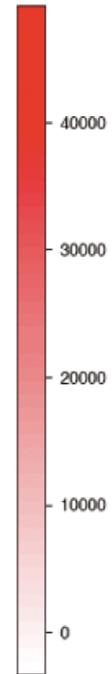
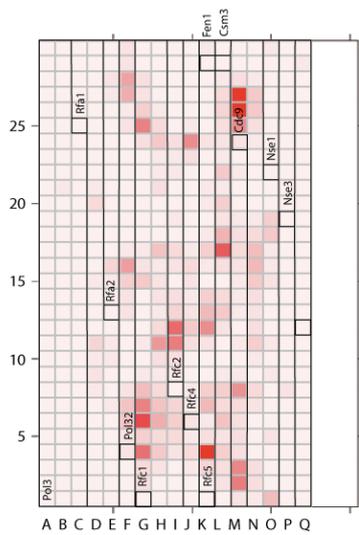
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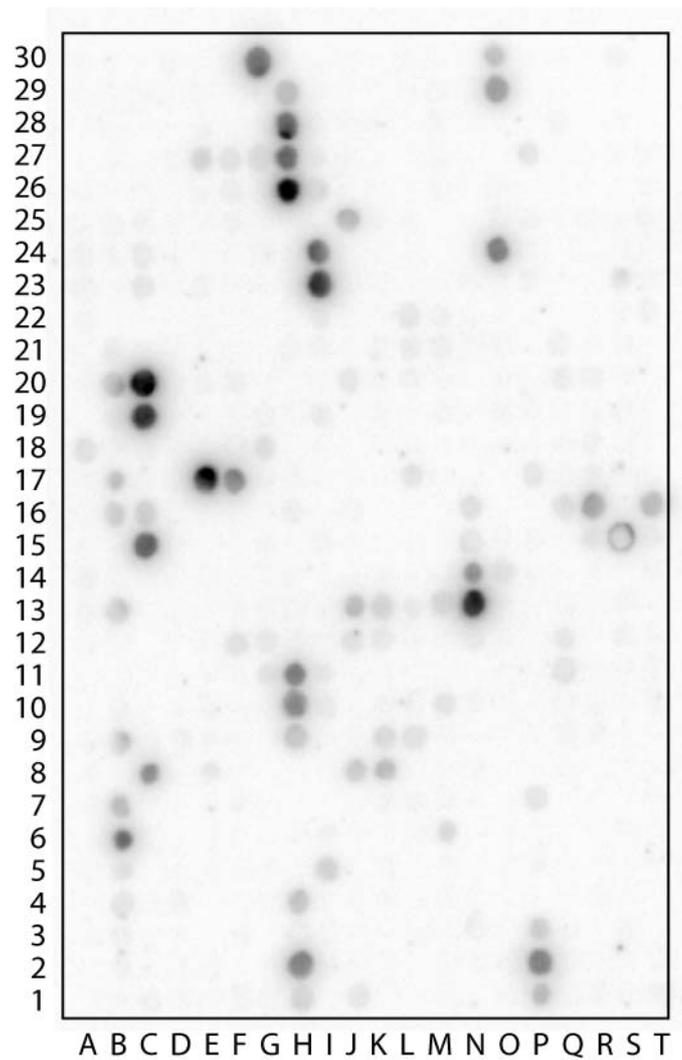


Figure 3. Membrane 1 phosphorylated with Cdk2-cyclinA. Raw data prior to λ phosphatase treatment. Membranes were incubated with 0.5 μ M Cdk2-cyclinA in the presence of 32 P γ ATP for 1 hour at room temperature. Membranes were washed and dried, and radiolabel incorporation visualised using a phosphorimager.

The data set appeared immediately interesting. Multiple peptides were observed to be phosphorylated, most often as expected in sets of two or more adjacent peptides.

Amongst the most strongly phosphorylated proteins were Orc1, Orc6 and Cdc6 on membrane 1, Mcm10 on membrane 2, Sld2 and Pol12 on membrane 5, and Pol32, Rfc1, Rfc2, Rfc5, Fen1, Csm3 and Cdc9 on membrane 6. In addition, the majority of proteins present on the arrays were phosphorylated to some extent at one or more locations. The experiment is expected to give a saturated set of possible kinase substrates, since peptides are presented to the kinase without consideration of the tertiary structure of the protein in which they are found *in vivo*. Other phosphorylated proteins include Sld3, Orc4, Orc5, Cdt1, Mcm2, Mcm3 and Mcm4 on membrane 1; Mcm7, Dbf4 and Pol1 on membrane 2; Dna2, Mrc1, Tof1 and Top2 on membrane 3; Sgs1 on membrane 4; Orc2, Dpb11, Pri1 and Pri2 on membrane 5; and Rfc4 on membrane 6.

Substrates of Cdc28 are known to include Orc2, Orc6 and Cdc6 and Mcm3, phosphorylation of which all contribute to the inhibition of pre-RC formation outside of G1 phase (Diffley, 2004; Liku et al., 2005), as well as Sld2 and Sld3, which are the minimal essential S-phase promoting CDK substrates (Zegerman & Diffley, 2007; Tanaka et al., 2007). The essential sites in Sld2 and Sld3 are known to be T84 in Sld2, and T600 and S622 in Sld3. The exact sites of phosphorylation in Orc2, Orc6 and Cdc6 are not known, but they all contain multiple putative S/T-P sites. The sites in Cdc6 have been shown to act in a partially redundant fashion, with mutations in various combinations of sites all contributing to the regulation of protein stability (Elsasser et al, 1999). Mcm3 has been shown to be phosphorylated in at least one of five strong consensus motifs, S761, S765, S781, T786 and S845 (Liku et al., 2005).

Analysis of peptide sequences on the phosphorylated arrays therefore allows both verification of phosphorylation of known sites, and the prediction of unknown sites. In Sld3, T600 and S622 are located on membrane 1 at peptides B16-18, and in Sld2, site T84 is located in membrane 5 at peptides G14-15. Unexpectedly, of the sites on membrane 1, B16 and 17 are only weakly phosphorylated and B18 not at all, perhaps indicating phosphorylation of T600 but not S622. On membrane 5, G15 was similarly weakly phosphorylated, but not G14. It therefore appears that not all known *in vivo* sites are being phosphorylated *in vitro*. There are several possible explanations for this. First, cyclinA-Cdk2 is not the enzyme directly responsible for Sld2 and Sld3 phosphorylation *in vivo*. Second, presentation of peptides outside the context of the full length protein may negatively affect kinase activity, for example by removing a distant binding site. Third, it cannot be expected that the essential *in vivo* substrates are necessarily the most readily phosphorylated either *in vivo* or *in vitro*.

In Orc2, phosphorylation is also relatively weak, but is observed at sites A1-2 and A17 (membrane 5). These contain the putative S/T-P phosphorylation sites S16, T24, T217 and T219 respectively, of which site S16 is contained in a strong S-P-x-K consensus. In Cdc6, phosphorylation is considerably stronger, including in particular peptides H26-29 and I23-24 (membrane 1). These correspond to S/T-P sites T7, T23, S43 and S372, all of which are contained in strong consensus sites. Orc6 was phosphorylated on peptides G30-H4 (membrane 1), corresponding to S106, T114, S116, S123, T146 and S172. Of these, S106, S116, S123 and T146 are all contained in a strong consensus sequence. However, another unexpected observation was the appearance of phosphorylation of Orc6 in peptides H9-11. These sites do not contain an S/T-P consensus. The reason for their phosphorylation in these experiments is

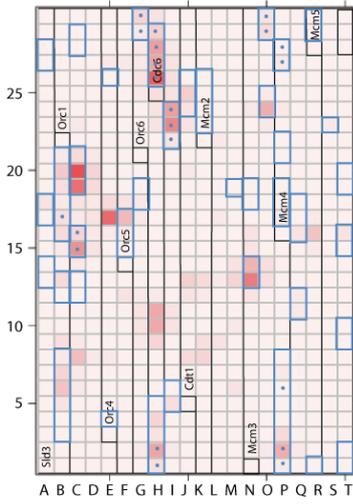
therefore unclear, since previous reports have shown an absolute requirement for the proline residue in the +1 position, and all known substrates conform to this consensus (Songyang et al, 1994). Mcm3 is phosphorylated in peptides containing sites S761, S765, S781 and T786, although not site S845. Mcm3 is also phosphorylated at two minimal consensus sites S167 and S695.

In order to examine the possible extent of phosphorylation of peptides which did not contain an S/T-P motif, the heat maps were re-annotated and overlaid with S/T-P motifs and stronger S/T-P-x-K/R motifs, marked with a blue box or a central blue dot respectively. These are shown in Figure 4. It should be noted that where an S/T-P or S/T-P-x-K/R motif is present only at a single site rather than a pair, it is either in the first or last 13 residues of a protein, or the S/T-P site falls exactly at residues 13-14 of the peptide in which it is contained. In this context the site is not repeated since only the proline is contained in the second peptide of the sequence.

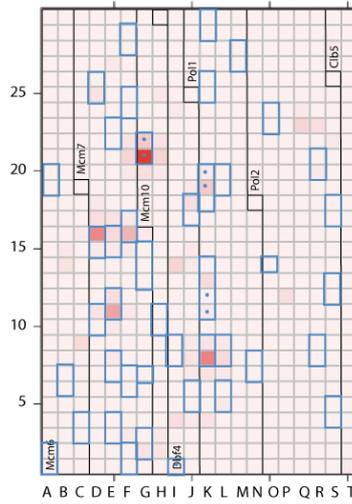
As well as giving an indication of the extent of non S/T-P motif phosphorylation, this analysis also gives a measure of the specificity of the kinase towards different S/T-P containing peptides. If CDK phosphorylation is dependent simply on the presence of an S/T-P or S/T-P-x-K/R consensus then the pattern of phosphorylation would be expected to coincide exactly with the presence of these motifs, with peptides containing multiple S/T-P-x-K/R or S/T-P motifs phosphorylated more strongly than those with only a single site, and a complete absence of phosphorylation in peptides which do not contain a consensus site. If other factors also contribute to kinase specificity, then a different pattern would be expected.

Figure 4. Annotation of phosphorylated arrays with CDK consensus motifs. Re-annotation of the CDK phosphorylated heat maps, with CDK consensus motifs (S/T-P) annotated with blue boxes and stronger consensus sites (S/T-P-x-K/R) with a blue dot inside the box.

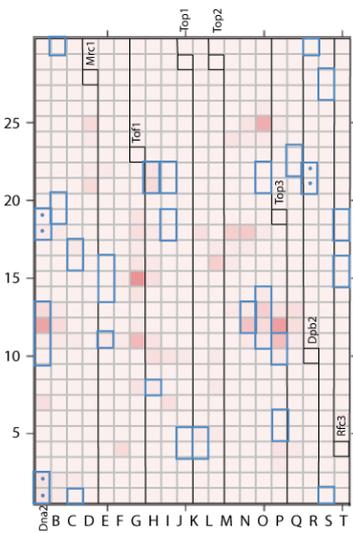
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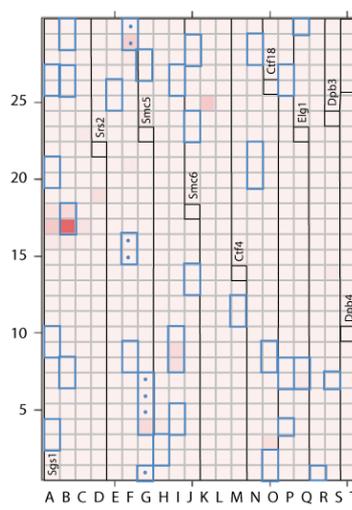
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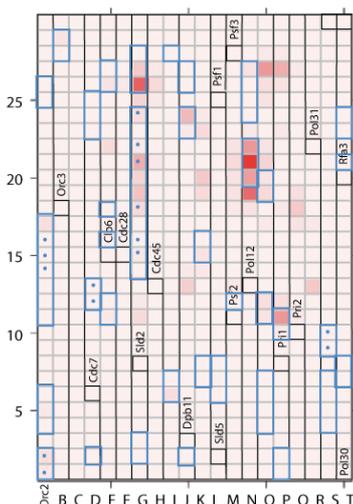
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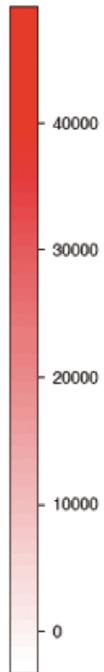
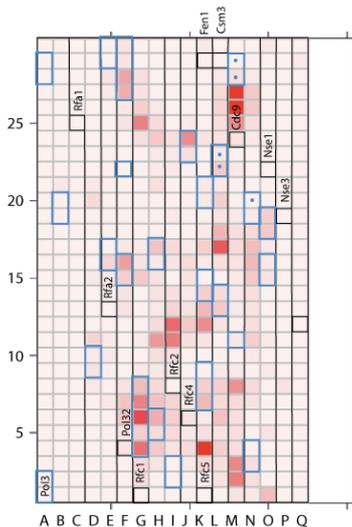
4.



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6.



It is immediately clear from these figures that the correlation of phosphorylation with the presence or absence of a consensus sequence is not direct. Many sites containing S/T-P motifs remain unphosphorylated above background levels and, whilst in the majority of cases the most strongly phosphorylated peptides do contain one or more S/T-P motifs, this is not always the case. Notable exceptions include the sites in Orc6 already mentioned, as well sites in Mrc1 on membrane 3, and Rfc1, Rfc2, Csm3 and Cdc9 on membrane 6.

Leaving aside sites showing strong phosphorylation in the absence of a consensus motif, several interesting potential substrates are revealed. These include Orc1, Mcm3, Mcm7 and Mcm10, Pol1, Dna2, Top2, Sgs1, Pol12, Pol32 and Rfc1. The reason for the specificity of phosphorylation of sites in these proteins over other S/T-P containing peptides is currently unclear, and further verification is necessary to determine whether CDK phosphorylation of these sites occurs *in vivo*. The lack of phosphorylation of essential sites in Sld2 and Sld3 makes it difficult to conclude directly from these experiments whether they represent physiologically important events. However, it is possible that CDK phosphorylation contributes to regulation of these proteins. It is interesting that phosphorylation of the strong consensus motifs in Mcm3 is specific for S761, S765, S781 and T786 but not S845, and this may represent genuine specificity of phosphorylation *in vivo*. The four phosphorylated sites are closely clustered around the nuclear localisation signal in the protein (Liku et al., 2005)

A selection of sites phosphorylated in the absence of an S/T-P motif is shown in Table 2, with serine and threonine residues marked in red.

Table 2. CDK phosphorylated sites in the absence of an S/T-P motif. Peptide sequences are listed, and serine and threonine residues marked in red.

Membrane	Site	Peptide sequence
1	E17	ETISSGSLTEVFEEKILLLLDSTTKTR
	E18	KILLLLDSTTKTRNEDSGEVDRESIT
	H 9	HESDPTSEEEPLGVQESRSGRTKQNK
	H10	VQESRSGRTKQNKAVGKPQSELKTAK
	H11	AVGKPQSELKTAKALRKRGRIPNSLL
3	G 8	QKPVVDQKNKKVIISEDFVQKSLSFLK
	G11	SRIQHGNDIAIEDLYTLKQNSSIKSF
	G14	NTIIDLEKRPEDEDEVENGDTSLVGV
	G15	DEVENGDTSLVGVFKHPSIIKSFASR
	G16	FKHPSIIKSFASRTDINDKFKEGNKT
	G18	VKILKSYKTVGSSKASITYMGKTRKL
	G19	KASITYMGKTRKLIAPKRKTEGSHRY
	M18	VYLNGKSLKIRNFKNYVELYLKSLEK
	N18	KIFHSLQGNDKDYIDLAFSKKKADDR
	O25	PEELYGTYEYLLGMRIWSLTKERYQK
4	A17	SEDAKRLQLSRDIRPQLSNMSIRIDS
	A18	RPQLSNMSIRIDSLEKEIHKAKKDGM
5	K19	ICPRGHKDDFKCKIKKPYYSISSEK
	K20	IKKPYYSISSEKQYQNNDPKIDKTI
	K23	MKDTKNELLQKIRETDSGRKKRSVSS
	M19	WLTTKELDRKIYEEKTHPDRFSELPW
	M20	EKTHPDRFSELPWNWLVLARILFNKA
	M21	NWLVLARILFNKAKDDFHDPIHELGR
	M22	KDDFHDPIHELRGKIQDLREIRQIKV
	O21	TVLIPSTKDAISNHAAYPQASLIRKA
	O22	HAAYPQASLIRKALQLPKRNFKCMAN
	O25	SNVDTFKDLKEVIKGGTTSSRYRLDR
	O26	KGGTTSSRYRLDRVSEHILQQRYYYP
	O27	VSEHILQQRYYPIFPGSIRTRIKPK
	P26	PFFVSIMLEDENPWEDDQHAIQTLLP
	P27	WEDDQHAIQTLLPALYDKQLIDSLKK
	Q17	IWAIDRLKILLEIESCLSRNKSIKEI
	Q18	ESCLSRNKSIKEIETFTIIKPQFQKL
Q19	ETFTIIPQFQKLLPFNTESLEDRKK	
R13	YGLEGNRINYKPWDCHTILSKPRPGR	
6	F6	FTEVKPVLFTDLIHHLKIGPSMAKKL
	F7	HHLKIGPSMAKKLMFDYYKQTTNAKY
	G15	PTLERGESEALAKRYGARVTKSISSK
	G16	RYGARVTKSISSKTSVVVLGDEAGPK
	G17	TSVVVLGDEAGPKKLEKIKQLKIKAI
	G24	RDNVVREEDKLWTVKYAPTNLQQVCG
	G25	VKYAPTNLQQVCGNKG SVMKLNWLA
	G26	NKG SVMKLNWLANWENS KKN SFKHA
	G27	NWENS KKN SFKHAGKDGS GVFRAAML
	G28	GKDGS GVFRAAMLYGPPGIGKTTAAH
	H7	NERNLPKMRPFDRVCLDIQFRRPDAN
	H8	VCLDIQFRRPDANSIKSRLMTIAIRE

H10	KFKLDPNVIDRLIQ T TRGDIRQVINL
H11	Q T TRGDIRQVINLL S TISTTTKTINH
H12	L S TISTTTKTINHENINEISKAWEKN
H21	W S LLPLHAVL S SVYPAS K VAGHMAGR
H24	AKYYRLLQEIHYH T RLGT S TDKIGLR
H25	T RLGT S TDKIGLRLDYLP T FRKRLD
I5	E E TQ S S T DLKKDKLIKQKAK P TKRK
I6	KLIKQKAK P TKRK T AT S KPGG S KKRK
I7	KQKAK P TKRK T AT S KPGG S KKRK T KA
I9	MFEGFGPNKKRK I SKLAAEQ S LAQQP
I10	S KLAAEQ S LAQQPWVEKYRPKNLDEV
I11	WVEKYRPKNLDEV T AQDHAV T VL K KT
I12	T AQDHAV T VL K KT L KSANLPHMLFYG
I13	L K SANLPHMLFYGPPG T G K T STILAL
I14	PPG T G K T STILAL T KELYGPDLM K SR
I16	I L ELNAS D ERGISIVREKVKNFAR L T
I17	I V REKVKNFAR L T V S K P S K HDL E N Y P
J11	I G K T S VHCLAHELLGR S YADGVLEL
J12	LLGR S YADGVLELN S DDRGIDVVRN
J13	NA S DDRGIDVVRNQIKHFAQKKLHLP
J14	QIKHFAQKKLHLP P PGKHKIVILDEAD
J15	PGKHKIVILDEAD S M T AGAAQ Q ALRR T
J16	S M T AGAAQ Q ALRR T MELY S N S TRFAFA
K 4	K S L S DQPRDLP H LLLYGPNG T G K K T R
K11	QMEQVDFQ D S KDGLAHRYK C VIINEA
K12	LAHRYK C VIINEAN S L T KDAQAALRR
K13	N S L T KDAQAALRR T MEKY S KNIRLIM
L2	FGRK V AID A S MSLYQFLIAVRQQDGG
L3	YQFLIAVRQQDGG Q L T NEAG E T T SHL
L4	Q L T NEAG E T T SHLMGMFYR T LRMIDN
L5	MGMFYR T LRMIDNGIKPCYVFDG K PP
L6	GIKPCYVFDG K PPDL K SH E L T KR S SR
L7	DL K SH E L T KR S SR R VE T E K KLAE A T T
L8	R V ET E K K LAE A T TELEKMKQERRLVK
L16	L V LRGLD L T IEQFV D LCIMLGCDYCE
L17	V D LCIMLGCDYCE S IRGVGPV T ALKL
L18	S IRGVGPV T ALKLIK T H G S IEKIVEF
L24	LCDDKK F SEER V K S GISRLKKGL K S G
L25	S GISRLKKGL K S GIQGRLDGFFQVVP
L26	I Q GRLDGFFQVVP K T KEQLAA A AKRA
L27	K T KEQLAA A AKRAQEN K KL N KN K NV
M2	GLDG S VVD P T IAD P T A I TARKRRP Q V
M3	P T A I TARKRRP Q V K L T A E KLL S DKGL
M4	K L T A E KLL S DKGLPYVLK N AH K RIRI
M5	PYVLK N AH K RIRI S SK N S Y DNLS N I
M6	S SK N S Y DNLS N I I QFYQLWAHEL F P
M7	I Q FYQLWAHEL F P K A K FKDFM K IC Q T
M8	K A K F KDFM K IC Q T V G K T DPV L REYRV

M25	MRLLTGCLLSSARPLKSRLPLLMSS
M26	RPLKSRLPLLMSSSLPSSAGKKPKQA
M27	SLPSSAGKKPKQATLARFFTSMKNKP
N11	LGENLLMKTISETCGKSMSQIKLKYK
N12	CGKSMSQIKLKYKDIGDLGEIAMGAR
N13	DIGDLGEIAMGARNVQPTMFKPKPLT
N14	NVQPTMFKPKPLTVGEVFKNLRAIAK
N15	VGEVFKNLRAIAKTQGKDSQLKKMKL
N16	TQGKDSQLKKMKLIKRMILTACKGIEA
N17	IKRMLTACKGIEAKFLIRSLESKLRI
N18	KFLIRSLESKLRIGLAEKTVLISLSK
O1	LDCEAVAWDKDQGKILPFQVLSTRKR

The initial control testing the non-specific binding of ^{32}P γATP to the membranes makes it unlikely that ^{32}P labelling of these sites is kinase independent, and furthermore, the signal was reduced following λ phosphatase treatment, arguing that phosphate incorporation is due to peptide phosphorylation. Phosphorylation of these peptides is therefore difficult to explain. They do not appear to contain any immediately apparent sequence similarity. It is possible that their phosphorylation is due to the activity of a contaminating kinase, although this is also unlikely since any contaminating activity should be present at much lower levels than CDK. Further control experiments such as re-phosphorylating the arrays in the presence of a CDK inhibitor would be possible to test this, but have not been attempted here. It is also possible that in the context of this assay, CDK is active against substrates which do not conform to its consensus. However, if this is the case then it is strange that many sites which do contain an S/T-P motif, which should be more readily phosphorylated, do not show phosphate incorporation. This includes peptides which contain known phosphorylation sites *in vivo*. A repeat experiment using a re-synthesised copy of membrane 6 gave an identical pattern of phosphorylation, arguing against inaccurate peptide synthesis as the cause (data not shown). It is also to be noted that amongst the

peptides listed above, four do not contain either a serine or a threonine residue (M21-22 on membrane 5, H7 and J14 on membrane 6). Any phosphate incorporation into these sites must therefore be through alternative residues. For example, it is known that λ phosphatase removes phosphate from phosphorylated histidine residues (Zhuo *et al.*, 1993).

From this experiment it was concluded that in this assay, CDK seems to show some specificity towards the phosphorylation of individual consensus motifs, resulting in the identification of several putative targets of the kinase, including both previously known and unknown substrates. The amount of specificity was unexpected, however, as was ^{32}P incorporation into peptides which do not contain S/T-P motifs. It remains possible that some of the results may be biased by the experimental technique. Although each peptide in the array is expected to behave in an identical manner, it is possible that the peptides differ in their accessibility to the kinase. It is also unclear to what extent the PEG membrane linker might interfere with kinase function. CDK may under these conditions phosphorylate some serine and threonine residues outside of its usual consensus sequence, the reason for which is unclear.

3.4 Cdc28-Clb5 phosphorylation of peptide arrays

Since cyclinA-Cdk2 is not itself the enzyme responsible for phosphorylation of the proteins on the array, and showed some slightly unexpected results, we wished to determine how the *S. cerevisiae* enzyme would perform in the same assay.

Endogenous Cdc28-Clb5, purified at the outset of the project, was therefore used to phosphorylate the peptide arrays.

Endogenous *S. cerevisiae* enzymes were purified using the Tandem Affinity Purification (TAP) tag method (Puig et al, 2001; Rigaut et al, 1999). The TAP tag used in these purifications was almost identical to that originally described (Rigaut et al, 1999), containing a calcium sensitive calmodulin binding peptide (CBP), two IgG binding units from *Staphylococcus aureus* proteinA and a TEV cleavage site for final protein elution. However, the TEV cleavage site was moved from between the calmodulin and IgG binding domains to the immediate C terminus of the tagged protein in order to ensure that purified proteins contained as little exogenous material as possible (Figure 5A). The modified TAP tag was termed TAP^{TCP}, and plasmids were a gift of D. Remus. Purification of TAP tagged proteins was as follows; proteins were bound to a calmodulin affinity resin in the presence of calcium and eluted from the resin in the presence of EGTA. Proteins were then bound to IgG sepharose and finally eluted by cleavage of the tag with TEV protease (Figure 5B). The TEV protease used in these experiments itself contained a hexa-His tag, and was therefore removed as a final step of the purification by binding to a nickel affinity resin.

TAP^{TCP} tagged Clb5 was overexpressed under the control of the *GALI-10* promoter, and was obtained by sub-cloning *CLB5* amplified by PCR from *S. cerevisiae* strain W303 genomic DNA into plasmid pLD52, a pRS306 based yeast transformation vector (Sikorski & Hieter, 1989) which contained *GALI-10 CDC6-TAP^{TCP}*. The *GALI-10* promoter is galactose inducible, being highly stimulated by the transcription factor Gal4 when cells are grown in the presence of galactose, but inhibited in the presence of glucose (Lohr et al, 1995). This therefore allows the controlled over-expression of proteins *in vivo*. It is less robustly repressed when cells are grown in the presence of raffinose, allowing rapid induction of protein expression. *CDC6* was

excised from between the *GALI-10* promoter and TAP^{TCP} tag in pLD52 and *CLB5* inserted in its place, creating plasmid pSS2. *CLB5* was sequenced and pSS2 transformed into *S. cerevisiae* strain ySC4, which contained a Pep4 protease deletion in order to minimise protein degradation in cell extracts when used for purification (Ammerer et al, 1986).

A selection of transformants was tested for expression of TAP tagged Clb5 after growth in galactose containing medium. A whole cell TCA precipitation was made from samples of 1×10^8 cells and proteins analysed by SDS-PAGE and Western blotting using an HRP coupled anti-peroxidase antibody, recognising the IgG binding section of the TAP tag. Of the positive transformants, one was selected and named ySS5. Protein over-expression was then followed over a 5 hour timecourse after addition of galactose to a cell culture grown to mid-log phase (approximately 2×10^7 cells ml^{-1}) in the presence of raffinose. Samples of 1×10^8 cells were taken at 30 minute intervals, TCA preps made and proteins analysed by SDS-PAGE and Western blotting as described (Figure 5C). Protein expression was observed to be maximal after approximately 1.5 hours.

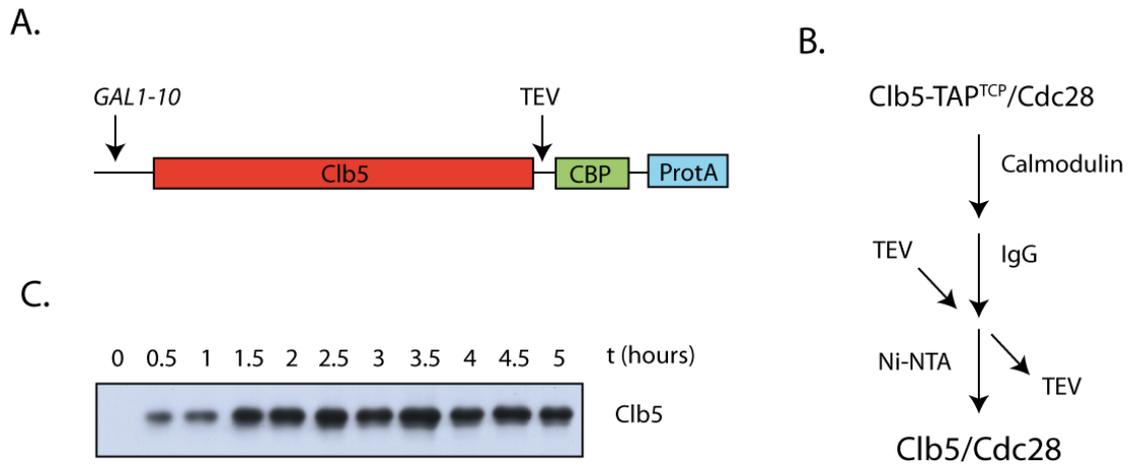


Figure 5. Purification of TAP^{TCP} tagged proteins and expression of Clb5-TAP

- A.** The TAP^{TCP} tag contains a calmodulin binding peptide (CBP) and two *S. aureus* proteinA (protA) IgG binding units, as well as a TEV cleavage site adjacent to the C terminus of the tagged protein (modified slightly from the original description) (Rigaut et al., 1999). Tagged *CLB5* was cloned into plasmid pLD52 downstream of the *GAL1-10* promoter.
- B.** TAP^{TCP} tagged proteins are purified by sequential binding and elution from calmodulin affinity resin and IgG sepharose. Final elution from IgG is by TEV protease cleavage. Following purification, Hexa-His tagged TEV is removed by binding to Ni-NTA agarose (Qiagen).
- C.** Overexpression profile of *GAL1-10 CLB5-TAP^{TCP}*. Samples of 1×10^8 cells were taken at 30 minute intervals after induction of protein expression, TCA preps made and proteins analysed by SDS-PAGE followed by Western blotting against the TAP^{TCP} tag using an HRP coupled anti-peroxidase antibody. Expression is maximal after approximately 1.5 hours.

Since Sic1 binds and inhibits the activity of Cdc28 through the G1 phase of the cell cycle (Schwob et al, 1994), *SIC1* was next deleted in strain ySS5 by integration of the *TRP1* marker into the *SIC1* locus after amplification by PCR from plasmid pRS304 (Sikorski & Hieter, 1989). Transformants were selected by growth on selective plates in the absence of tryptophan and correct integration verified by colony PCR of the *SIC1* locus. One positive clone was selected and named ySS8.

Purification of Clb5-Cdc28 was then carried out according to the scheme outlined above and described in Materials and Methods. 8 litre cultures of both ySS5 and ySS8 were grown in raffinose to mid log phase, and galactose added to induce protein expression for 2.5 hours. A cell extract was made and TAP tagged proteins bound to calmodulin affinity resin (Stratagene) in the presence of calcium. Bound protein was eluted in a calcium free buffer and subsequently re-bound to IgG beads and eluted with TEV protease. Protease was removed by binding to a nickel affinity resin.

Protein purification at each step was followed by Western blotting against Clb5 and Cdc28 (Figure 6A and C) and silver staining (Figure 6B and D). Cdc28 purified with Clb5 as a complex. Clb5 shows two major degradation products and, in the absence of Sic1, partial phosphorylation which can be removed by treatment with λ phosphatase (Figure 6E). Two contaminating bands were observed which were common to the purification from both ySS5 and ySS8.

The activity of Cdc28-Clb5 towards histone H1 was tested in a series of kinase assays (Figure 6F). 5 μ l, approximately 100ng, of kinase purified from either ySS5 or ySS8 was used to phosphorylate 20 μ g histone H1 in the presence of 32 P γ ATP. Control

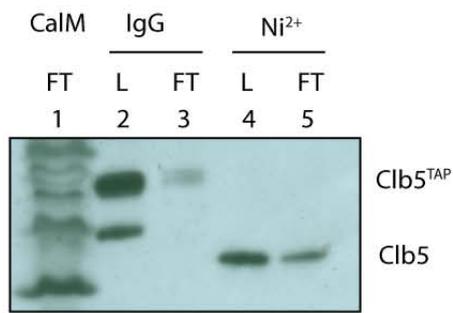
reactions were carried out in the absence of kinase. Purified histone H1 was a gift from A. Errico. Reactions were allowed to proceed for 20 minutes at 30°C before stopping by the addition of an equal volume of 2x Laemmli buffer and boiling for 10 minutes. A quarter of the total reaction was separated by SDS-PAGE and proteins visualised by Coomassie staining. Stained gels were dried and radiolabel visualised by autoradiography.

Figure 6F shows that Cdc28-Clb5 was highly active against histone H1 only in the absence of Sic1. Phosphorylation was sufficient to cause saturated incorporation of ³²P into histone H1, resulting in an upward shift in the resolving position of the protein in SDS-PAGE.

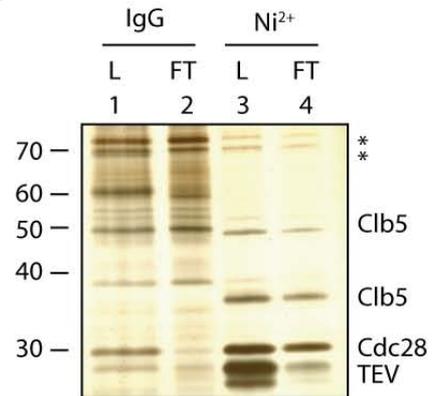
Figure 6. Cdc28-Clb5 purification and activity

- A.** Clb5-Cdc28 purification from ySS5. Clb5 is followed at each step of the purification by Western blotting using a Clb5 polyclonal antibody (Santacruz). Lanes show calmodulin flow through (lane 1), IgG input and flow through (Lanes 2 and 3), and nickel resin input and flow through (Lanes 4 and 5). TAP^{TCP} tagged and untagged Clb5 are indicated.
- B.** Silver stain of Clb5 purification from ySS5. Lanes are as described above, but do not include the calmodulin flow through. Clb5 and its major degradation products are indicated, as are Cdc28 and TEV protease. Contaminating bands are indicated with an asterisk.
- C.** Clb5-Cdc28 purification in the absence of Sic1 from ySS8. Clb5 and Cdc28 are followed at each step of the purification by Western blotting using a polyclonal antibody against Clb5 (Santacruz) and a monoclonal antibody against the PSTAIRE loop of Cdc28. Lanes are as described above with the addition of the calmodulin input material (lane 1). Clb5-TAP^{TCP}, untagged Clb5 and Cdc28 are indicated.
- D.** Silver stain of Clb5-Cdc28 purification from ySS8. Lanes are as described above but do not include either calmodulin input or flow through. Clb5 and its major degradation product are indicated, as are Cdc28 and TEV protease. Contaminating bands are indicated with an asterisk.
- E.** Clb5 purified in the absence of Sic1 is phosphorylated. Lanes show input, and Clb5-Cdc28 either treated or mock treated with λ phosphatase
- F.** Clb5-Cdc28 can phosphorylate histone H1 *in vitro* in the absence of Sic1. Coomassie stain and autoradiographs are shown in upper and lower panels respectively.

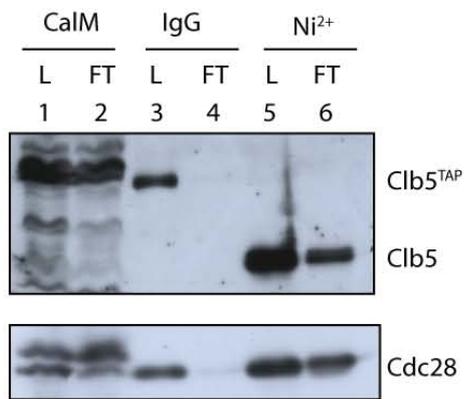
A.



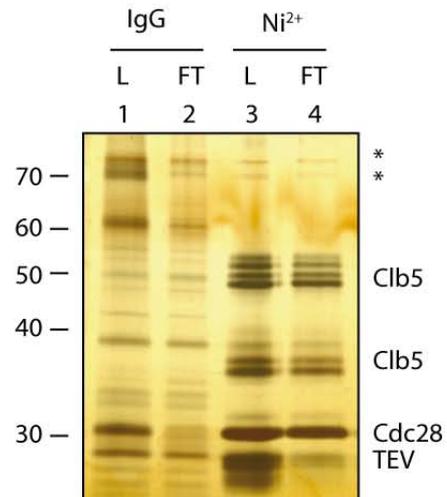
B.



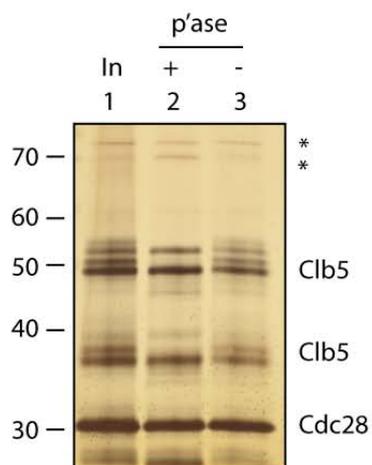
C.



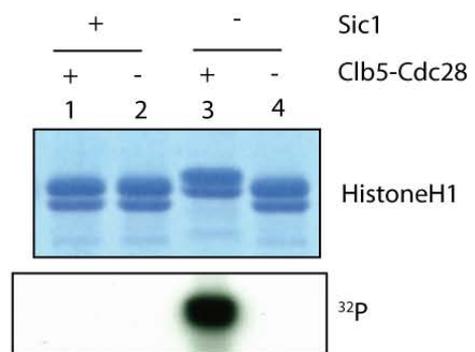
D.



E.



F.



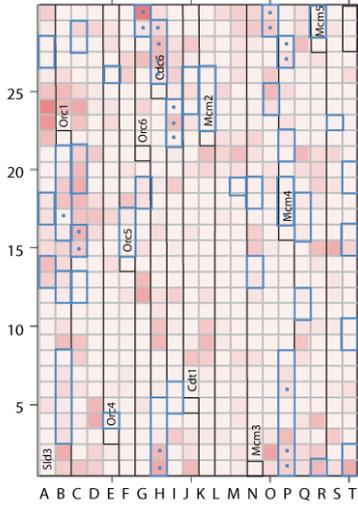
Cdc28-Clb5 purified in the absence of Sic1 was therefore used in the array phosphorylation experiments. Array phosphorylation was carried out as before, with slight changes in the salt and ATP concentrations in the reaction buffer. Between Cdk2-cyclinA and Cdc28-Clb5 phosphorylation experiments, radiolabel remaining on the arrays was allowed to decay to negligible levels over a period of approximately 6 months. Approximately 200ng of purified enzyme was used per membrane.

Reactions were allowed to proceed for 1 hour at 30°C and were stopped with 1% SDS followed by washing, drying, visualisation, quantification, λ phosphatase treatment, re-quantification and data analysis as before.

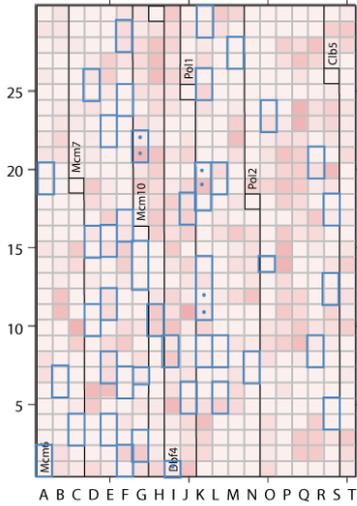
Heat maps are shown in Figure 7, showing CDK consensus sites as in Figure 4. Once again, for comparison the raw data is shown for membrane 1 prior to phosphatase treatment, in Figure 8.

Figure 7. *Cdc28-Clb5 phosphorylation of peptide arrays.* Heat maps following phosphorylation of the membranes with Cdc28-Clb5 are shown, along with CDK consensus sites annotated as in Figure 3.

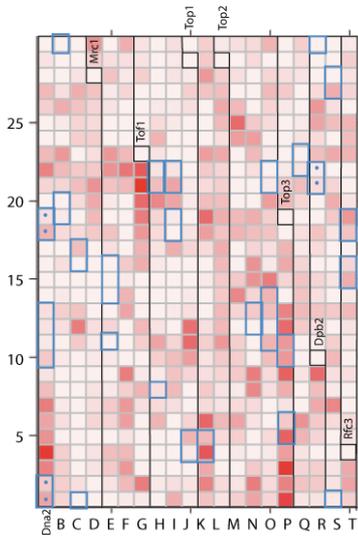
1.



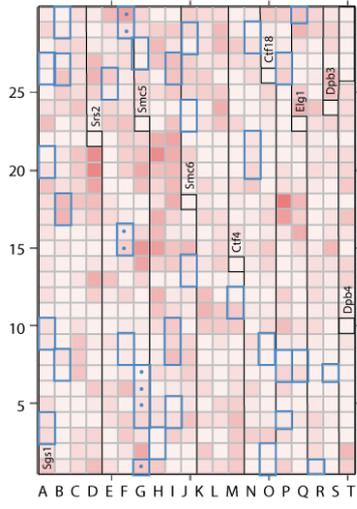
2.



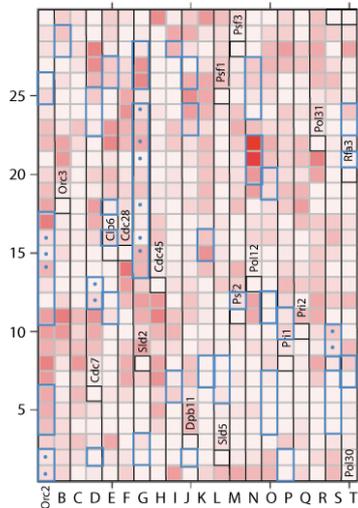
3.



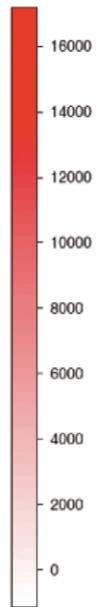
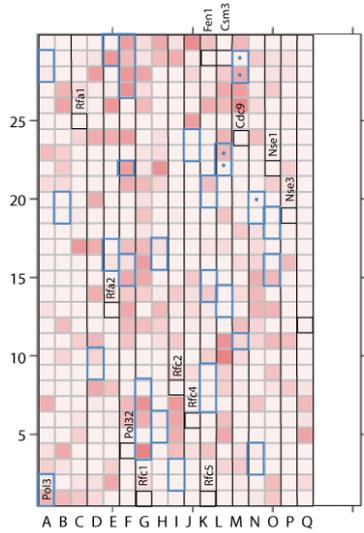
4.



5.



6.



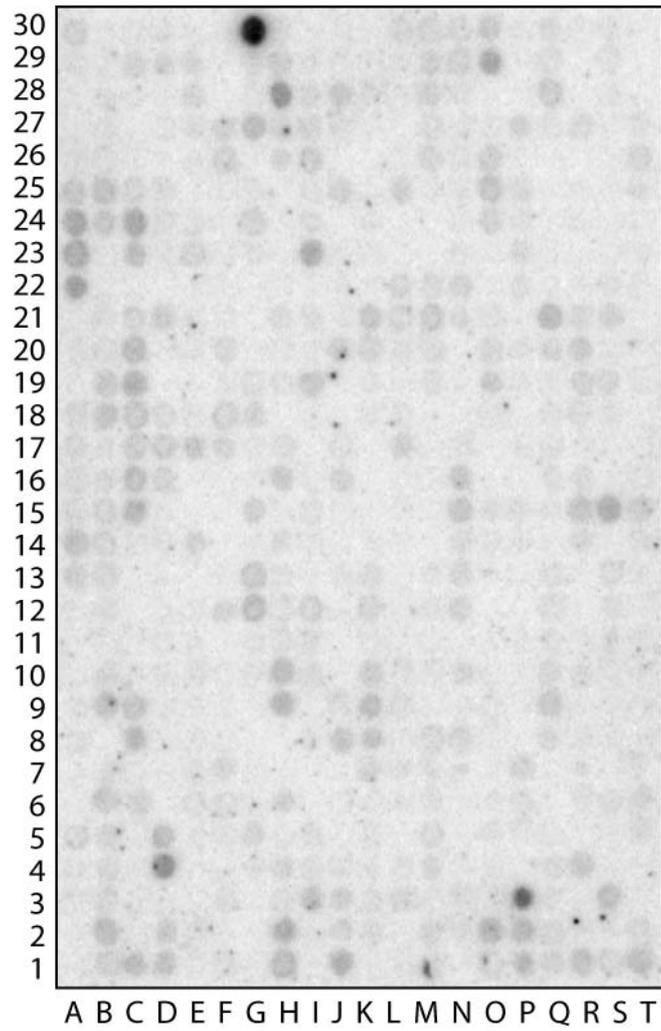


Figure 8. Membrane 1 phosphorylated with Cdc28-Clb5. Raw data prior to λ phosphatase treatment. Membranes were incubated with Cdc28-Clb5 in the presence of ^{32}P γATP for 1 hour at 30°C. Membranes were washed and dried, and radiolabel incorporation visualised using a phosphorimager

In this experiment, however, the pattern of phosphorylation differed even more markedly from the pattern of S/T-P consensus motifs. Levels of overall phosphorylation were lower, presumably due to the lower enzyme concentrations used, and large numbers of peptides with no S/T-P motif were apparently phosphorylated. There does not appear to be any enrichment for phosphorylation of peptides which do contain a consensus motif. It was therefore very difficult to determine genuine CDK phosphorylation events from background events. Again, it is possible that a contaminating enzyme is responsible for the activity, although it is strange that CDK phosphorylation events are not more prominent, given its activity against histone H1. Again, no control experiment, such as phosphorylation of the arrays with Cdc28-Clb5 purified in the presence of Sic1, was attempted. It is also possible that there is now some background ^{32}P γATP binding which was not seen in the initial control experiment.

To some extent, the pattern of phosphorylation overlaps with that of cyclinA-Cdk2. On membrane 1, for example, some sites phosphorylated in Sld3, Orc1, Orc6, Cdc6, Cdt1 and Mcm3 containing S/T-P motifs are the same in both experiments. In addition, on this membrane several of the sites apparently phosphorylated in the absence of a CDK consensus also remain the same. For example, the sites in Orc6 at H9-11, and in Cdt1 at J8, K8-9 and K12. This makes the activity of a contaminating kinase unlikely, since it would not be the same activity in both preparations, one from *E. coli* and the other from yeast. On membrane 2, the sites in Mcm10 at G21-22 containing a consensus are phosphorylated in both experiments, as are the sites in Sgs1 at B17-18 on membrane 4. On membrane 5, sites in Sld2 (E19, O29, O22-23 and O26-27) are common to both experiments, as are the sites in Pol12 at N20-22.

The peptide at N21 on this membrane is the strongest phosphorylated in both experiments. On membrane 6, the pattern of phosphorylation in Rfc1 at sites G4-8 remains broadly the same, with the strongest phosphorylation in peptides G4 and G6-7. In addition, the three peptides in Cdc9 most strongly phosphorylated by cyclinA-Cdk2 in the absence of a CDK consensus, at sites M26-27, are also phosphorylated by Clb5-Cdc28, as is the peptide K4 in Rfc5. Phosphorylation of the essential sites in Sld2 and Sld3 is no more prominent than before, although the T84 containing peptides in Sld2 are phosphorylated which were not previously.

Several proteins were chosen to attempt to validate CDK phosphorylation *in vivo*. These were Rfc1, Pol32, Cdc9, Sld2, Sld3 and Orc2. On the arrays, both Rfc1 and Pol32 are phosphorylated at sites containing S/T-P motifs in experiments with both cyclinA-Cdk2 and Clb5-Cdc28, whilst Cdc9 is phosphorylated in both experiments at sites that do not contain an S/T-P consensus. Sld3, Sld2 and Orc2 are known to be substrates of CDK *in vivo* and therefore act as positive controls. Of the three test proteins, both Pol32 and Cdc9 have been previously reported as substrates of Cdc28-Clb2 (Ubersax et al., 2003).

Strains in which these proteins had been tagged with 13 Myc were therefore arrested in either α factor or nocodazole to block the cells in G1 phase or mitosis. Proteins from a sample of 1×10^8 cells were then TCA precipitated, samples separated by SDS-PAGE and Myc tagged proteins identified by Western blotting with the monoclonal 9E10 antibody in order to look for a phosphorylation mediated shift specifically in mitosis, when CDK activity is high (Figure 9).

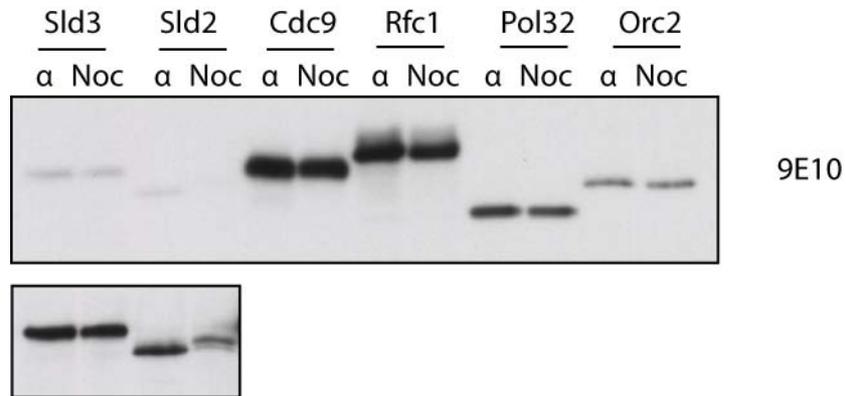


Figure 9. Validation of CDK substrates. 200ml cultures of strains containing 13xmyc tagged copies of Sld3, Sld2, Cdc9, Rfc1, Pol32 or Orc2 were arrested in G1 or mitosis with α factor (α) or nocodazole (Noc), total protein from a sample of 1×10^8 cells was TCA precipitated, and samples separated by SDS-PAGE and analysed by Western blotting to identify a shift in myc-tagged proteins when arrested in mitosis. Longer exposures are shown in the cases of Sld3 and Sld2. Only Sld2 was seen to shift clearly in the presence of CDK.

Of the six proteins, only Sld2 was seen to shift specifically in the presence of nocodazole but not α factor. The lack of shift does not necessarily imply a lack of phosphorylation, however, and both Sld3 and Orc2 are known to be phosphorylated *in vivo*. Orc2 is known to give a phosphorylation dependent shift in SDS-PAGE when not tagged (Van *et al.*, 2001). Attempts were therefore also made to examine the possible phosphorylation of Rfc1 by CDK *in vitro*, by pulling down Myc-tagged Rfc1 from extracts made of cultures arrested either in G1 or mitosis followed by protein analysis by SDS-PAGE and Western blotting against both the myc tag and using an antibody recognising phosphorylated S/T-P motifs. However, although Rfc1 was pulled down specifically from the Rfc1-myc tagged strain, no specific signal was seen from the pSP antibody in the asynchronous or nocodazole arrested cultures. Instead, only a general background signal was observed from all three cultures (data not shown). The relevance of any of the CDK substrates identified in these experiments *in vitro* therefore remains unclear. Further experiments are required to validate the potential sites of phosphorylation *in vivo* and to determine their possible effects on the process of DNA replication.

3.5 Purification of Cdc7-Dbf4

Although interpretation of the CDK array phosphorylation results proved complicated, we were also interested at the outset of the project in identifying Cdc7-Dbf4 substrates using the array phosphorylation technique. Endogenous Cdc7-Dbf4 was first purified in parallel to Clb5-Cdc28. As described for *CLB5*, *DBF4* was subcloned into pLD52, sequenced and transformed into ySC4, making strain ySS4.

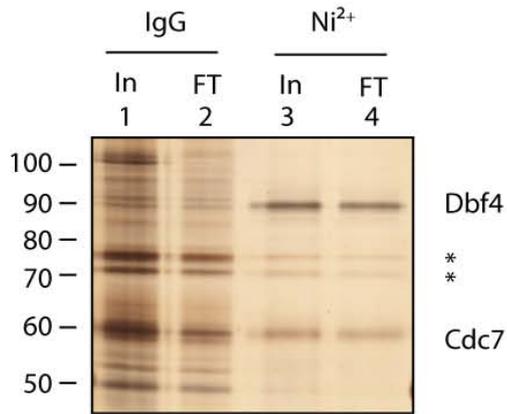
Protein expression was once again checked over a 5 hour timecourse, and protein purified from an 8 litre culture. Cells were grown to mid-log phase in raffinose before galactose addition, protein induction for 2.5 hours and extract production as before. Purification was carried out exactly as described previously. Figure 10A shows the result of Cdc7-Dbf4 purification performed in this way, illustrating the load and flow through fractions for the IgG and nickel affinity steps of the purification after separation by SDS-PAGE and silver staining. Cdc7 purified with Dbf4 as a stoichiometric complex. Two contaminants are present which run at the same size by SDS-PAGE as those seen in the Clb5-Cdc28 purification.

The activity of the purified kinase was tested in a series of reactions with individual Mcm2-7 complex subunits and Cdt1, each purified to homogeneity from *E. coli* (gift of D. Remus). Each reaction was performed with 2µg of substrate in a 20µl volume of reaction buffer with 5µl, approximately 100ng, purified kinase in the presence of ³²P γATP. Control reactions were carried out in the absence of kinase. Reactions were allowed to proceed for 30 minutes at 30°C and were stopped by the addition of an equal volume of 2x Laemmli buffer followed by boiling for 10 minutes. A quarter of the total reaction was separated by SDS-PAGE followed by Coomassie staining and gel drying (Figure 10B). Phosphorylated proteins were visualised by autoradiography and were quantified using ImageJ software (NIH, open source) to determine the strength of each phosphorylated band (Figure 10C).

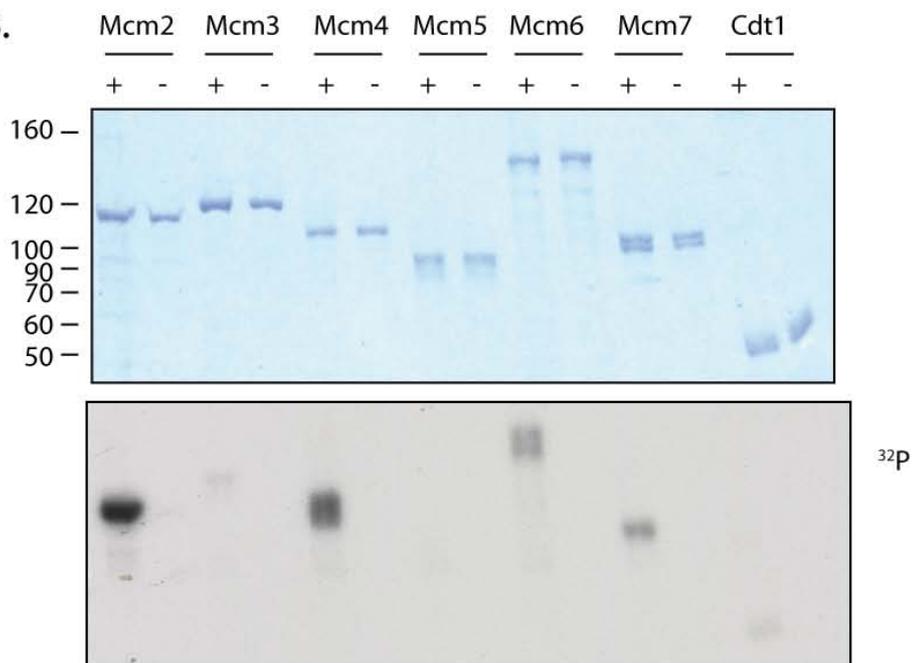
Figure 10. Purification of Cdc7-Dbf4 from *S. cerevisiae*

- A.** Purification of TAP^{TCP} tagged Dbf4 from *S. cerevisiae*. Lanes show input (lane 1), flow through (lane 2) and elution (lane 3) from the IgG column, and flow through from the nickel agarose binding step (lane 4). Cdc7 and Dbf4 are indicated, and contaminating bands marked with asterisk.
- B.** Phosphorylation of individual Mcm subunits and Cdt1 by purified Cdc7-Dbf4. 2µg of each Mcm subunit was incubated with 100ng of purified Cdc7-Dbf4 in a total reaction volume of 20µl at 30°C for 30 minutes in the presence of ³²P γATP. Control reactions were performed in the absence of kinase. Reactions were stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes, and a quarter of the total reaction separated by SDS-PAGE followed by Coomassie staining (upper panel). Gels were dried and visualised by autoradiography (lower panel).
- C.** Phosphorylation of each MCM subunit in the presence of Cdc7-Dbf4 was quantified using ImageJ software (NIH, open source). Phosphorylation is measured in arbitrary units and is plotted alongside background signal observed in the absence of kinase.

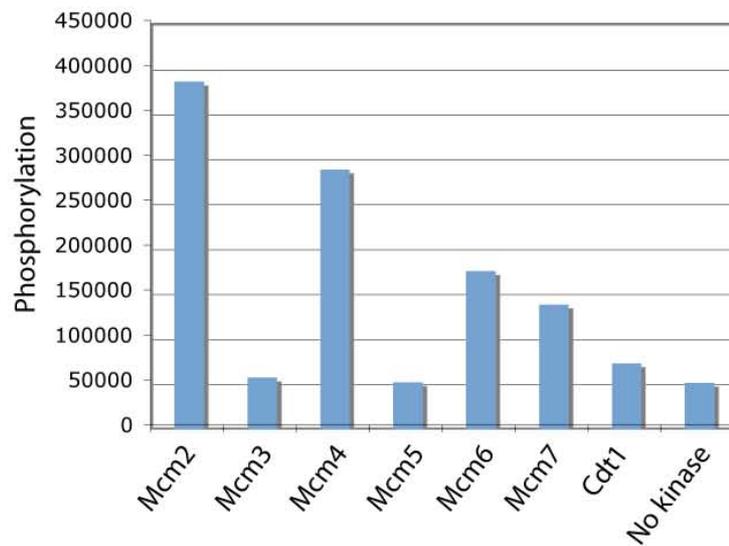
A.



B.



C.



The principle substrates of Cdc7-Dbf4 in these reactions were Mcm2, 4, 6 and 7, with very weak phosphorylation of Mcm3 and Cdt1 also observed. Mcm5 was not phosphorylated at all, in agreement with previous reports (Weinreich and Stillman, 1999). Although both Mcm4 and Mcm6 clearly shifted when phosphorylated by Cdc7, neither was seen to shift by Coomassie staining of the final product, indicating that only a small fraction of the total population was phosphorylated in these assays.

Since the activity of the enzyme in these assays was low, we also set out to purify larger quantities of enzyme in order to obtain sufficient amounts to phosphorylate the peptide arrays. For this purpose we utilized the Invitrogen BactoBac baculovirus protein expression system to co-express Cdc7 and Dbf4 in insect cells. Viruses were produced containing either Cdc7 or hexa-His tagged Dbf4 and subjected to three rounds of amplification in Sf9 cells. Hi5 cells were used for final protein expression, and a virus titration and timecourse used to determine optimal protein expression conditions. Batch purifications were performed from twenty 150mm petri dishes containing Hi5 cells at approximately 60% confluence. Viruses were mixed at a ratio of 4:1 Cdc7:Dbf4 in order to induce stoichiometric levels of protein expression, determined empirically, and induction carried out for 96 hours.

A nuclear extract was prepared as described in Materials and Methods and used for the initial step of purification on a 1ml HisTrap column using an AKTA Prime FPLC machine (GE Healthcare). Extract was loaded onto the HisTrap column and washed with buffer containing 40mM imidazole to remove background contaminants, followed by elution of bound proteins over a 20ml gradient between 40mM and 1M imidazole, continuously collecting 500µl fractions. Peak protein containing fractions

were identified by UV absorbance, samples separated by SDS-PAGE and visualised by Coomassie staining (figure 11A, left hand panel).

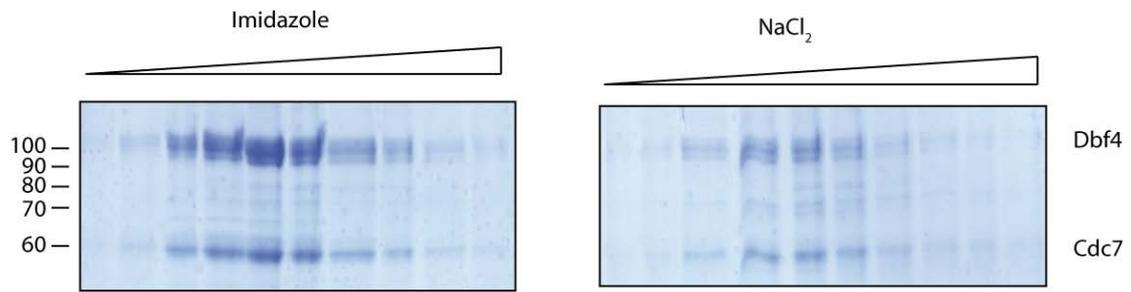
Peak fractions were pooled and dialyzed into a buffer containing 100mM NaCl before a second round of purification by ion exchange chromatography on Q sepharose, again using an AKTA Prime FPLC machine. Proteins were loaded onto a 1ml Q sepharose column and eluted over a 20ml gradient from 100mM to 1M NaCl, collecting 500 μ l fractions as before. Peak protein containing fractions were once again identified (figure 11A, right hand panel), pooled and dialysed into buffer containing 100mM NaCl before flash freezing in liquid nitrogen and storing at -80°C. Final protein concentration was determined to be approximately 0.4mg ml⁻¹ by comparison to BSA standards after SDS-PAGE and Coomassie staining.

Dbf4 purified as a phosphoprotein, showing a visible shift by SDS-PAGE which could be collapsed by treatment with λ phosphatase (figure 11B). In order to test the effect of Dbf4 autophosphorylation on enzyme activity, 200 μ l samples were therefore either treated or mock treated with λ phosphatase and re-purified on a 100 μ l MonoQ column using a SMART FPLC machine (Amersham Pharmacia Biotech AB). 50 μ l fractions were continuously collected over a 2ml gradient between 100mM and 1M NaCl. Samples of peak fractions identified by UV absorbance were separated by SDS-PAGE and proteins visualised by Coomassie staining (Figure 11C).

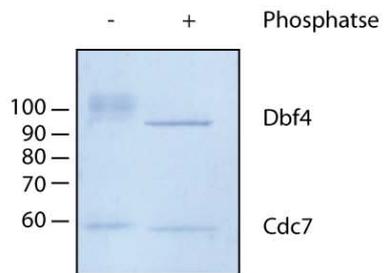
Figure 11. Purification of Cdc7-Dbf4 from insect cells

- A.** Cdc7-Dbf4 was purified from a nuclear extract of Hi5 cells by sequential HisTrap (left panel) and Q sepharose (right panel) chromatographic steps. Samples of peak fractions identified by UV absorbance in each case were separated by SDS-PAGE and protein visualised by Coomassie staining. Peak fractions were pooled, dialysed, flash frozen in liquid nitrogen and stored at -80°C.
- B.** Samples of purified Cdc7-Dbf4 were either mock treated (-) or treated with λ phosphatase (+) for 30 minutes at 30°C. A sample of each reaction was separated by SDS-PAGE and proteins visualised by Coomassie staining.
- C.** 200 μ l aliquots of purified Cdc7-Dbf4 were either mock treated (Mock) or treated with λ phosphatase and subsequently re-purified on a 100 μ l MonoQ column. Samples of peak fractions were separated by SDS-PAGE and proteins visualised by Coomassie staining. Peak fractions were dialysed, flash frozen in liquid nitrogen and stored at -80°C

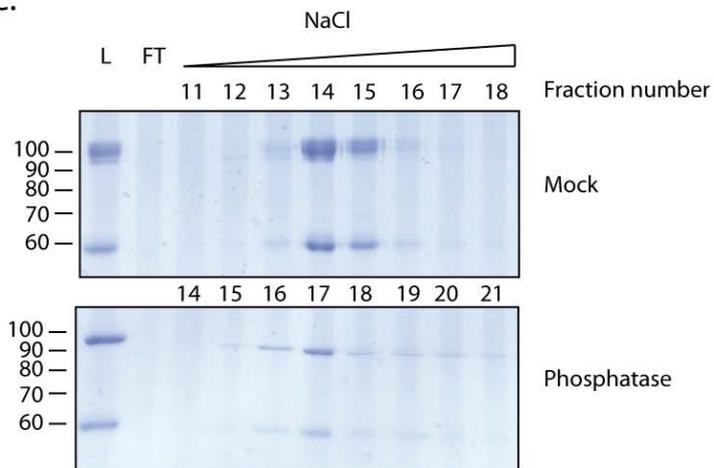
A.



B.



C.



The initial experiments designed to test the activity of the insect cell purified Cdc7-Dbf4 encountered the problem that strong Dbf4 autophosphorylation was seen which coincided with the size of phosphorylated Mcm2 and Mcm4, making analysis of substrate phosphorylation difficult. To circumvent this problem, an N terminal truncation of Mcm2 termed Mcm2-N, known at the time to contain sites of Cdc7 phosphorylation in the human protein and recently demonstrated also with the *S. cerevisiae* homologue (Bruck & Kaplan, 2009; Cho et al, 2006; Ishimi et al, 2001) was purified after expression in *E. coli*. A strain expressing GST tagged Mcm2 residues 1-194, termed Mcm2-N, was a gift of L. Drury. Protein expression was induced with 1mM IPTG in a 1 litre culture overnight at 24°C, followed by extract production as described in Materials and Methods. Mcm2-N was purified in two steps over GSTrap and gel filtration columns. At each step, peak fractions were identified by UV absorbance and verified by SDS-PAGE and Coomassie staining (Figure 12A). Fraction 19 of the gel filtration step was determined to be approximately 90% pure and was therefore dialysed, flash frozen in liquid nitrogen and stored at -80°C. Protein concentration was approximately 1mg ml⁻¹.

In order to determine whether phosphorylation of Dbf4 during purification affected the activity of the enzyme, λ phosphatase and mock treated enzymes were used to phosphorylate Mcm2-N. Enzyme concentrations were first equalised. The two purified complexes were titrated and their relative concentrations determined by silver staining of samples after separation by SDS-PAGE (Figure 12B). It was approximated that mock treated and re-purified Cdc7-Dbf4 was present at twice the concentration of phosphatase treated enzyme. Approximately 100ng of each enzyme was then used to phosphorylate 0.5 μ g Mcm2-N in 20 μ l reactions in the presence of

^{32}P γATP . Phosphorylation was allowed to continue for half an hour at 30°C before stopping by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. One quarter of the total reaction was separated by SDS-PAGE followed by silver staining, gel drying and visualisation of phosphorylated proteins using a phosphorimager (Figure 12C).

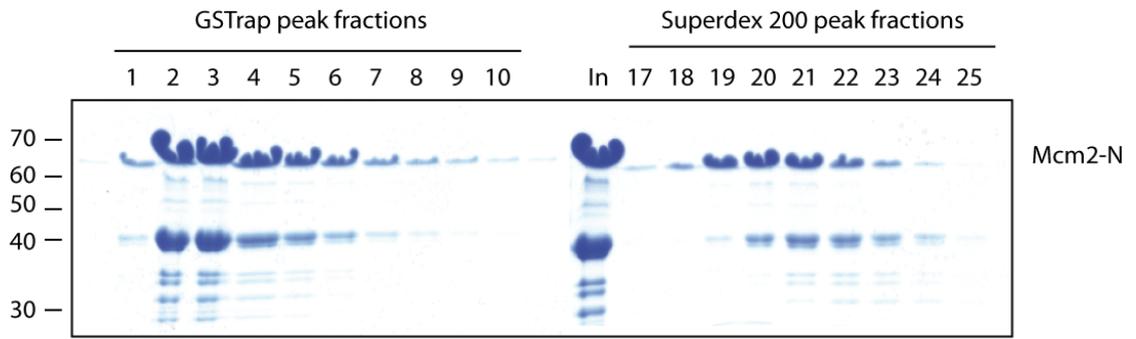
Phosphatase treatment of the enzyme appeared to have little effect on its ability to phosphorylate Mcm2-N in this assay. The population of mock treated Dbf4 is shifted to a higher molecular weight in the gel, visible by both silver staining and ^{32}P incorporation, since it is already phosphorylated at the start of the experiment. This indicates that phosphorylation of Dbf4 in the cell extracts is not due to autophosphorylation, since if this were the case no increase in shift would be expected following *in vitro* phosphorylation. Dbf4 phosphorylation in Hi5 cell extracts could be due to CDK activity, since virally infected insect cells arrest in G2-M (Braunagel et al, 1998). Dbf4 has previously been reported to be a CDK substrate, containing several minimal CDK consensus sites and two strong CDK consensus sequences at its N terminus (Ubersax *et al.*, 2003).

Since de-phosphorylation of purified Dbf4 seemed to have little effect on the activity of the enzyme, most further experiments were performed with crude, untreated enzyme.

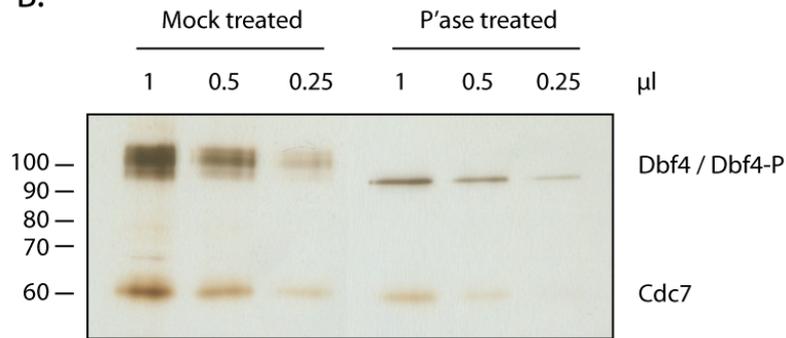
Figure 12. Purification and phosphorylation of Mcm2-N

- A.** An N terminal fragment of Mcm2, Mcm2-N, was purified over 2 steps using GSTrap and Superdex 200 columns. Peak fractions were identified by UV absorbance and samples separated by SDS-PAGE. Proteins were visualised by Coomassie staining. Fraction 19 was determined to be approximately 90% pure and was dialysed, flash frozen in liquid nitrogen and stored at -80°C.
- B.** Phosphatase treated and mock treated Cdc7 were titrated over twofold dilutions in order to normalise the concentrations of the two enzymes. Relative enzyme concentrations were approximated by silver staining of titrated samples. Mock treated enzyme was determined to be present at approximately twice the concentration of the phosphatase treated enzyme.
- C.** The activity of mock and phosphatase treated Cdc7-Dbf4 was compared in a phosphorylation assay with the N terminal fragment of Mcm2. 100ng of each enzyme was used to phosphorylate 0.5µg Mcm2-N in the presence of ^{32}P γ ATP for 30 minutes at 30°C. Samples of each reaction were separated by SDS-PAGE and proteins visualised by silver staining (left panel), and phosphorylated proteins visualised using a phosphorimager after gel-drying (right panel).

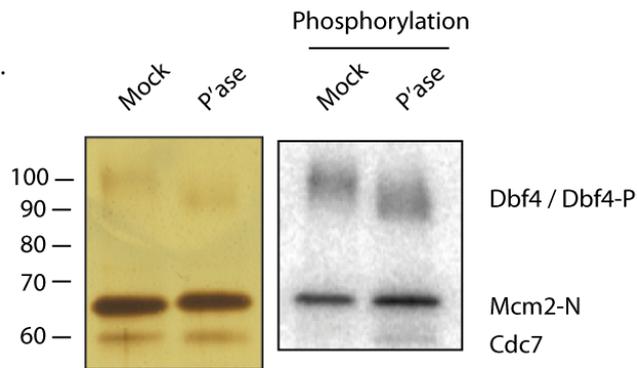
A.



B.



C.



A timecourse of Mcm2-N phosphorylation by Cdc7-Dbf4 was next tested.

Approximately 0.8 μ g of purified Cdc7-Dbf4 was incubated with 5 μ g Mcm2-N for varying times in individual reaction volumes of 20 μ l. Each reaction was stopped after the appropriate time by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. One quarter of each reaction was separated by SDS-PAGE and proteins visualised by Coomassie staining (Figure 13A, upper panel). Stained gels were dried and radiolabelled proteins visualised using a phosphorimager (Figure 13A, lower panel). Mcm2-N and Dbf4 phosphorylation at each time point were quantified using ImageQuant software (Figure 13B). Mcm2-N phosphorylation at the 5 minute timepoint was anomalously low. However, maximal phosphorylation of Mcm2-N was observed between 2 and 5 minutes, and coincided with maximal autophosphorylation of Dbf4.

In order to investigate whether Dbf4 autophosphorylation had any effect on the activity of the kinase, an experiment was set up in which Mcm2-N was phosphorylated for 10 minutes after prior phosphorylation of Dbf4 for varying time periods. For this experiment, λ phosphatase treated Cdc7-Dbf4 was used as a starting material. Each reaction was set up in a total volume of 20 μ l, and 0.4 μ g Cdc7-Dbf4 was pre-incubated with ATP in each reaction vessel for the required time before the addition of 5 μ g Mcm2-N substrate peptide. Phosphorylation of Mcm2-N was subsequently stopped 10 minutes after substrate addition with Laemmli buffer as described previously. Samples of each reaction were separated by SDS-PAGE and proteins visualised by Coomassie staining (Figure 13C, upper panel). The Coomassie stained gel was dried and phosphorylated proteins visualised using a phosphorimager

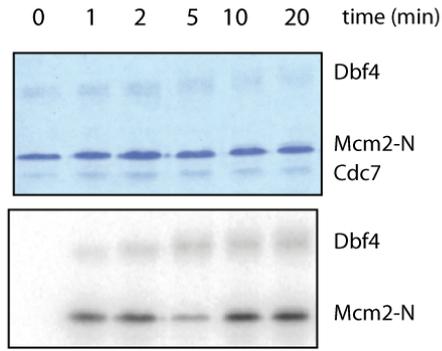
(Figure 13C, lower panel). Mcm2-N phosphorylation was quantitated using ImageQuant software (Figure 13D).

A clear decrease in the activity of Cdc7 towards Mcm2-N was observed after increasing periods of pre-phosphorylation of the kinase, although activity was never abolished. This indicates that autophosphorylation of Dbf4 inhibits the activity of the kinase, although perhaps not completely. It is unknown at this stage whether this represents a mechanism of regulation of the kinase *in vivo*, and it is possible that dephosphorylation of the kinase is required to trigger its activity at the onset of S phase. This also provides further evidence that phosphorylation of Dbf4 in the insect cell extracts is not due to autophosphorylation, since the purified phosphorylated protein retains its activity against Mcm2-N (Figure 12C).

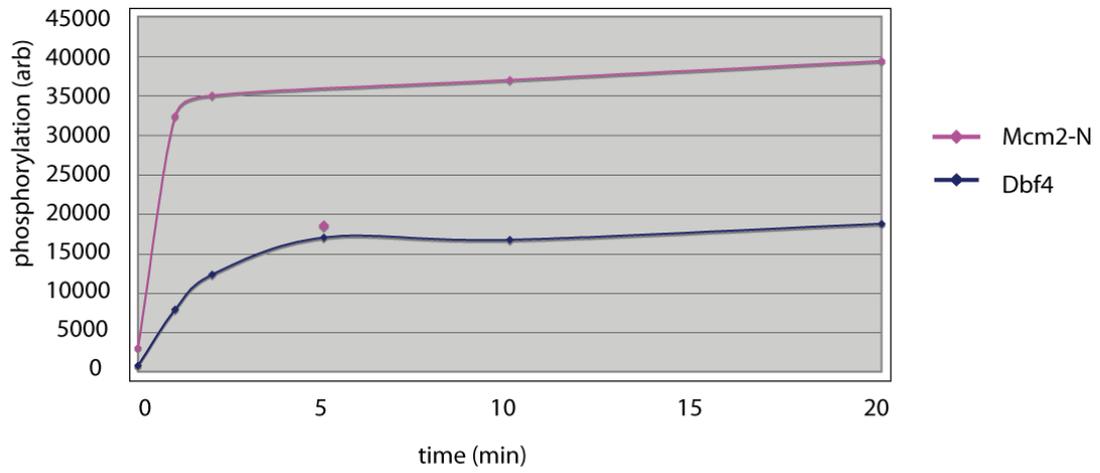
Figure 13. Activity of Cdc7-Dbf4

- A. Timecourse of Mcm2-N phosphorylation by Cdc7-Dbf4. After incubation for varying time periods, reactions were stopped and a sample separated by SDS-PAGE. Gels were Coomassie stained (upper panel), dried, and phosphorylated proteins visualised using a phosphorimager (lower panel)
- B. ^{32}P incorporation into Mcm2-N (pink) and Dbf4 (blue) was quantified at each timepoint using ImageQuant software. Phosphorylation, measured in arbitrary units, was plotted. Maximal phosphorylation of both Mcm2-N and Dbf4 was seen after 5-10 minutes.
- C. Cdc7-Dbf4 phosphorylation of Mcm2-N after pre-phosphorylation of Dbf4 for varying time periods. Cdc7-Dbf4 was incubated with ATP in 20 μl reaction volumes for varying time periods before the addition of Mcm2-N substrate peptide. Mcm2-N phosphorylation was allowed to continue for 10 minutes before stopping the reactions as above, separating proteins by SDS-PAGE and visualising by Coomassie staining (upper panel). The Coomassie stained gel was dried and phosphorylated proteins visualised using a phosphorimager (lower panel).
- D. Mcm2-N phosphorylation at each time point was quantified using ImageQuant software and plotted against the time of Dbf4 pre-phosphorylation.

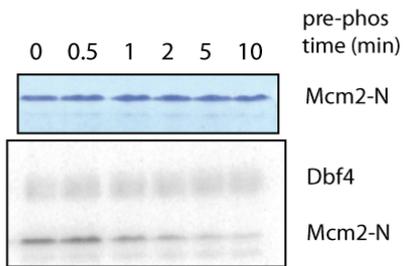
A.



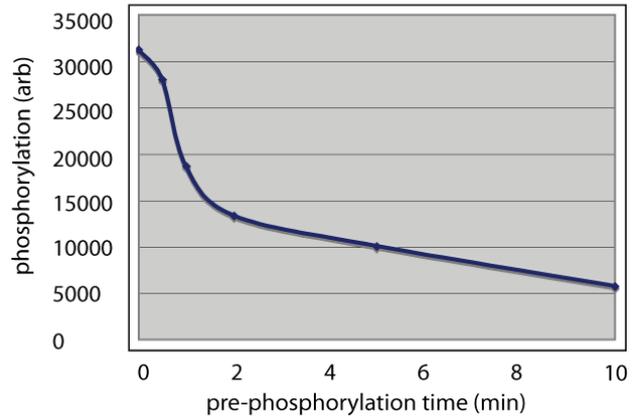
B.



C.



D.



3.6 Peptide array phosphorylation with Cdc7-Dbf4

We next used purified Cdc7-Dbf4 to phosphorylate the peptide arrays. Arrays were re-synthesised and the experiment carried out using the same protocol as described previously. Buffer was used as described for Cdc28-Clb5, now containing approximately 33 μ g Cdc7-Dbf4 in 2.5ml buffer per membrane. ATP was added to each aliquot of buffer immediately prior to addition to the membrane, in order to prevent inhibition of Cdc7 activity before peptides had been phosphorylated.

Reactions were allowed to proceed for 1 hour at 30°C and were stopped by heating to 65°C in 1% SDS for 1 hour followed by overnight incubation in 0.5% phosphoric acid, methanol washes, drying and visualisation by phosphorimager. Treatment with λ phosphatase and data analysis was carried out as described previously, and the results are shown in Figure 14. As before, raw data for membrane 1 prior to phosphatase treatment is shown in Figure 15.

Figure 14. Array phosphorylation with Cdc7-Dbf4. Arrays were phosphorylated and λ phosphatase treated as described. Heat maps are displayed with protein positions marked and the scale indicated on the right hand side.

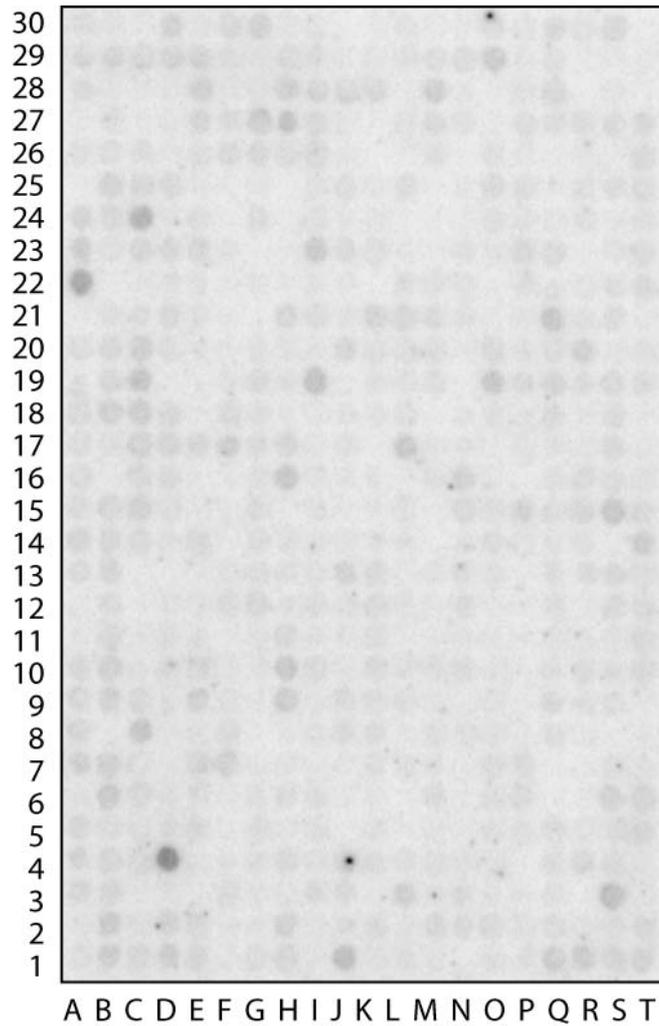


Figure 15. Membrane 1 phosphorylation with Cdc7-Dbf4. Raw data prior to λ phosphatase treatment. Membranes were incubated with 33 μ g Cdc7-Dbf4 in the presence of 32 P γ ATP for 1 hour at 30°C. Membranes were washed and dried, and radiolabel incorporation visualised using a phosphorimager.

Unfortunately, it was once again difficult to determine specific phosphorylation events in this experiment from general background levels of ^{32}P . A slightly more robust appearing pattern of phosphate incorporation was observed prior to λ phosphatase treatment, but this showed several similarities to the pattern of apparent phosphorylation of sites which did not contain an S-T/P motif after phosphorylation with Cdc28-Clb5, strengthening the argument that background ATP binding is observed in these experiments which was not seen in the initial control experiment. Very little of this signal was removed by λ phosphatase, and it could not therefore be attributed to peptide phosphorylation. The results after analysis showed general ^{32}P incorporation above background levels across membranes 1 and 6, whilst only a few isolated sites showed ^{32}P incorporation above background levels on membranes 2, 3, 4 and 5. There was no enrichment for phosphorylation of known sites in the N termini of Mcm2 and 4.

Peptides showing ^{32}P incorporation above an arbitrary threshold of 4000 are listed in Table 3. Of those sites listed, just under a third contain the reported S/T-D/E consensus sequence and therefore agree with previous reports of Cdc7-Dbf4 selectivity (Cho et al, 2006; Masai et al, 2006; Montagnoli et al, 2006). However, one of the peptides, membrane 1 site T5, does not contain a serine or threonine residue.

Table 3. Peptides phosphorylated by Cdc7-Dbf4. Peptides with ^{32}P incorporation over 4000 units. Serine and threonine residues are marked in red, and S/T-D/E consensus sites in green.

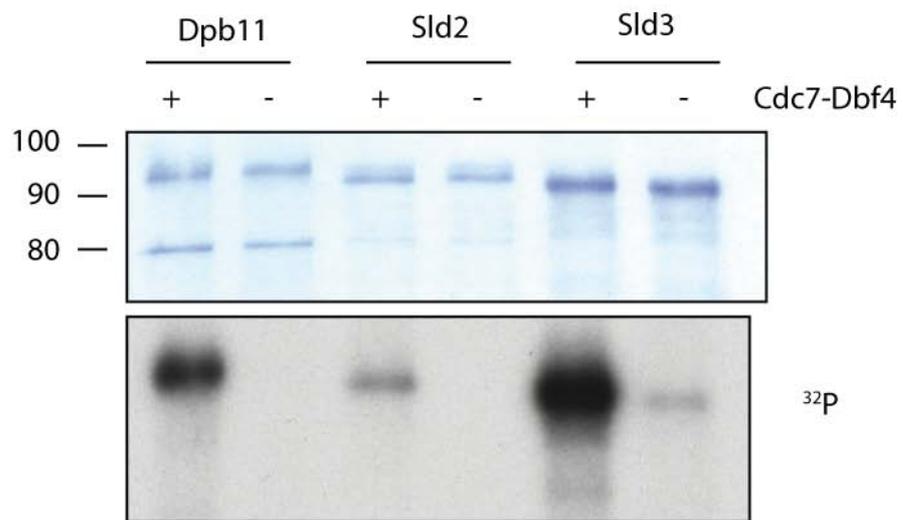
Membrane	Site	Peptide sequence
1	A5	MKSRKEYFLLEEYGPFWVKWPYNYF
	A7	NGYSLPERRTEVVTTVERERAKRETL
	A9	KTWDELKFKELLHLWSEEPKGSCKLE
	A29	DAIEASNGTIQEHHKNILDKSKEASL
	B6	SRSSIINSVPSSPALRRVDANLFSRK
	B7	ALRRVDANLFSRKSIASPTPELLNSR
	B29	LNNVELWALTYLRWFEVNPLAHYRQ
	C17	KLGKDDIDASVQPPPKRGRKPKDPS
	C18	PPKKRGRKPKDPSKPRQMLLISSCRA
	C20	NNTPVIRKFTKKNVARAKKKYTPFSK
	C29	SDSATTIYVAGTPGVGKTLTVREVVK
	D29	EQLRIISWDFVLNQLLDAGILFKQTM
	E28	LSDPRSNLNRHIRMNFETFRSLPTLK
	H27	RNLFDAPATPPRPLKRKKLQFTDVT
	H28	PLKRKKLQFTDVTPESSPEKLQFGSQ
	R1	SKSQILQYVHKITPRGVYTSKGSSA
	S6	TAITQVAKRISILSRAQSANNNDKDP
	T5	MVLADGGVVCIDEFDKMRDEDRVAIH
3	I7	IVSRLFSDERIQLLSNLPGKIGSKYSL
	I10	LKVLEQYSDDKTLVIEGKSRRQKKFN
	J9	PDDQILSKSDAA YFKDLNNASDKLK
4	G15	KPKTRNRKSKRGDKVKVEEVIDLKSE
6	A10	IDAEQSVLNGIKDENTSTVVRFFGVT
	A23	SSCQLEVSINYNLIAHPAEGDWSHT
	B10	ETRRRLAVYCLKDAYLPLRLMEKLM
	B19	TTLCNKATVERLNLKIDEDYVITPNG
	B21	DYFVTTKRRRGILPIILDELISARKR
	B22	PIILDELISARKRAKKDLRDEKDPFK
	B27	AYGRTMILKTKTAVQEKYCIKNGYKH
	C10	TNPQPHAVLAERMKRREGVGPVVGDR
	C19	PLRKGEGPLCSNCLARSGELYIKALY
	C27	IFTNKQRYDNPTGGVYQVYNTRKSDG
	D27	IPEAYALKGWYDSKGRNANFITLKQE
	E29	LFVKDDNDTSSGSSPLQRILEFCKKQ
	F30	KQETPSSNKRLKKQGTLESFFKRKAK
	G2	MVNISDFFGKNKKSVRSSSTRPTRQV
	G21	RKLEEQHNIATKEAELLVKKEEERSK
	G26	NKGSVMKLNWLANWENSCKNSFKHA
	H23	INFATAWLQNSKSAKYRLLQEIHYH
	H30	TTAIKKIPATVKSFGTRKYNMTHP
	K30	MGIKGLNAIIEHVPSAIRKSDIKSF
	M12	PMVEEHVTSAEERPIVADSFAQDKRN
	M18	TFRVQGPVGLEENEKLLLGWLDHR
	N30	DFIQDLDTTKNLILDCEAVAWDKDQG
	P16	ESCREENEETGENSLSQIWHVDCFKH
	P22	VARKMVRYILSRGESQNSIITRNKLQ

Interpretation of the results therefore once again proved difficult, and will be discussed further below. However, in addition to the peptide array phosphorylation experiment, phosphorylation of Sld2, Sld3, Dpb11 and Mcm10 by Cdc7-Dbf4 was also tested *in vitro* with full length proteins. Mcm10, purified as a hexa-His tagged protein after expression in *E. coli*, was a gift of B. Pfander. Sld2, Sld3 and Dpb11 were also purified after expression in *E. coli*, as part of this study. Sld3 and Dpb11 were both hexa-His tagged, and purified by sequential HisTrap and gel filtration chromatographic steps as described in Materials and Methods. Sld2 was insoluble as a His tagged protein and was instead tagged with GST and purified in a single step using a GSTrap column (GE Healthcare). Phosphorylation of Sld2, Sld3 and Dpb11 was carried out using endogenously purified Cdc7-Dbf4, whilst phosphorylation of Mcm10 was carried out with enzyme purified from Hi5 cells. Each reaction was carried out in a total reaction volume of 20 μ l, with approximately 4 μ g Sld2, Sld3 and Dpb11 and 10 μ g Mcm10. Approximately 1 μ g insect cell purified Cdc7-Dbf4 was used for phosphorylation of Mcm10, and for phosphorylation of Sld2, Sld3 and Dpb11, 100ng of endogenously purified enzyme was used. Control reactions were carried out either in the absence of kinase (in the case of Sld2, Sld3 and Dpb11) or in the absence of substrate (in the case of Mcm10). Reactions were each allowed to continue for 30 minutes at 30°C. After this time, reactions were stopped and proteins analysed by SDS-PAGE and Coomassie staining. Stained gels were dried and phosphorylated proteins visualised either by autoradiography (Sld2, Sld3 and Dpb11) or using a phosphorimager (Mcm10). The results are shown in Figure 16. As can be seen, phosphorylation of all four proteins, particularly Dpb11, Sld3 and Mcm10, occurs in the context of the full length proteins.

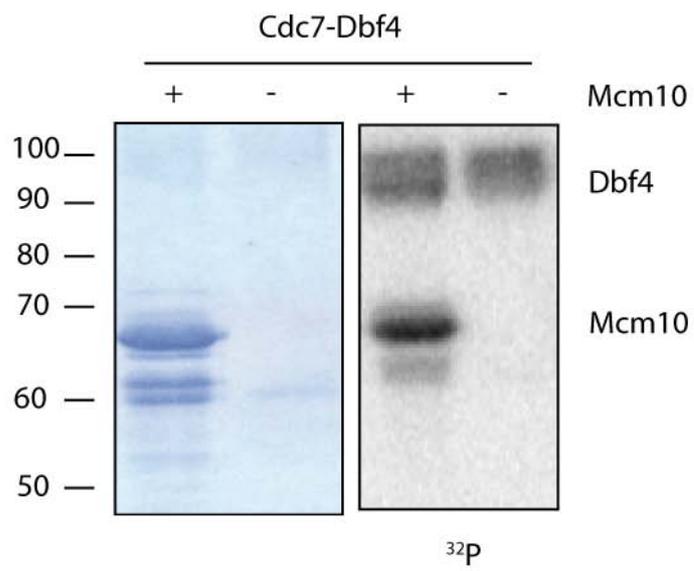
Figure 16. *Cdc7-Dbf4 phosphorylation of Sld2, Sld3, Dpb11 and Mcm10*

- A.** Phosphorylation of Sld2, Sld3 and Dpb11 by Cdc7-Dbf4. Endogenous Cdc7 was used to phosphorylate Sld2, Sld3 and Dpb11 in 20 μ l reactions for 30 minutes at 30°C. Reactions were stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. Samples were separated by SDS-PAGE and proteins visualised by Coomassie staining (upper panel). Stained gels were dried and phosphorylated proteins visualised by autoradiography (lower panel). Control reactions were carried out in the absence of kinase.
- B.** Phosphorylation of Mcm10 by Cdc7-Dbf4. Insect cell purified Cdc7 was used to phosphorylate Mcm10 in a 20 μ l reactions for 30 minutes at 30°C. The reaction was stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. Samples were separated by SDS-PAGE and proteins visualised by Coomassie staining (upper panel). Stained gels were dried and phosphorylated proteins visualised using a phosphorimager (lower panel). A control reaction was carried out in the absence of substrate.

A.



B.



Several *in vitro* substrates of Cdc7-Dbf4 are therefore known; Mcm2, Mcm3, Mcm4, Mcm6, Mcm7, Pol1 (Weinreich & Stillman, 1999), Cdc45 (Nougarede et al, 2000), Mcm10, Sld3, Dpb11, Sld2 and both Cdc7 and Dbf4 themselves. Of these, as mentioned, no enrichment was observed on the membranes for phosphorylation of the N termini of Mcm2 or Mcm4 above the general levels of peptide ³²P incorporation seen on membrane 1. Similarly, Mcm6, Mcm7, Mcm10 and Dbf4 did not show phosphorylation on membrane 2, with the exception of a single site in Mcm10, H7. This peptide contains two serine residues, although no S/T-D/E consensus. Sld2 and Dpb11 were not phosphorylated on membrane 5, and Cdc45 only at very low levels at peptides H18, I16 and I18. H18 and I16 both contain S-E sites, as well as multiple other serine and threonine residues. I18 contains one serine and two threonine residues. However, phosphorylation of three sites did not occur in pairs of peptides.

An attempt was made to stimulate phosphorylation of the N termini of Mcm2 and Mcm4 by pre-phosphorylation of a freshly synthesised copy of membrane 1 with CDK in the absence of ³²P, followed by removal of CDK and phosphorylation with Cdc7 in the presence of ³²P (see Materials and Methods). Stimulation of phosphorylation of these regions might be expected since they contain several S-S-P motifs, candidates for priming by CDK phosphorylation. Stimulation of Cdc7 phosphorylation of Mcm2 and Mcm4 by prior phosphorylation with CDK has been previously reported (Masai et al, 2000; Masai et al, 2006; Cho et al, 2006) . However, no significant stimulation was seen, a result not entirely unexpected since no phosphorylation of these sites by CDK was seen in the earlier experiment.

One plausible explanation for the lack of Cdc7-Dbf4 activity in the array phosphorylation experiments despite the activity of the enzyme against purified full length proteins is the suggestion that Cdc7-Dbf4 requires a distant binding site for efficient substrate phosphorylation (Sheu and Stillman, 2006; Bruck and Kaplan, 2009). Although it is not clear whether this is required for phosphorylation of every substrate, it would certainly affect the results of phosphorylation of individual peptides of substrates in which it is required. Coupled with low efficiency of substrate phosphorylation due to auto-phosphorylation and inhibition of Dbf4, this makes the density of genuine phosphorylation events is very low. Attempts to identify Cdc7-Dbf4 binding peptides by a Western blot approach (see materials and methods) unfortunately also proved unsuccessful due to high background levels.

The peptide array approach therefore unfortunately proved unproductive in the identification of Cdc7-Dbf4 kinase substrates. Some conclusions could be drawn regarding potential substrates of CDK, however, and Cdc7 phosphorylation of Sld2, Sld3, Dpb11 and Mcm10, and Cdc7-Dbf4 auto-inhibition were all demonstrated with full length or truncated proteins in solution. The implications of these findings will be explored further in the Discussion. However, an alternative approach to the identification of kinase substrates was chosen to continue the study, involving phosphorylation of a complete pre-RC assembled *in vitro*. The results of these experiments will be described in the following chapter.

Chapter 4: Phosphorylation of Orc1 by Casein Kinase II

4.1 Introduction

As an alternative strategy to identify substrates of both CDK and Cdc7, we decided to phosphorylate pre-replicative complexes assembled *in vitro*. Assembly of Pre-RCs in a cell free yeast extract and their subsequent purification has been described previously (Bowers et al, 2004; Seki & Diffley, 2000). Phosphorylation of these complexes *in vitro* with purified kinases has several advantages over the peptide array technique described in the previous chapter. Proteins are full length and therefore contain all tertiary structural elements and distant binding sites required for kinase recruitment and activity. In addition, since the proteins are present in the context of a fully formed pre-RC, the quaternary structure of the complex is also taken into account. On the other hand, the technique has the disadvantage that the set of substrates tested and their concentrations are more limited, being restricted only to pre-RC components and not their downstream partners. Ultimately, reconstitution of DNA replication from a eukaryotic origin *in vitro* will be necessary in order to fully determine the functions of each of the components involved. Successful phosphorylation of assembled pre-replicative complexes will be a necessary step in this reconstitution.

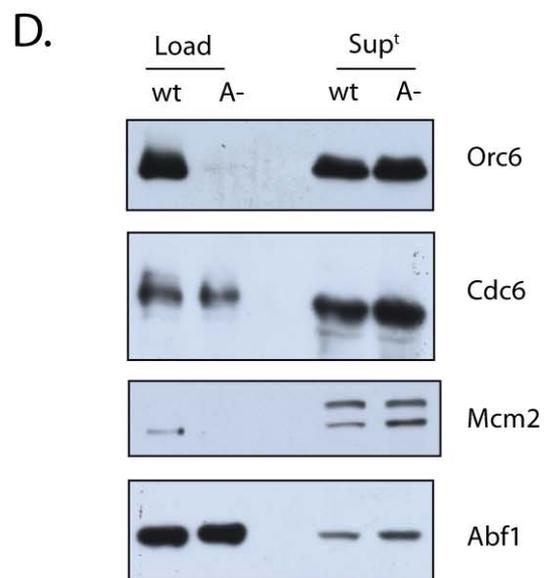
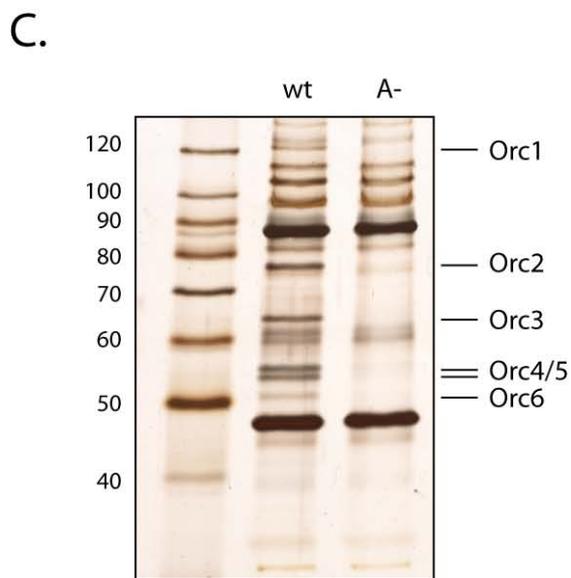
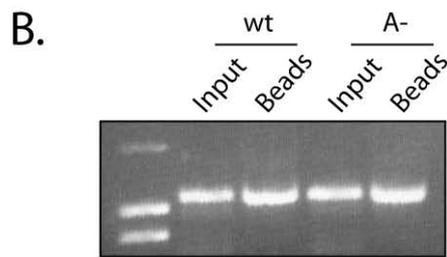
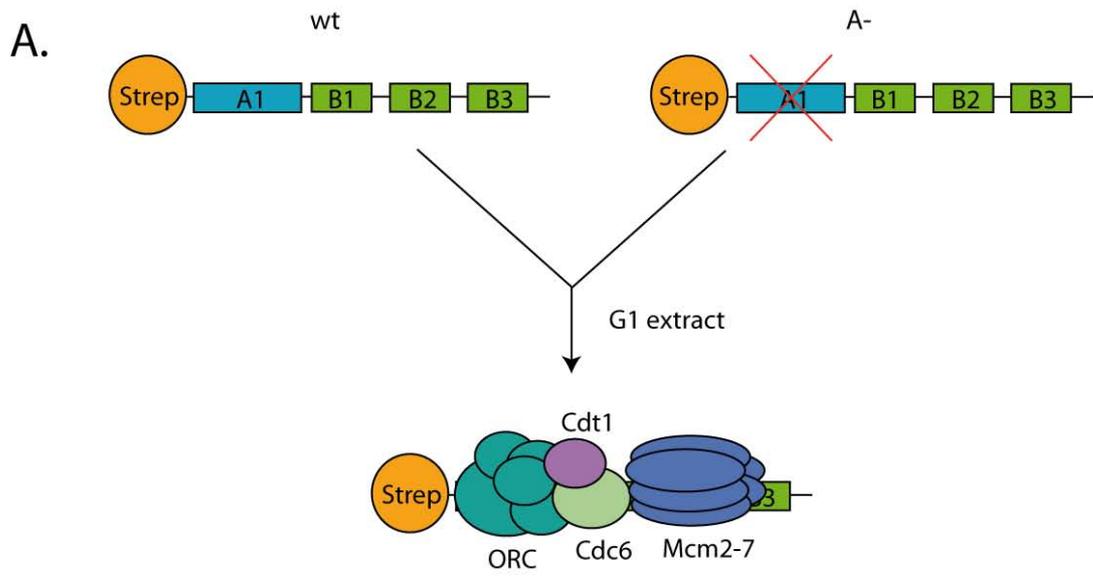
4.2 The loading assay

The previously described *in vitro* assembly of pre-RCs involves the incubation of ARS containing DNA in a G1 phase extract of *S. cerevisiae* in the presence of ATP (Bowers et al, 2004; Seki & Diffley, 2000). For this purpose, a strain of *S. cerevisiae* (ySC17) is used that overexpresses all six subunits of ORC as well as Cdc6 in order to promote complex formation *in vitro* (Bowers et al, 2004). *S. cerevisiae* strain ySC17 also contains a Pep4 deletion in order to minimize protein degradation in the extract. The process is termed the 'loading assay', since MCM complexes are loaded onto the DNA. As a control reaction, DNA is used which contains a XhoI linker substitution in the A element of the ARS (Marahrens & Stillman, 1992) in order to prevent ORC binding and downstream complex formation. Fragments of DNA approximately 1kb in length are amplified by PCR from plasmid templates, using a biotinylated 3' primer such that all product DNA molecules are biotinylated at the 3' end in order to subsequently isolate DNA bound complexes. DNA is bound to streptavidin beads, and bead bound DNA incubated in extract diluted twofold in a loading buffer containing ATP, an ATP regenerating system comprising creatine phosphate and creatine phosphokinase, and poly(dIdC) competitor DNA. DNA is incubated in the extract for 20 minutes at 24°C, isolated, washed, resuspended in Laemmli buffer and bound proteins analysed by SDS-PAGE followed by silver staining and Western blotting. Paramagnetic streptavidin beads (dynabeads M280, Invitrogen) are used in order to enable isolation of the DNA between steps using a magnetic rack (Invitrogen).

Previous experiments have demonstrated that this results in sequence specific ORC binding and salt-stable loading of the MCM complex (Bowers et al, 2004). The principle of the assay is demonstrated in Figure 17A. Figure 17B shows the successful binding of wild-type (wt) and ACS mutant (A-) DNA to streptavidin beads. Amplification of wt and A- DNA was carried out in eight separate PCR reactions per template, products purified, combined and incubated with magnetic streptavidin beads for 2 hours at room temperature. Beads were washed and resuspended in a final storage buffer volume of 200µl. 0.5% of input and bead bound DNA was then analysed by agarose gel electrophoresis after first denaturing the biotin-streptavidin interaction by boiling bead bound DNA in 1% SDS. Figures 17C and D demonstrate a successful loading assay performed with this DNA. Protein overexpression and extract production in a G1 phase arrested culture of ySC17 was carried out as described in Materials and Methods. Assembly of the pre-RC was then carried out in 20µl aliquots of extract diluted twofold in reaction buffer. Buffer was removed from 10µl of bead bound DNA per reaction and was incubated with extract at 24°C for 20 minutes with agitation. Beads were then isolated, washed three times in a low salt buffer and resuspended in a final volume of 40µl Laemmli buffer. DNA bound proteins from a quarter of the total reaction were separated by SDS-PAGE and analysed by silver staining (Figure 17C) or Western blotting against Orc6, Cdc6, Mcm2 and Abf1 (Figure 17D). Silver staining shows clear binding of the ORC complex specifically to wild type DNA, whilst the Western blots show in addition specific loading of the MCM complex, albeit at sub-stoichiometric levels. Cdc6 appears to bind non-specifically, which may be due to precipitation in the reaction. Abf1 acts as a loading control as it binds in an ORC independent manner to the B3 element of the ACS (Diffley et al., 1988).

Figure 17. The loading assay

- A.** Principle of the loading assay. Streptavidin bound, biotinylated DNA containing either wild type (wt) ARS1 or ARS1 containing a linker substitution in the ACS (A-) is incubated in a G1 arrested extract of strain ySC17, containing elevated levels of ORC and Cdc6. Pre-RCs are assembled which can be subsequently purified.
- B.** DNA binding to streptavidin beads. ARS1 wild type (wt) and A box mutated (A-) sequences were amplified by PCR from plasmid templates using a biotinylated 3' primer such that all PCR products were biotinylated at the 3' end. DNA was purified and bound to magnetic streptavidin beads for 2 hours at room temperature. 0.5% of input and bead bound DNA was analysed by agarose gel electrophoresis after first denaturing the biotin-streptavidin interaction by boiling the bead bound DNA in 1% SDS.
- C.** Silver stain of assembled pre-RCs. Loading assays were carried out with wt and A- DNA as described in Materials and Methods. 25% of bead bound material was separated by SDS-PAGE and proteins visualised by silver staining. Subunits of ORC are indicated.
- D.** Western blots of DNA bound (Load) and unbound (Sup^t) material from the loading assay described in (C). Primary antibodies were used against Orc6, Cdc6, Mcm2 and Abf1.



4.3 Phosphorylation of the pre-replicative complex

We were therefore interested in the result of phosphorylation of pre-replicative complexes formed in this way with CDK, Cdc7 or both enzymes together. Pre-RCs were assembled, purified, resuspended in phosphorylation buffer and incubated for 1 hour at 30°C either alone or in the presence of approximately 100ng of endogenously purified Cdc7-Dbf4, 20ng of endogenously purified Cdc28-Clb5 (in the absence of Sic1), or both enzymes together. Different quantities of each enzyme were used due to differences in activities. Reactions were all carried out in a total volume of 20µl and were stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. A quarter of the total volume of each reaction was separated by SDS-PAGE and proteins visualised by silver staining. The silver stained gel was dried and radiolabelled proteins visualised by autoradiography (Figure 18)

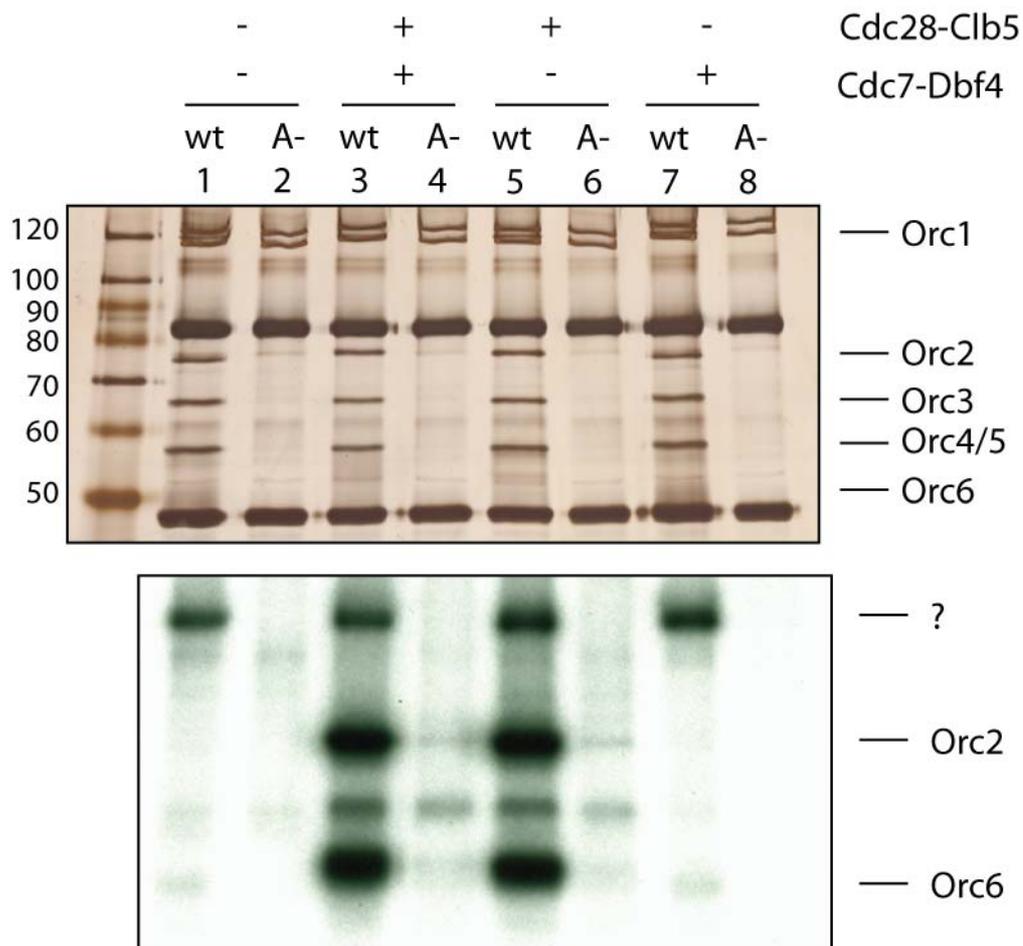


Figure 18. Pre-replicative complex phosphorylation

Four sets of loading assays were performed with wt and A- DNA and were subsequently incubated for 1 hour at 30°C in the presence of ^{32}P γATP either alone (lanes 1 and 2) with both Cdc7-Dbf4 and Cdc28-Clb5 (lanes 3 and 4), Cdc28-Clb5 alone (Lanes 5 and 6) or Cdc7-Dbf4 alone together (lanes 7 and 8). Samples of each reaction were separated by SDS-PAGE and analysed by silver staining (upper panel). Silver stained gels were dried and phosphorylated proteins visualised by autoradiography (lower panel). Phosphorylation of Orc2 and Orc6 by CDK, as well as an unidentified protein present on the wt DNA in all four experiments, is indicated.

The silver stain shows specific binding of the ORC complex to the wt DNA as expected. In the case of reactions containing Cdc28, strong phosphorylation was seen of proteins running at the sizes of Orc2 and Orc6, both known *in vivo* targets of CDK. No additional phosphorylated bands were seen specifically in reactions containing Cdc7, a result that can be explained by the low abundance of its predicted substrates, subunits of the Mcm2-7 complex, and the relatively low activity of the enzyme. It was later shown that the 1 hour 30°C incubation during the phosphorylation reaction reduces DNA bound MCM to levels undetectable by Western blotting. Any remaining Cdc7 activity is therefore overshadowed by other phosphorylation events. Unexpectedly, however, wt DNA specific phosphorylation of another band was also seen in all four reactions, including the control reaction performed in the absence of exogenously added kinase. It therefore appeared that a protein kinase present in the assay which was either binding specifically to the pre-RC, or active against a protein present specifically on the wild type DNA. We were therefore interested in identifying this kinase and its substrate, in order to determine whether it plays a role in the process of pre-replicative complex formation.

4.4 The phosphorylated protein is Orc1

We began by identifying the phosphorylated substrate. For this purpose, ySC17 extract used in the loading assay was combined in a 1:1 ratio with extracts from strains in which one of three candidate proteins had been TAP^{TCP} tagged, in order to cause a shift in the phosphorylated band after ³²P incorporation. The three candidates chosen were Orc1, Mcm2 and Mcm4, all of which resolve in SDS-PAGE at the

approximate position of the unidentified phosphorylated band. Extracts were mixed in order to maintain the high levels of ORC and Cdc6 present in ySC17.

Loading assays with both wt and A- DNA were therefore carried out using these extracts and were phosphorylated in the absence of exogenously added kinase as described above. The results are shown in Figure 19. A shift in the phosphorylated band was clearly seen when TAP^{TCP} tagged Orc1 was included in the reaction (the lower phosphorylated band in this reaction is likely to be untagged Orc1 from the ySC17 extract). By contrast, no shift in the phosphorylated protein was observed when Mcm2 or Mcm4 were tagged. An overall reduction in the intensity of the phosphorylated band in the latter two cases is likely to be due to the reduction in the quantity of ORC present in the reaction. This experiment therefore demonstrates that the substrate of the unexpected phosphorylation event in the loading assay is Orc1

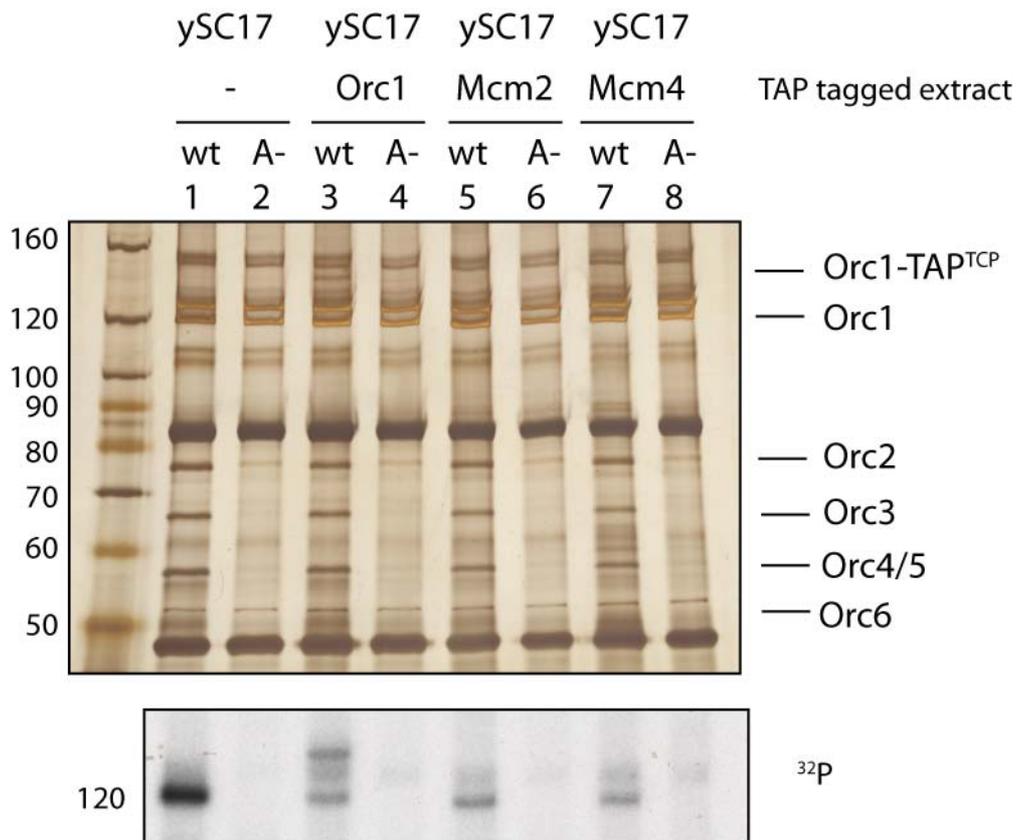


Figure 19. *Orc1 is the substrate of phosphorylation*

Loading assays with wt and A- DNA were performed either with ySC17 extract alone (lanes 1-2), or with a mixture of ySC17 and extracts in which one of three candidate substrate proteins, Orc1 (lanes 3-4), Mcm2 (lanes 5-6) or Mcm4 (lanes 6-7), had been TAP tagged. Loaded proteins were subsequently phosphorylated in the absence of exogenously added kinase. 25% of the total DNA bound material was separated by SDS-PAGE and proteins visualised by silver staining (lower panel). Members of the ORC are indicated. The silver stained gel was dried and ^{32}P incorporation visualised by autoradiography (lower panel). A clear shift in the phosphorylated band was seen when Orc1-TAP was included in the reaction, demonstrating that Orc1 is the substrate of phosphorylation.

4.5 Identification of potential Orc1 phosphorylating kinases

An experiment was next carried out to identify possible kinases responsible for Orc1 phosphorylation. Two approaches were taken. First, ORC was purified in a single step from an extract of the Orc1-TAP^{TCP} tagged strain used in the previous experiment, yDR11, which overexpresses all six ORC subunits. Extract from a 10 litre culture of this strain after induction of protein expression during a G1 phase arrest was a gift of D. Remus. Tagged proteins were bound to calmodulin affinity resin and eluted in 200µl fractions, resulting in purification of all six ORC subunits, as well as an N terminal degradation product of Orc1. Samples of each elution fraction were separated by SDS-PAGE and proteins visualised by Coomassie staining (Figure 20A). When tested, this single step purified ORC showed a phosphorylation activity against Orc1 when incubated with ³²P γATP, although ORC purified to homogeneity (purified by gel filtration, TEV cleavage and ion exchange after calmodulin step, gift of D. Remus) did not. Samples of each complex were taken and incubated in 20µl reaction volumes in the presence of ³²P at 30°C for 30 minutes. Reactions were stopped with Laemmli buffer, proteins separated by SDS-PAGE and visualised by silver staining. The silver stained gel was dried and ³²P incorporation visualised by autoradiography (Figure 20B). Interestingly, the N terminal degradation product of Orc1 remained unphosphorylated, indicating that phosphorylation occurs in the N terminus of the protein.

In order to identify kinases co-purifying with ORC, the peak fraction (fraction 4) of the Orc1 purification was concentrated tenfold and half of the total protein separated by SDS-PAGE. The gel was stained with Coomassie and the ORC containing lane

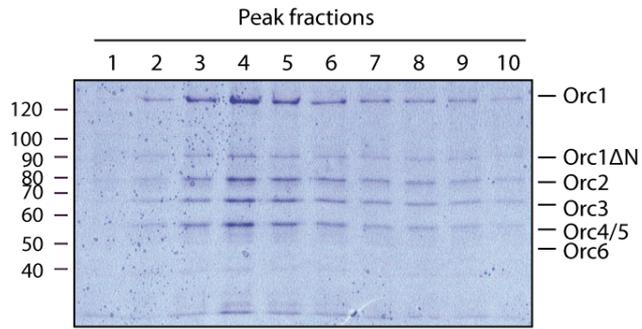
cut into slices and analysed by mass-spectrometry. The six subunits of the ORC were identified as well as five protein kinases; all four subunits of casein kinase II (CKII), Ypk1, Gin4 and the two calcium/calmodulin dependent kinases Cmk1 and Cmk2 (Figure 20C). A complete list of proteins identified by mass spectrometry in this assay is given in Appendix 2.

In parallel with this experiment, loading assays were also analysed by mass-spectrometry after scaling up of reactions with both wild type and A- DNA ten-fold. This was intended to determine whether any of the kinases identified as co-purifying with ORC also co-purified with the pre-RC. However, no kinases were identified in this way, presumably due to the low abundance of the enzyme binding to the pre-RC. A list of proteins identified as binding specifically to wild type DNA is given in Appendix 3.

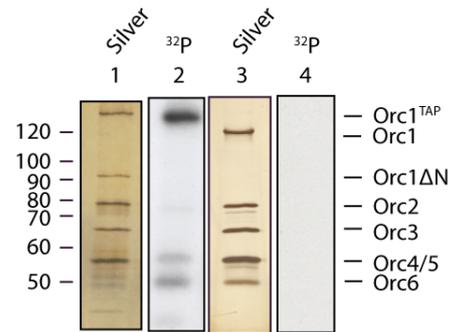
Figure 20. ORC purification and kinase identification

- A.** ORC was purified in a single step from a 10 litre extract of strain yDR11 which over-expresses all six subunits of the complex with a TAP^{TCP} tag on Orc1. Tagged protein was bound to calmodulin affinity resin and eluted in ten 200µl fractions in the absence of calcium. Samples of each fraction were separated by SDS-PAGE and proteins visualised by Coomassie staining. Subunits of ORC as well as an N terminal degradation product of Orc1 are indicated.
- B.** ORC was shown to purify with a kinase activity which phosphorylates Orc1. Samples of the complex purified in a single calmodulin affinity step as well as ORC purified to homogeneity (gift of D. Remus) were tested for kinase activity by incubation with ³²P for 30 minutes at 30°C . Samples of each reaction were separated by SDS-PAGE and visualised by silver staining (panels 1 and 3). Silver stained gels were dried and phosphorylated proteins visualised by autoradiography (panels 2 and 4).
- C.** Fraction 4 of the crude ORC purification was concentrated tenfold, proteins separated by SDS-PAGE and analysed by mass-spectrometry. Proteins identified included the six subunits of ORC as well as five protein kinases; all four subunits of CKII, Ypk1, Gin4 and both *S. cerevisiae* calcium dependent kinases, Cmk1 and Cmk2. The table gives the number of peptides identified as well as the percentage confidence of protein identification, generated by Scaffold software (Proteome Software Inc.) A complete list of identified proteins is given in Appendix 2.

A.



B.



C.

Protein	No. of unique peptides	Confidence
Orc1	23	>95%
Orc2	9	>95%
Orc3	15	>95%
Orc4	21	>95%
Orc5	11	>95%
Orc6	12	>95%
Cmk1	13	>95%
Cmk2	9	>95%
Cka1 (CKII α)	3	>95%
Ckb1 (CKII β)	1	80-94%
Cka2 (CKII α')	3	50-79%
Ckb2 (CKII β')	2	50-79%
Ypk1	1	50-79%
Gin4	4	50-79%

Of the five kinases identified, casein kinase II (CKII) is a tetrameric protein which has been isolated from a large number of eukaryotic organisms (Hathaway & Traugh, 1982). It was one of the first identified protein kinases and has an $\alpha_2\beta_2$ structure in which the α subunits contain the catalytic activity whilst the β subunits are regulatory, the functions of which are unclear (Domanska et al., 2005; Kubinski et al., 2007). In *S. cerevisiae*, although not in all eukaryotes, two isoforms of both α and β subunits exist, α , α' , β and β' , forming a tetramer which can be purified as a catalytically active enzyme (Bidwai et al, 1994; Padmanabha & Glover, 1987). The α and α' subunits are polypeptides of 44 and 35kDa respectively and are encoded by the genes Cka1 and Cka2 (Reed et al, 1994). The two α subunits are redundant but deletion of both genes is lethal (Padmanabha et al, 1990). Lethality can be rescued with the *Drosophila* α subunit, implying a conservation of function across evolution (Padmanabha et al, 1990). Active enzyme may consist of $\alpha\alpha'\beta\beta'$, $\alpha_2\beta\beta'$, $\alpha'_2\beta\beta'$ or the α' subunit alone (Domanska et al, 2005), although when present it appears that the β subunits are both required to function together (Kubinski et al, 2007). The different complexes show slightly different substrate selectivities and sensitivities to inhibitors (Domanska et al, 2005).

The enzyme is constitutively active (Poole et al, 2005) and has been shown to phosphorylate a large number of substrates in a variety of processes including tRNA and rRNA synthesis, apoptosis, cell survival and transformation (Meggio and Pinna, 2003). It has a preference for substrates rich in acidic residues, particularly at the +1 and +3 positions, and its broad substrate spectrum and constitutive activity have led to the suggestion that it plays a general role in protein structure, stabilising the unfolding of α helices (Meggio & Pinna, 2003). In yeast, a temperature-sensitive allele of Cka2

in the absence of Cka1 results in arrest in either G1 or anaphase at the non-permissive temperature, implying at least two essential functions (Hanna et al, 1995). CKII in mammalian cells has been linked to a variety of anti-apoptotic, pro-survival pathways and is elevated in a variety of tumours. It is therefore under investigation as a potential anti-tumour therapeutic target (Sarno & Pinna, 2008).

Ypk1 is a budding yeast kinase homologous to mammalian Serum and Glucocorticoid Inducible Kinase (SGK) and is involved in the sphingolipid mediated second messenger signalling pathway (Spiegel and Milstien, 2002). It is regulated by an activating phosphorylation by the upstream kinase Pkh1 (Sun et al, 2000) and has been shown to be involved in receptor endocytosis (deHart et al, 2002). The kinase is redundant with its homologue Ypk2 (Casamayor et al, 1999). The two kinases have also been implicated in the maintenance of cell wall integrity (Roelants et al, 2002) and show different localisations, with Ypk1 reported to remain largely cytosolic whilst Ypk2 enters the nucleus (Roelants et al, 2002). Ypk1 has been shown to be synthetically lethal with the phospho-binding 14-3-3 protein Bmh2 (Gelperin et al, 2002), and 14-3-3 proteins have been speculated to play a role in pre-RC formation (Zannis-Hadjopoulos et al, 2008). Any interaction between Ypk1 activity and 14-3-3 binding is therefore potentially interesting. It was notable that Bmh2 was identified in the mass spectrometric analysis of both the ORC purification and the wt, but not the A-, loading assay (Appendix 2 and 3).

Gin4 is a kinase which has been reported to be involved in the switch from polar to isotropic bud growth at the mitotic transition in *S. cerevisiae* (Altman & Kellogg, 1997). It is activated by phosphorylation by Cdc28-Clb2 and functions in a pathway

with the protein Ncp1 (Altman & Kellogg, 1997). Gin4 deletions are viable but have a pronounced elongated bud phenotype (Altman & Kellogg, 1997). Gin4 may also be involved in the regulation of the transition to mitosis, affecting the degradation of the *S. cerevisiae* Wee1 homologue Swe1 (Asano et al, 2006; Okuzaki et al, 2003). It has been shown to interact directly with septins at the bud neck (Barral et al, 1999).

Finally, Cmk1 and 2 are two calmodulin dependent kinase in *S. cerevisiae* which are homologous to the broad specificity mammalian CaM kinase II family (Ohya et al, 1991). The activity of Cmk1 is not absolutely dependent on the presence of calcium and calmodulin, but is strongly stimulated in their presence (Ohya et al, 1991).

Neither Cmk1 nor Cmk2 are essential, since a double deletion remains viable, and their functions are unknown. Calmodulin is an essential protein in *S. cerevisiae* but its function is not dependent on binding to calcium, since calcium binding deficient mutants are viable (Geiser et al, 1993).

The activities of Gin4, Cmk1 and Cmk2 in the loading assay are unlikely, since CDK activity required for Gin4 activation is absent in the G1 phase extracts used for the assay, and no calcium is present in the reaction buffer. However, at least in the cases of Cmk1 and Cmk2, activity cannot be ruled out at this stage. The purification of Cmk1 and Cmk2 can be explained by the fact that both kinases bind calmodulin (Ohya et al, 1991). Similarly, the purification of Gin4 may be explained by the presence of septins Cdc3 and Cdc12 in the purification (see Appendix 2).

4.6 Cka1 and Ypk1 are present in the loading assay

In order to determine whether any of these kinases bind specifically to the pre-RC in the loading assay, strains were generated in the ySC17 background in which Cka1, Ypk1, Gin4 or Cmk1 were each tagged with nine tandem copies of the Myc tag. 9-Myc sequences coupled to a hygromycin resistance marker were amplified by PCR from plasmid pYM20 (Janke et al, 2004) using primers containing regions of homology to the C termini of each of the target kinases. PCR products were transformed into *S. cerevisiae* strain ySC17 and transformants selected by growth in the presence of hygromycin. Correct integration was verified by analysis of TCA precipitated cell extracts by SDS-PAGE and Western blotting against the Myc tag using the monoclonal 9E10 antibody. The result is shown in Figure 21A, demonstrating the sizes at which each of the tagged proteins resolves by SDS-PAGE. Gin4 seems to be present at a considerably lower abundance in the cell than the other kinases, requiring a 20-fold longer Western blot exposure before it became visible. In each case, smaller tagged products were present in some clones due to spontaneous deletion of tandem Myc repeats during the transformation process. Individual transformants of *CKA1-MYC*, *YPK1-MYC*, *GIN4-MYC*, and *CMK1-MYC* strains were selected and named ySS9, 10, 11 and 12 respectively. A G1 phase extract overexpressing ORC and Cdc6 was made from each as described for ySC17.

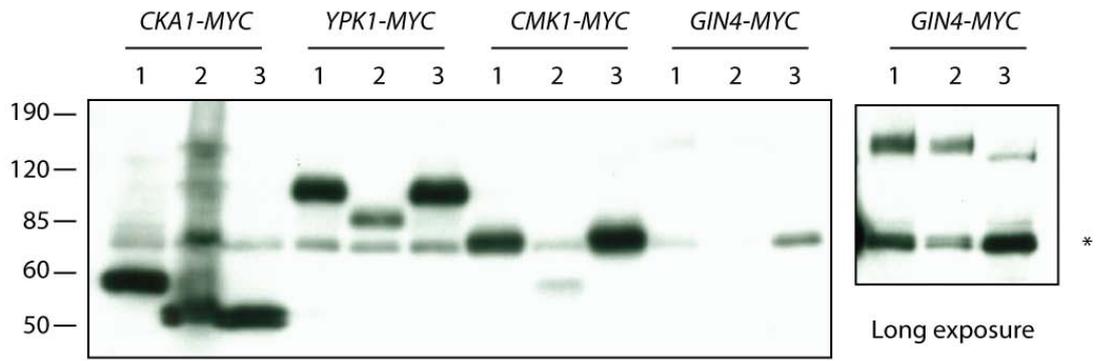
Loading assays were then performed with extracts from each strain and DNA bound proteins analysed by SDS-PAGE followed by silver staining and Western blotting against the Myc tag (Figure 21B). Cka1 was seen to bind specifically to the wt DNA, whilst Ypk1 was present on both wt and A- DNA. Cka1 did also showed some non-

specific binding to the A- DNA, however, and there was some variability between experiments. In some cases, the non-specific binding of Cka1 appeared approximately equal to the specific binding (data not shown). Neither Gin4 nor Cmk1 were seen to bind to either wt or A- DNA. Gin4, however, already a low abundance protein, was not visible by Western blotting in the cell extract (data not shown), and may be insoluble under the conditions used for extract production.

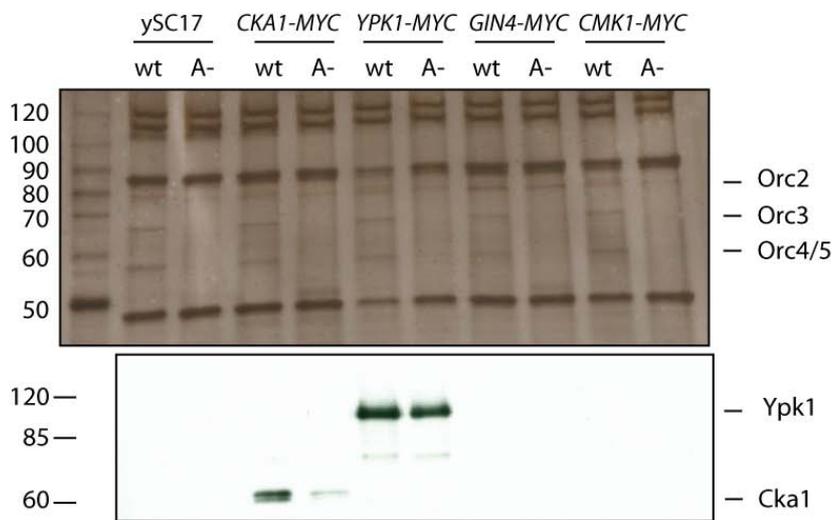
Figure 21. *Cka1* and *Ypk1* are present in the loading assay

- A.** *Cka1* (lanes 1-3), *Ypk1* (lanes 4-6), *Cmk1* (lanes 7-9) and *Gin4* (lanes 10-12) were tagged with 9 tandem repeats of the Myc tag in a ySC17 background. After transformation, three transformants were selected and the presence of tagged proteins verified by making a TCA precipitation of cell contents and analysing a sample by SDS-PAGE followed by Western blotting using the monoclonal 9E10 antibody against the Myc tag. Shorter products arise due to spontaneous deletion of tandem Myc repeats during transformation. A 20-fold longer exposure of the Western blot is shown for the three *GIN4-MYC* clones. A cross reacting band is marked with an asterisk.
- B.** Loading assays were performed with wt and A- DNA and extract from ySC17 or from each of the four Myc-tagged strains. 25% of DNA bound material was separated by SDS-PAGE and proteins visualised by silver staining (upper panel) or Western blotting against the Myc tag (lower panel)

A.



B.



4.7 Casein kinase II can phosphorylate Orc1 *in vitro*

We next tested whether any of the four myc-tagged kinases were capable of phosphorylating Orc1 *in vitro*. For this purpose, full length Orc1 was cloned and expressed as a hexa-His tagged protein in *E. coli*, and purified in a single step using nickel-NTA agarose. Relatively pure protein results from this single step purification, and is shown in Figure 22A. Next, monoclonal 9E11 anti-myc antibody was crosslinked to proteinA beads in order to purify Myc-tagged proteins from ySS9, 10, 11 and 12 extracts. 1mg of purified antibody was bound to 100µl bed volume of proteinA agarose and crosslinked with DMP as described in Materials and Methods. Samples of input and unbound antibody, as well as bead bound protein released from beads by boiling in Laemmli buffer both before and after DMP crosslinking, were separated by SDS-PAGE and visualised by Western blotting (Figure 22B). The crosslinking procedure successfully renders the antibody-proteinA interaction resistant to denaturation in SDS.

Proteins were then immunoprecipitated from 100µl aliquots of each extract, using 20µl of 9E11 bound proteinA beads or an equivalent quantity of beads containing no crosslinked antibody. Beads were incubated in extract for 1 hour at 4°C, after which time bead bound proteins were isolated, washed and resuspended in a final volume of 50µl. Samples were taken of input, unbound and bead bound fractions, separated by SDS-PAGE and used for Western blotting against the Myc tag (Figure 22C).

Cka1, Ypk1 and Cmk1 were all purified from the extract as expected. However, once again, no Myc tagged protein was visible by Western blotting in the input or bead

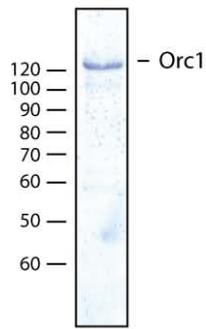
bound fractions of the *GIN4-MYC* extract. Only Cka1, Ypk1 and Cmk1 were therefore used in the subsequent experiment. Bead bound kinases were titrated and analysed by Western blotting in order to approximate relative enzyme concentrations, and Ypk1 and Gin4 containing beads diluted in order to equalise the concentrations of the three proteins (data not shown).

Purified kinases were then used to phosphorylate Orc1 *in vitro*. The following reactions were performed; 2µl of purified Orc1 in the absence of proteinA beads, Orc1 in the presence of 10µl of proteinA beads containing proteins purified from ySC17, *CKA1-MYC*, *YPK1-MYC* and *CMK1-MYC* strains, 10µl of proteinA beads containing proteins purified from ySC17, *CKA1-MYC*, *YPK1-MYC* and *CMK1-MYC* strains in the absence of Orc1, and Orc1 in the presence of twofold titrations (10, 5 and 2.5µl each) proteinA beads containing proteins purified from ySC17, *CKA1-MYC*, *YPK1-MYC* and *CMK1-MYC* strains. Reactions were performed in the same buffer conditions used in the loading assay phosphorylation experiments. Reactions were performed for 1 hour at 30°C and were stopped by the addition of an equal volume of Laemmli buffer and boiling for 10 minutes. 25% of each reaction was separated by SDS-PAGE and analysed by silver staining and Western blotting against Myc-tagged proteins (Figure 22D, upper and middle panels). Silver stained gels were dried and radiolabel incorporation visualised using a phosphorimager (figure 22D, lower panel). The results demonstrate that Cka1 but not Ypk1 or Cmk1 are capable of phosphorylating Orc1 *in vitro*. A small amount of Cka1 and Cmk1 autophosphorylation was also seen.

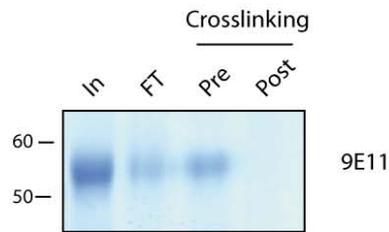
Figure 22. *Cka1* can phosphorylate *Orc1* *in vitro*

- A.** *Orc1* was expressed as a hexa-His tagged protein in *E. coli* and purified in a single step using nickel-NTA agarose. A sample of purified protein was separated by SDS-PAGE and visualised by Coomassie staining.
- B.** Monoclonal 9E11 anti-Myc antibody was crosslinked to proteinA agarose using DMP as described in Materials and Methods. Samples of input (In) and unbound (FT) antibody as well as bead bound antibody before (Pre) and after (Post) crosslinking were separated by SDS-PAGE after boiling in Laemmli buffer and visualised by Coomassie staining. Crosslinking renders the antibody-ProteinA interaction resistant to denaturation by SDS.
- C.** 9E11 crosslinked proteinA beads (9E11) or proteinA beads containing no antibody (Mock) were used to pull down Myc tagged proteins from *ySC17*, *CKA1-MYC*, *YPK1-MYC*, *GIN4-MYC* and *CMK1-MYC* strains. Samples of input (In), flow through (Sup^l) and bead bound (Pull down) proteins in each case were separated by SDS-PAGE and analysed by Western blotting using monoclonal 9E10 antibody.
- D.** Bead bound kinases were used to phosphorylate *Orc1 in vitro*. Reactions were as follows; 2µl of purified *Orc1* in the absence of proteinA beads (lane 1), *Orc1* in the presence of 10µl of proteinA beads containing proteins purified from *ySC17*, *CKA1-MYC*, *YPK1-MYC* and *CMK1-MYC* strains (lanes 2-5), 10µl of proteinA beads containing *Cka1*, *Ypk1* and *Cmk1* in the absence of *Orc1* (6-8), and *Orc1* in the presence of twofold titrations (10, 5 and 2.5µl each) proteinA beads containing proteins purified from *ySC17*, *CKA1-MYC*, *YPK1-MYC* and *CMK1-MYC* strains (lanes 9-20). 25% of each reaction was separated by SDS-PAGE followed by silver staining (upper panel) and Western blotting against the Myc tag (middle panel). Silver stained gels were dried and ³²P incorporation visualised using a phosphorimager (lower panel).

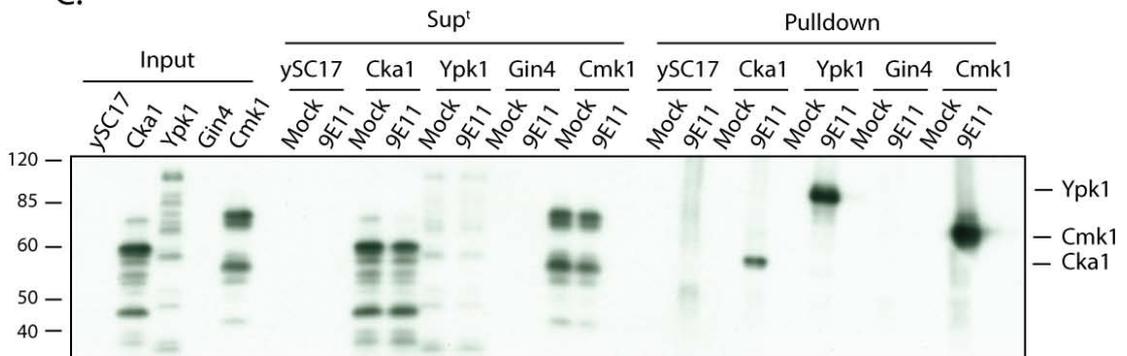
A.



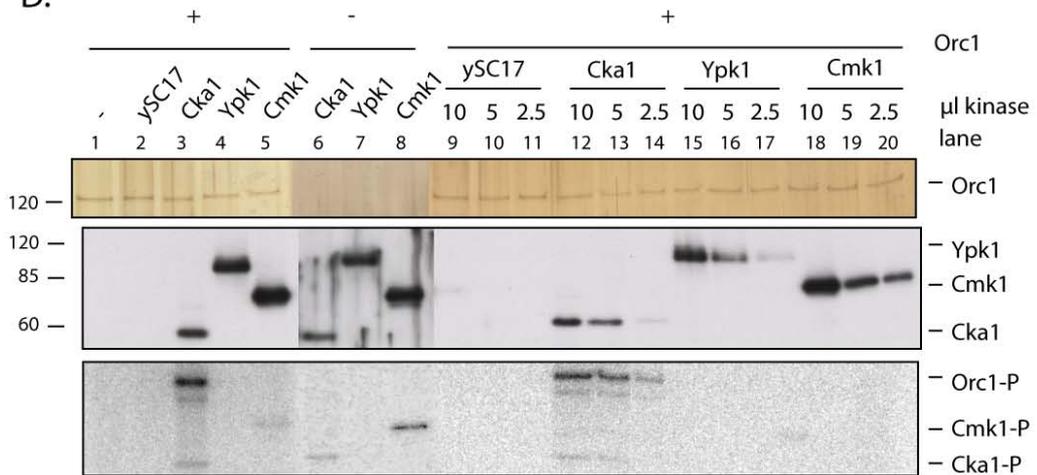
B.



C.



D.



4.8 CKII is responsible for kinase activity in the loading assay

The above experiments provided strong evidence that CKII is the kinase responsible for Orc1 phosphorylation in the loading assay. Gin4, Cmk1 and Cmk2 are likely to be inactive in the conditions used in the assay, and only Cka1 and Ypk1 appear to be present on the loaded DNA. Of these, only Cka1 is capable of phosphorylating Orc1 *in vitro*. Nevertheless, we wished to categorically rule out the involvement of the remaining kinases in Orc1 phosphorylation. Since Gin4, Cmk1 and Cmk2 are non-essential proteins, it is possible to verify that they are not involved in Orc1 phosphorylation by creating deletion strains.

To this end, strains were constructed in which either Gin4 or both Cmk1 and Cmk2 had been deleted in the ySC17 strain background. In addition, strains in which Cka2 and Ypk2 had been deleted were also created in the *CKA1-MYC* and *YPK1-MYC* backgrounds respectively. Either hygromycin or nourseothricin resistance markers were amplified by PCR from plasmids pFA6-hphNT1 or pFA6-natNT2 (Janke et al, 2004) respectively, depending on the strain to be transformed, using primers containing regions of homology to the relevant genes. PCR products were transformed into the relevant background strains and transformants selected by growth in the presence of antibiotic. Successful integration and deletion was confirmed by colony PCR of the relevant locus. Cmk1 and Cmk2 deletions were made sequentially using hygromycin and nourseothricin markers. *YPK1-MYC ΔYPK2*, *CKA1-MY CΔCKA2*, *ΔGIN4* and *ΔCMK1ΔCMK2* strains were named respectively ySS13, 14, 15 and 16.

Extracts were made from each of the deletion strains as described for ySC17 and used for loading assays which were then phosphorylated in the absence of exogenously added kinase. Samples of each reaction were separated by SDS-PAGE and proteins analysed by silver staining (Figure 23A, upper panel) and Western blotting against Orc6 to verify levels of specifically bound ORC (Figure 23A, middle panel). The silver stained gel was dried and ^{32}P incorporation visualised using a phosphorimager (Figure 23A, lower panel).

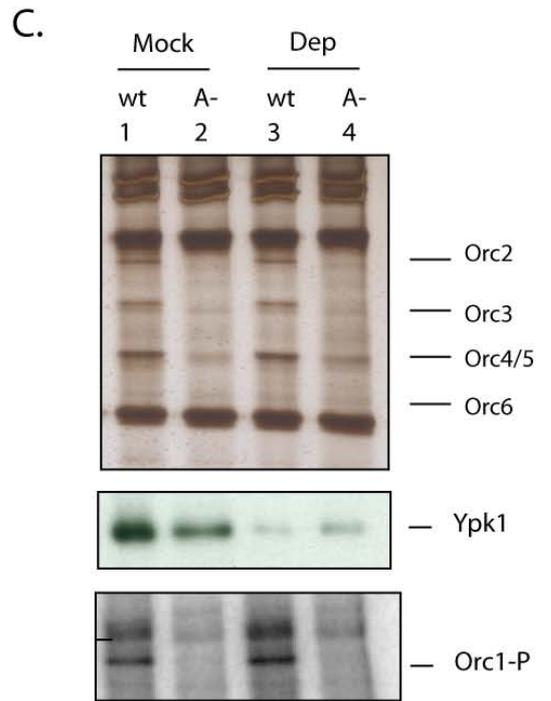
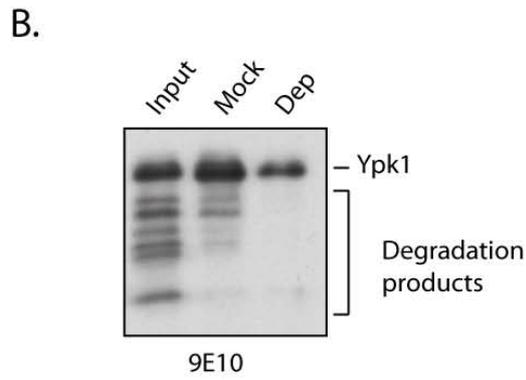
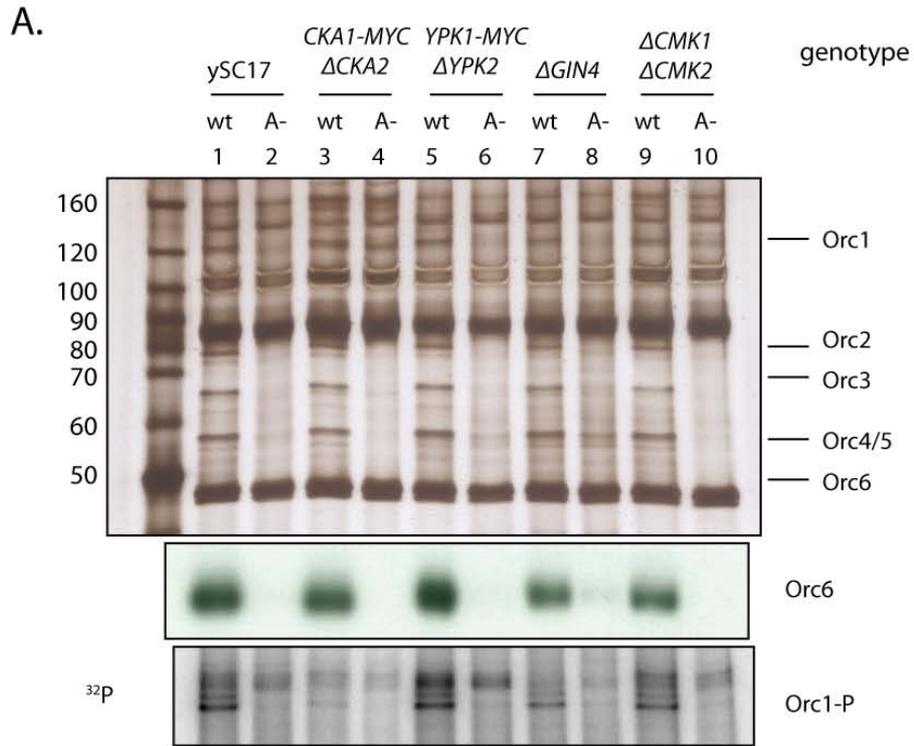
ORC binding to wild type DNA was as expected in each of these strains. Although there was a partial reduction in ORC DNA binding, visible by Western blotting, and Orc1 phosphorylation in both the ΔGIN4 and $\Delta\text{CMK1}\Delta\text{CMK2}$ strains, neither showed a complete loss of Orc1 phosphorylation, hence ruling out these three kinases as responsible for Orc1 phosphorylation. Interestingly, the $\text{CKA1-Myc } \Delta\text{CKA2}$ strain showed a sharp reduction in the level of Orc1 phosphorylation, providing further evidence that CKII is the kinase responsible for the phosphorylation of Orc1. It appears that the C terminal Myc tag interferes with the interaction of Cka1 with its substrates.

In addition, an attempt was made to deplete Ypk1 kinase from the $\text{YPK1-MYC } \Delta\text{YPK2}$ strain. A 100 μl aliquot of proteinA beads coupled to 9E11 or an equivalent volume of beads containing no antibody was taken, pelleted and buffer removed. Beads were resuspended in a 200 μl aliquot of $\text{YPK1-MYC } \Delta\text{YPK2}$ extract and incubated with rotation for one hour at 4°C. Beads were then removed and the extract subjected to two further rounds of depletion by the same protocol. Samples of input and depleted extract were analysed by SDS-PAGE and Western blotting against the Myc tag

(figure 23B). The technique was only partially successful, resulting in a decrease of approximately 50% of tagged protein in the extract. However, a loading assay performed with mock treated or depleted extract showed a clear reduction in the amount of detectable DNA bound Ypk1 following protein depletion (figure 20C, upper and middle panel). This was not, however, associated with a reduction in Orc1 phosphorylation when the loaded proteins were subsequently phosphorylated in the absence of exogenously added kinase (Figure 23C, lower panel). This therefore further argues against the possibility of Ypk1 being responsible for Orc1 phosphorylation in the loading assay.

Figure 23. Effect of kinase deletion or depletion on Orc1 phosphorylation

- A.** Extracts from ySC17 (lanes 1-2), *CKA1-MYC ΔCKA2* (lanes 3-4), *YPK1-MYC ΔYPK2* (lanes 5-6), *ΔGIN4* (lanes 7-8) and *ΔCKM1ΔCMK2* (lanes 9-10) strains were used in loading assays, and purified pre-RCs subsequently phosphorylated in the absence of exogenous kinase. 25% of each reaction was separated by SDS-PAGE and proteins visualised by silver staining (upper panel) and Western blotting against Orc6 (middle panel). The silver stained gel was dried and ³²P incorporation visualised using a phosphorimager (lower panel).
- B.** Ypk1 was depleted from the *YPK1-MYC ΔYPK2* strain by three successive rounds of incubation with 9E11 bound proteinA beads or beads containing no antibody. Samples of input and depleted (Dep) or mock depleted (Mock) extracts were separated by SDS-PAGE and analysed by Western blotting against the Myc tag. Depletion was only partially successful, showing a reduction of approximately 50% of the total tagged protein.
- C.** Mock depleted (lanes 1-2) and depleted (lanes 3-4) extracts were used for loading assays and were subsequently phosphorylated in the absence of exogenous kinase. 25% of each reaction was separated by SDS-PAGE and proteins visualised by silver staining (upper panel) and Western blotting against the Myc tag (middle panel). The silver stained gel was dried and ³²P incorporation visualised using a phosphorimager (lower panel).



4.9 The role of Orc1 phosphorylation *in vivo*

From these experiments it was therefore concluded that CKII is the enzyme responsible for the *in vitro* phosphorylation of Orc1 in the loading assay. However, it is still unclear whether this event plays a role in DNA replication *in vivo*. Since the *CKA1-MYC ΔCKA2* strain showed a dramatic reduction in Orc1 phosphorylation observed in the loading assay, we wondered whether this defect was associated with a reduction in the efficiency of MCM loading *in vitro* or in the rate of S phase progression *in vivo*. Loading assays were therefore performed with both this strain and ySC17 without subsequent phosphorylation in order to maintain Mcm2-7 complex association with the DNA. Samples from supernatant and DNA bound fractions in each case were taken and analysed by SDS-PAGE followed by silver staining (Figure 24A) and Western blotting against both Orc6 and Mcm2 (Figure 24B, left panel). DNA bound samples were also titrated in twofold dilutions of 10, 5 and 2.5µl and were similarly analysed by Western blotting in order to compare relative protein amounts present in each reaction (Figure 24B, right panel).

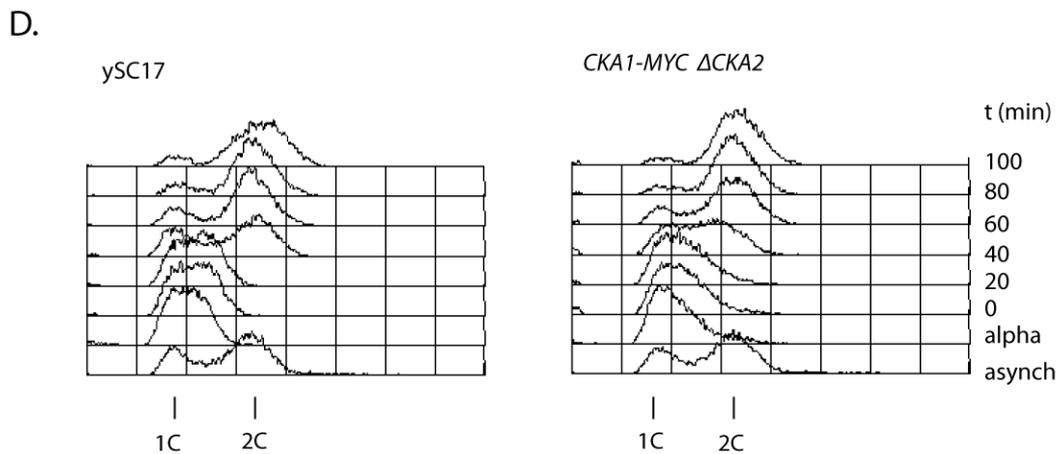
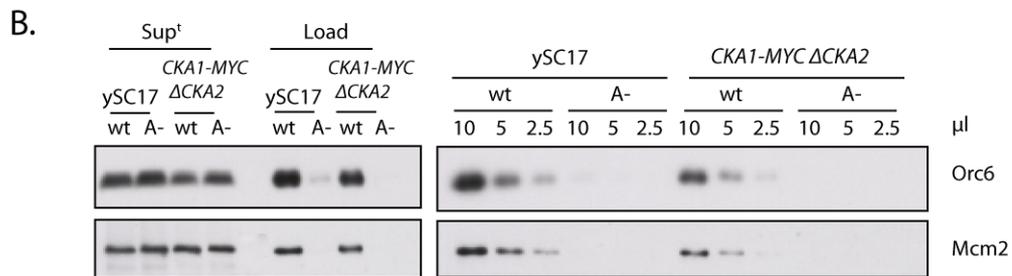
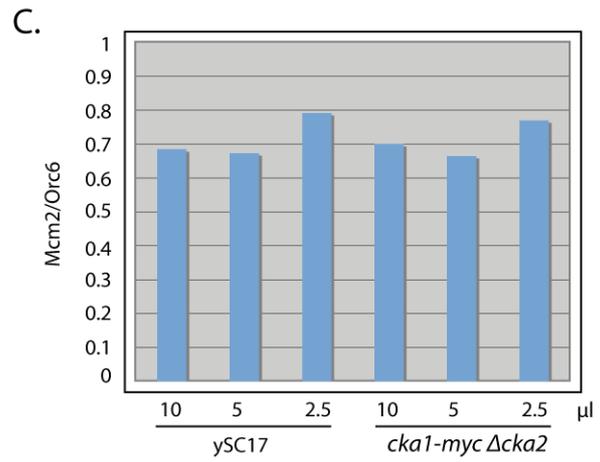
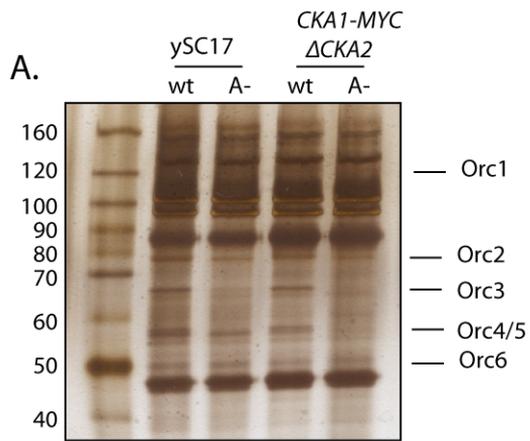
In both cases, sequence specific ORC binding and Mcm2-7 loading was observed. Since the *CKA1-MYC ΔCKA2* strain extract appeared to bind approximately 50% less ORC to the DNA than strain ySC17, signal intensities of Orc6 and Mcm2 bands in the titration Western blot were quantified using imageJ software (NIH, open source). Loaded MCM protein was then normalised relative to the amount of DNA bound ORC for each point in the titration (Figure 24C). The amount of MCM loaded per DNA bound ORC molecule appeared to remain constant between the two extracts, demonstrating that the reduction in Orc1 phosphorylation does not affect the

efficiency of Mcm2-7 complex loading *in vitro*. No test was made of the salt stability of the loaded complex, however. It also remains possible that Orc1 phosphorylation plays a role in ORC binding to the DNA, resulting in the observed reduction in ORC DNA binding observed in the *CKA1-MYC ΔCKA2* strain extract. The *CKA1-MYC ΔCKA2* strain is viable, however, so either sufficient ORC phosphorylation remains for function *in vivo*, or Orc1 phosphorylation does not have an essential function. Other essential functions of CKII must also be unaffected by Myc tagging of the Cka1 subunit.

In a final experiment, we also tested the rate of DNA replication in both ySC17 and the *CKA1-MYC ΔCKA2* strain. Cultures were arrested in G1 phase with α factor followed by release into a mitotic arrest with nocodazole. Samples were taken at 20 minute time points after release, as well as during the G1 arrest, and were analysed for DNA content by FACS analysis, as described in Materials and Methods (Figure 24D). No defect in the rate of DNA synthesis was observed in the *CKA1-MYC ΔCKA2* strain, however, and although a subset of the population in each case failed to escape from the G1 arrest, replicating cells in both cases had completed DNA replication by approximately 40-60 minutes after release.

Figure 24. The role of *Orc1* phosphorylation in vivo

- A.** Loading assays were performed with ySC17 extract and extract from the *CKA1-MYC ΔCAK2* strain. 25% of each reaction was separated by SDS-PAGE and visualised by silver staining.
- B.** Supernatant (Sup^l) and bead bound (Load) fractions were also analysed by Western blotting against Orc6 and Mcm2 (left hand panels). Twofold titrations (10, 5 and 2.5μl samples) of DNA bound samples were similarly analysed by SDS-PAGE and visualised by Western blotting (right hand panels).
- C.** Orc6 and Mcm2 signals were quantified at each point in the titration using ImageJ software (NIH, open source). The amount of loaded MCM complex was normalised relative to the amount of DNA bound ORC.
- D.** FACS analysis of DNA content of ySC17 or *CKA1-MYC ΔCAK2* cells either growing asynchronously (asynch), arrested in α factor (alpha), or at 20 minute time points after release from α factor into nocodazole.



Although CKII phosphorylation of Orc1 is clear *in vitro*, therefore, these experiments have so far failed to demonstrate a role for the event *in vivo*. However, Orc1 phosphorylation *in vitro* is not completely abolished in the *CKA1-MYC ΔCKA2* strain, so it remains possible that complete loss of kinase function, for example by using a temperature-sensitive mutant, may reveal an essential function that is not uncovered here. We have also not successfully demonstrated Cdc7 mediated phosphorylation of members of the Mcm2-7 complex, or other pre-RC factors, which we initially set out to do. On the other hand, CDK activity, albeit in a negative regulatory role, was clearly demonstrated. Recent developments in reconstitution of the pre-RC using purified components (Remus et al, in press) will facilitate the study of Cdc7 function, by increasing both the purity and the concentration of its substrates.

Chapter 5: Discussion

5.1 Peptide array phosphorylation experiments

In the first approach taken here to identify novel substrates and functions of CDK and Cdc7, enzymes were purified and used to phosphorylate arrays of peptides *in vitro*. Arrays contained complete sequences of all essential replication proteins in *S. cerevisiae*, as well as some proteins involved in the regulation of genome stability. Proteins were split into sets of 26mer peptides attached to a solid cellulose support. Three experiments were performed in which arrays were phosphorylated with human Cdk2-cyclinA, *S. cerevisiae* Cdc28-Clb5 and *S. cerevisiae* Cdc7-Dbf4. At the outset of the project, the essential S phase promoting substrates of CDK were unknown, as were the reported essential Cdc7 phosphorylation sites in the N termini of Mcm2 and Mcm4 and the requirement for a distant binding site for recruitment of the kinase. (Tak et al, 2006; Tanaka et al, 2007; Zegerman and Diffley, 2007; Sheu and Stillman, 2006; Bruck and Kaplan, 2009). Also unknown was the requirement for acidic or phosphorylated residues in the +1 position for Cdc7 phosphorylation (Cho et al, 2006; Masai et al, 2006; Montagnoli et al, 2006; Bruck and Kaplan, 2009). The array experiments were therefore designed with two aims; to identify substrates for both kinases, and to determine a possible substrate consensus for Cdc7.

The approach was met with limited success, however. In the first experiment, phosphorylation of arrays with human Cdk2-cyclinA revealed preferential phosphorylation of several proteins, including Sld3, Orc1, Orc4, Orc5, Orc6, Cdc6, Cdt1, Mcm3, Mcm7, Mcm10, Top2, Sld2, Pol12, Pri1, Pol32, Rfc1, Rfc2, Rfc4, Rfc5,

Fen 1 and Cdc9, as well as sites of phosphorylation in multiple other proteins. Analysis of phosphorylated peptides revealed the unexpected finding that some of the phosphorylated peptides did not contain an S/T-P consensus sequence, a requirement for substrate phosphorylation by CDK (Songyang et al, 1994). Many peptides that did contain an S/T-P consensus, on the other hand, remained unphosphorylated. In addition, known sites of phosphorylation in Sld2 and Sld3 either showed low levels of phosphorylation or none at all relative to background signal. The reason for these observations still remains unclear. It is possible that peptides were behaving on the array in unpredictable ways, with some sites accessible to the kinase whilst others are not. It is also possible that CDK was phosphorylating residues outside of its normal consensus in these experiments. Despite this, however, phosphorylation of S/T-P consensus sites in proteins including Orc1, Cdc6, Mcm7, Mcm10, Top2, Sgs1, Sld2, Pol12, Pol32, Rfc1 and Rfc4 was revealed, the *in vivo* significance of which remains to be investigated. Although verification of several substrates that appeared to be phosphorylated on the arrays was attempted, only Sld2 was confirmed.

In the second experiment, arrays were phosphorylated with endogenous Cdc28-C1b5. Although the purified enzyme was highly active against histone H1, the relatively low yields of purification of this enzyme meant that a low concentration of enzyme was used. The results of the experiment revealed an even less predictable pattern of phosphorylation than human Cdk2-cyclinA, with no apparent enrichment for phosphorylation of peptides containing an S/T-P consensus. Although to some extent the pattern could be seen to overlap with that produced in the earlier experiment, the random appearance of phosphorylation events in this experiment made interpretation extremely difficult. In some cases, ³²P incorporation was seen in peptides that did not

contain a serine or threonine residue. The results of this experiment may be attributed to either CDK phosphorylation outside of its consensus, a contributing phosphorylation by a contaminating kinase, or a background binding of ^{32}P γATP that was not observed in control experiments.

What conclusions can therefore be drawn from these experiments about the substrates of CDK involved in replication? To some extent, the experiments were superseded over the course of the study by the identification of phosphorylation of Sld2 and Sld3 (Tak et al, 2006; Tanaka et al, 2007; Zegerman and Diffley, 2007), at sites which were in any case not identified in this study. The lack of phosphorylation of known sites coupled with the apparent phosphorylation of sites that cannot be substrates *in vivo* makes it difficult to determine whether the CDK phosphorylation events observed here are physiologically relevant. However, some of the S/T-P and S/T-P-x-K/R phosphorylation events are potentially interesting. Two sites in membrane 2 strongly phosphorylated by Cdk2-cyclinA, peptides G21-22, for example, represent phosphorylation of Mcm10 at residue S63, an S-P-x-K motif. This falls within a conserved N terminal domain which has been reported to be responsible both for oligomerisation (Robertson et al., 2008) and binding to Dbf4, stimulating phosphorylation of the Mcm2-7 complex (Lee et al., 2003). It is therefore possible that either one of these may be affected by phosphorylation of the protein. An EM structure of Mcm10 revealed a ring shaped hexamer with similarities to the structures of SV40 TAg and the archaeal MCM proteins (Okorokov et al., 2007), leading to the speculation that it may encircle the DNA and couple helicase and polymerase components at the fork. CDK phosphorylation may therefore promote this oligomerisation and thereby stimulate replication fork formation. Alternatively,

phosphorylation may promote binding to Dbf4, hence providing a mechanism of CDK stimulation of Cdc7 activity aside from priming SSP motif phosphorylation (Lee et al., 2003).

Rfc1 also appears to be phosphorylated in several N terminal minimal CDK consensus sites. The structure of RFC in complex with PCNA contained an N terminal truncation of Rfc1 which was functional for clamp loading (Bowman et al., 2004), so the precise function of the N terminal domain is unknown. It may be involved in protein-protein interactions, however, and has been reported to bind DNA (Allen et al., 1998). Another potential substrate was the Pol12 subunit of Pol α , which was strongly phosphorylated at several N terminal minimal consensus motifs. Phosphorylation of Pol12 in a cell cycle dependent manner not dependent on Cdc7 has previously been demonstrated (Foiani et al., 1995). Phosphorylation is dependent on the interaction between Pol12 and Pol1, but does not affect formation of the complex (Ferrari et al., 1996). Although Pol12 is essential for DNA replication *in vivo*, it does not seem to affect polymerase or primase activities *in vitro* (Foiani et al., 1994; Brooke et al., 1991 a,b). The human protein has been shown to form a tight complex with SV40 TAg, and it may play a similar role in tethering the polymerase to a full replication fork (Collins et al., 1993). The *S. pombe* protein has also been shown to bind Orc2 (Uchiyama & Wang, 2004). However, any direct regulatory role for phosphorylation of the protein, for example in regulating the interactions between other Pol α subunits or replication fork proteins remains to be elucidated.

Other possible substrate revealed in the Cdk2-cyclinA array phosphorylation experiment included Pol1, Dna2, Top2, Sgs1, Smc5, Pol32 and Rfc4, all of which

were phosphorylated by Cdk2-cyclinA within CDK consensus motifs. However, since none of these substrates has so far been verified *in vivo*, discussion of possible functions of phosphorylation events remain entirely speculative. Phosphorylation events may alter the protein structure or stability, create or destroy protein-protein interactions or directly alter enzymatic activities.

In the third experiment, arrays were phosphorylated with *S. cerevisiae* Cdc7-Dbf4 purified after co-expression in insect cells using a baculovirus protein expression system. Activity of this kinase was verified by phosphorylation of the N terminus of Mcm2. However, the array phosphorylation experiment revealed only very low or no activity of Cdc7-Dbf4. Prior to phosphatase treatment of the membranes, the pattern of phosphate incorporation resembled that seen when arrays were phosphorylated by Cdc28-Clb5, creating an argument that in neither experiment was ³²P incorporation dependent on kinase activity. Only a small amount of this signal was removed by treatment with λ phosphatase. Of those peptides that showed radiolabel incorporation above an arbitrarily chosen threshold, approximately a third contained the reported S/T-D/E consensus sequence (Cho et al, 2006; Masai et al, 2006; Montagnoli et al, 2006; Bruck and Kaplan, 2009). Once again, no enrichment for known substrates was observed. Neither could phosphorylation of SSP motifs be stimulated by prior phosphorylation of the membrane with CDK. It therefore seems likely that Cdc7-Dbf4 is essentially inactive for peptide phosphorylation in this system. There are two contributing explanations for this. The first is that Cdc7-Dbf4 has an auto-inhibitory activity, which therefore limits the activity of the kinase *in vitro* (Figure 13). The second is that recruitment motifs were absent from the peptides, which have been shown to enhance phosphorylation of at least two substrates *in vitro* (Sheu and

Stillman, 2006; Bruck and Kaplan, 2009). Low levels of kinase activity coupled to low efficiency of substrate recognition results in only minimal levels of substrate phosphorylation.

Cdc7 array phosphorylation therefore revealed little about the function of the kinase.

Neither known nor novel substrates appeared to be efficiently phosphorylated.

However, the observation that the kinase has auto-inhibitory activity is interesting, and merits further study. It is possible that the cell regulates Dbf4 or Cdc7

autophosphorylation, and that this contributes to the cell cycle dependent regulation of

the kinase. One possibility is that CDK phosphorylation of either Dbf4 or Cdc7

promotes de-phosphorylation by the activity of a protein phosphatase. A phosphatase

with activity against Cdc7 and Dbf4 remains to be identified. Dbf4 contains multiple

S/T-D/E sites throughout its sequence, explaining the large shift in the resolving

position of the protein in SDS-PAGE. It also contains two strong CDK consensus

sites at its extreme N terminus, residues S3 and S11, as well as three other minimal

S/T-P sequences. Cdc7 on the other hand contains only four S/T-D/E motifs, all at its

C terminus. It contains four CDK consensus sites, including one at the C terminus

amongst the S/T-D/E sites. Interestingly, Cdc7 contains only one strong CDK

consensus, and this is found in an S-S-P motif at the N terminus of the protein. CDK

phosphorylation of Cdc7 may therefore potentiate rather than prevent

autophosphorylation (Appendix 1, membranes 2 and 5). The C terminus of Cdc7 has

been reported to be responsible for binding to Dbf4 (Jackson et al., 1993). No

phosphorylation of sites in either Cdc7 or Dbf4 was seen when peptide arrays were

phosphorylated with Cdk2-cyclinA, although Dbf4 but not Cdc7 phosphorylation by

CDK has been reported previously (Ubersax et al., 2003).

An alternative possibility for regulation of Cdc7-Dbf4 autophosphorylation is that some other Cdc7-Dbf4 interacting factor prevents Cdc7 and Dbf4 autophosphorylation in either a constitutive or cell cycle dependent manner. Such a factor may be identified by mass-spectrometric analysis of endogenous enzyme, or by pull down from a whole cell extract with larger quantities of purified kinase. This approach may also reveal new substrates of the kinase that contain a docking site for Dbf4, which may be an important avenue for future research. Identification of a conserved docking sequence for Cdc7-Dbf4 would enable prediction of kinase substrates *in silico*.

In addition to the array phosphorylation experiment performed with Cdc7-Dbf4, phosphorylation of Sld2, Sld3, Dpb11 and Mcm10 was also demonstrated with full-length proteins *in vitro*. S/T-D/E sites are found throughout Mcm10, Sld3 and Dpb11, and in the C terminal half of Sld2. An interesting observation is the presence of three S-S-P motifs (S127, S137 and S171) clustered in the N terminal half of Sld2 in the vicinity of the essential CDK phosphorylation site T84. As for Mcm10, it is interesting to speculate that Sld2 may coordinate the activity of the two kinases; Sld2 phosphorylation by Cdc7 after phosphorylation by CDK may contribute to regulation of Sld2 function. Although CDK phosphorylation of Sld2 and Sld3 has been shown to promote binding of both proteins to Dpb11, further functional significance of phosphorylation is unknown, as is the consequence of binding to Dpb11. Also unclear is whether both Sld2 and Sld3 bind Dpb11 in a ternary complex. The precise functions of all three proteins in replication fork formation remain to be determined.

Future *in vitro* approaches to the identification of substrates of both Cdc7 and CDK could involve phosphorylation of complete set of full length proteins in solution. Not

only would this allow a comprehensive list of replication substrates to be built up, but experiments could also investigate changes in protein-protein interactions or enzyme activities resulting from phosphorylation. One possibility is that Cdc7 phosphorylation of the Mcm2-7 subunits promotes their interaction with Cdc45 and the GINS complex, leading to activation of the helicase (Masai et al, 2006). The *mcm5-bob1* bypass of kinase function suggests that this may be the only essential function of the kinase, although other subsidiary functions cannot be ruled out. Stimulation of Cdc7 activity by prior substrate phosphorylation by CDK, for example in Sld2, could also be investigated. Such an approach would lack the benefits of a high throughput screen however, and limits the number of potential substrates which could be tested. Achieving quantitative phosphorylation of Cdc7-Dbf4 substrates may be aided by the development of a more active form of purified Cdc7-Dbf4. Identification of the residues in Dbf4 or Cdc7 which are important for auto-inhibition of the kinase *in vitro* will enable a constitutively active form of the kinase to be purified.

Previous approaches to proteomic scale identification of protein kinase substrates have included the use of a bulky ATP analogue sensitive Cdc28 mutant to selectively radiolabel CDK substrates in *S cerevisiae* extracts (Ubersax et al., 2003). 360 CDK targets were identified in this way out of 695 proteins tested (approximately 10% of the yeast proteome), of which 181 were strongly phosphorylated. This screen detected phosphorylation of Sld2, although it did not detect phosphorylation of Sld3. Of the putative substrates of Cdk2-cyclinA detected on the peptide array, only Pol1, Dna2 and Pol32 were detected in the earlier screen. Another report used arrays of approximately 4000 GST tagged full length *S. cerevisiae* proteins printed onto chips to

identify substrate sets for 78 different yeast kinases (Ptacek et al., 2005). This screen identified 43 substrates of Cdc28-Clb5, of which 10 overlapped with the Cdc28 mutant study (Ubersax et al., 2003). Differences between the two screen may result from slightly different selectivities of the wild-type and mutant kinases, and the fact that one screen was performed in cell extracts whilst the other was performed on an array. Much further work is required to build a complete picture of the 'kinome' of eukaryotic organisms, and to understand the functional significance of phosphorylation events.

5.2 Pre-replicative complex phosphorylation

As an alternative approach to kinase substrate identification, pre-replicative complexes which had been assembled *in vitro* using cell free yeast extracts (Seki and Diffley, 2000; Bowers et al., 2004) were also phosphorylated with both endogenous Cdc28-Clb5 and Cdc7-Dbf4 kinases. Although this approach too failed to identify Cdc7 substrates, other laboratories have demonstrated phosphorylation of loaded Mcm2-7 complexes (Francis et al., 2009). Cdc28 was observed to phosphorylate its known substrates Orc2 and Orc6. However, an unexpected phosphorylation of Orc1 was also observed, catalysed by a kinase which was purified with the pre-replicative complex. This kinase was shown to be casein kinase II (CKII), a broad specificity, constitutively active kinase with a variety of functions *in vivo* (Poole et al., 2005; Meggio and Pinna., 2003). Like Cdc7, CKII phosphorylates acidic substrates (Meggio and Pinna, 2003; Sato et al., 1997).

What could be the consequence of Orc1 phosphorylation by CKII? CKII has been shown to influence protein function in a variety of ways. Phosphorylation of Akt/PKB in Jurkat cells appears to enhance its catalytic activity (Maira et al., 2005), phosphorylation of the PTEN phosphatase appears to affect the stability and cellular localisation of the protein (Al-Khoury et al., 2005; Vazquez et al., 2001; Das et al., 2003), phosphorylation of the NF κ B inhibitor I κ B α enhances its degradation (Shen et al., 2001), whilst phosphorylation of the haematopoietic lineage cell-specific protein 1 (HS1) inhibits its cleavage by caspases (Ruzzene et al., 2002).

An extract of *S. cerevisiae* in which CKII phosphorylation of Orc1 was severely reduced *in vitro* by deletion of the α' subunit in combination with a C terminal 9xMyc tag on the α subunit failed to show a specific defect in Mcm2-7 loading *in vitro*, although ORC binding to the DNA was slightly reduced. Neither did the strain show a reduction in the rate of DNA synthesis *in vivo*. More experiments are required to verify Orc1 phosphorylation by CKII *in vivo*, as well as the sites of phosphorylation. Interestingly, an N terminal truncation of Orc1 was not phosphorylated *in vitro*, implying that CKII phosphorylation is in the N terminus of the protein. The N terminus of Orc1 (residues 1-234) contains a protein binding Bromo-Adjacent Homology (BAH) domain, and has been shown to be required for the silencing function of the protein, but is dispensable for its replication function (Bell et al., 1995; Zhang et al., 2002). The predicted size of the N terminal truncation in these experiments according to its resolving position in SDS-PAGE is consistent with a deletion slightly larger than this, although the exact position of the cleavage remains to be determined.

CKII has been shown to have a strong preference for acidic residues in the +1 and +3 positions (Meggio and Pinna., 2003). Interestingly, Orc1 contains a cluster of four such sequences at positions S237, T247, S258 and S264 (array 1 sites C10-C13), lying just downstream of the BAH domain in a region predicted to be disordered (Duncker et al., 2009). These are therefore strong candidates for CKII phosphorylation. An alignment of *S. cerevisiae*, *S. pombe*, *Drosophila*, mouse and human Orc1 homologues reveals that these motifs are conserved in the N termini of each protein. The alignment is shown in Figure 25, which also marks the position of weaker S/T-D/E and S/T-x-x-D/E motifs (Meggio and Pinna, 2003). The *S. cerevisiae* BAH domain is predicted to be contained within residues 48-188 of the protein, whilst the AAA+ domain is at the C terminus, residues 471-628. The region of the N-terminus known to be non-essential for replication extends to residue 234 (Duncker et al., 2009; Bell et al., 1995; Zhang et al., 2002). Of the conserved S/T-D/E-x-D/E sites in the Orc1 homologues, most are found within the 1-234 region, with *S. pombe*, *Drosophila*, and mouse proteins all containing consensus motifs in this area. However, the mouse and human proteins contain one and two consensus motifs respectively downstream of *S. cerevisiae* region 1-234. It could be however that any role for CKII phosphorylation of Orc1 is in the silencing function of the protein. ORC binds to the *HMR* and *HML E* and *I* silencer regions to control silencing at the mating type locus, acting to recruit a complex of Sir1, Sir2, Sir3 and Sir4 (Loo and Rine, 1995; Bell et al., 1995; Zhang et al., 2002). Higher eukaryotes lack homologues of the *S. cerevisiae* Sir proteins, but ORC has been shown to bind heterochromatin protein HP1 (Auth et al., 2006). Mutation of the four potential CKII phosphorylation sites in *S. cerevisiae* Orc1 to alanine would reveal whether serine or threonine residues at these positions are essential, and whether they have any effect on either silencing or replication. If the

sites are non essential, loading assay phosphorylation experiments similar to those described here would determine whether they are indeed the sites of CKII phosphorylation, and if so, would allow a more robust determination of the effect of CKII phosphorylation of Orc1 on replication.

Figure 25. Alignment of *S. cerevisiae*, *S. pombe*, *Drosophila*, mouse and human *Orc1* homologues. S/T-D/E-x-D/E consensus sites are highlighted in yellow., whilst the predicted BAH domain and AAA+ domain of *S. cerevisiae* Orc1 are highlighted in red and green respectively. Weaker potential CKII consensus sequences S/T-D/E or S/T-x-x-D/E are also given in green letters. The region of Orc1 conserved between ORC subunits extends from the AAA+ domain to the C terminal end of the protein (Duncker et al., 2009).

Mouse ---MPSYLTRQKTRQTFSSWVGRPLPNRKQFQQMYREICMKINDGS-EIHIKVGQFVLIQG 56
 Human ---MAHYPTRLKTRKTYSWVGRPLLDRLKHYQTYREMVCVKTEGCSTEIHIQIGQFVLIQG 57
 Drosophila ---MVNKENARSVGQQVKWIGSQDELPPVKNLEHKNVYFYQKCIYGPLTSLVGFILVSN 57
 Pombe -----MPPRKSLSRSQLLING 15
 Cerevisiae MAKTLKDLQGWEEIITTDEQGNIIDGGQKRLRRRGAKEHYLKRSSDGIKLGRGDSVVMHN 60
 . : : .

Mouse EDNKKP-----YVAKLIELFQNGAEVPPKCC-ARVQWFVRFLEIPVSKRHLLGRSPPAQ 109
 Human DDDENP-----YVAKLLELFEDDSDPPPKKR-ARVQWFVRFCEVPACKRHLLGRKPGAQ 110
 Drosophila ADAAEPTVSGCDVARILHMYELRELTDREPCRAIVQWYSWPKAIPHKNYDDDEVAIDFS 117
 Pombe -----ID 17
 Cerevisiae EAAGTYS-----VYMI 71

Mouse EIFWYDCSDWDNKINVETIIGPVQVVALAPEEVIIPVDQKSEE-----TLFVKLS 158
 Human EIFWYDYPACDSNINAETIIGLVRVILAPKDVVPTNLKNEK-----TLFVKLS 159
 Drosophila LEVIEEHRPYDNDVALGAIYRKCIVLEGTSKTSAEIEILKRHANKLKSTACPMFVSRYRFV 177
 Pombe KSLLSDDSDADSDDIDEEEVYGVWTEEPFQKEAGR-----SYRSL 57
 Cerevisiae QELRLNLTNNVVELWALTYLRWFVFNPLAHYRQFNPDANILN-----RPLNYNK 121
 . : .

Mouse WNKKDFAPLPPEVLAALRE-QEDSP-----EWQKPLKAKIKNVKSPARNTTEQE 206
 Human WNEKKFRPLSSELFAELNKPQESAA-----KCQKPVRAKSKSAESPSWTPAEHV 208
 Drosophila KVKRSYRLIPLEIHLEQPEDNARPTRSSRKSLSLAHRESKRSISARHDDTAGNGSSVEKR 237
 Pombe KKNDVIYRVGDDITVHDGDSSFYLG-----VICKLYEKAIKDKHSGKKYV 101
 Cerevisiae LFSPTANKNELYLTAELAEQLFNFR-----VANVMDGSRNVLKGNVDE 167
 . : .

Mouse VKGIKSNHSTSKFHQTPANIVIPNAKKSLELDGLGTRKPNTRWSKSSCDSLQYQKTSK 266
 Human AKRIESRHSASKSRQTPHPLTPRARKLELGNLG-----NPQMSQQTSCASLDSPGRIK 263
 Drosophila RRASMAASSSVFIDVNSFICENKVSPKIVGGRSVVRLSEKKNAPENANYPASPLTE 297
 Pombe EAIWYSRAYAKRMEIKPEYLLPDRHINEVYVSCGR-----DENLTSCIEHCNVYSEAE 155
 Cerevisiae ERDFTVRYICEPTGEKFDINIEDVKAYIKKVEPREAQEYKDLTLPSKKKEIKRGPQK 227
 :

Mouse RRAAFSETTSPPKKPNKPREIKPSSALETRVKNQGT-----QPFCAKSSV 311
 Human RKVAFSEITSPKRSQPKLQTLSPALKAPEKTRETGLSYTEDD-----KKASPEHRI 316
 Drosophila KNAKVETPKSRASAARRNLSLDRGADTTADSDCLNYSIVQQTDPDKTPSNDMKIKLRL 357
 Pombe FFSKFPAGIPTKRKDLFPCNFFIRRGVHLKVN-----KYTEPLDW 195
 Cerevisiae DKATQTAQISDAE TRATDI TDNE DGNEDESD-----YESPSSIDV 268
 . : .

Mouse VLRARNPAMTTTKLG----VDNTLSPINRGLRSSVVPSSGGLTPVYIR----RKAKEQET 362
 Human ILRTRIAASKTIDIR----EERTLTPISGGQRSSVPSVILKPENIKK----RDAKEAKA 368
 Drosophila SERRRSVRLASMDVDPLSLEEAQVQEPNAQGRKRLGVANGDIYHTPTKKSKEPLESAAATE 417
 Pombe SYIAHNLER-----IEDLLVEMEENLRPTKKSAG-----SRGR 228
 Cerevisiae SEDMDSGEISADELEEEDEEEDEEEDEEKEARHTNSPRKRGRKIKLGK----DDIDASV 323
 :

Mouse HKEPIRT-SRVHRKSSLLTLKRIRQQCLLDGDDR----DQEEE-----ESVDSESEEE 411
 Human QNEATSTPHRIRRKSSVLTMRIRQQQLRFLGNSKS----DQEEKEILPAAEISDSSSDEE 424
 Drosophila QTPSTRRKSILKSATSRLAEGTPRRSILHSNIVEQRFEDDEIISTPKRGRSKTVQDND 477
 Pombe GRPRKYPLPNVESKESSSKVNKDFDLQD-----DSESSED 266
 Cerevisiae QPPPKRGRKPKDPSKPRQMLLISSCRANNTPVIR-----KFTKNVARAK 369
 . : .

Mouse DEFISSLPTRNSLGQSR-----TRQTPSKSPQKNPKRTPHRATPQIRD----- 455
 Human EASTPPLRRAPRTVSRNLRSSSLKSLHTLTKVPKSLKPRTPRCAAPQIRS----- 476
 Drosophila EDYSPKKSQKTPTRTRRSSTTTKTATTPSKGITATATPMTPSQMKKIRAGELSPSMQ 537
 Pombe NLTIQPQTPRRRHKRSR-----HNSSNLASTPKRNGYKQPLQITPLIRM----- 311
 Cerevisiae KKYTPFSKRFKSIAAIPDLTSLPEFYGNSSSELMASRFENKLTQKHQIVET----- 421
 . : .

5.3 Final comments

An important goal for the future of the eukaryotic DNA replication field is the reconstitution *in vitro* of replication from an ARS. Pre-RC formation has been successfully achieved using both cell extracts and purified *S. cerevisiae* proteins (Seki and Diffley, 2000; Bowers et al., 2004; Kawasaki et al., 2006; Remus et al., submitted). The next step in this process is achieving quantitative phosphorylation of the Mcm2-7 subunits with Cdc7-Dbf4, as well as any other important substrates of the kinase. Identification of essential and non-essential Cdc7-Dbf4 substrates is therefore an important future goal of DNA replication research. In conjunction with Sld2 and Sld3 phosphorylation by Cdc28, phosphorylation of Cdc7-Dbf4 substrates will allow pre-RC 'activation' and will enable the subsequent assembly of downstream factors, DNA unwinding and replication initiation. Analysis of intermediates of this process, using mutant proteins and different combinations of downstream replication factors, will allow determination of the function of each component of the complex. Although much work lies ahead before the process can be fully understood, these are exciting times for research into DNA replication, as genetically identified components become increasingly well biochemically characterised.

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Appendices

1. Peptides present in the peptide array

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Appendix 1. Peptides on each membrane in the peptide array. Table 1 is reproduced, giving the positions of each of the proteins in the array. Peptide locations and sequences are then tabulated. The name of each protein is given at the location of the first peptide, and the membrane number at the top of every page. Potential kinase consensus motifs are indicated according to the colour scheme below:

Serine/threonine:	S/T
Minimal CDK consensus:	S/T-P
Full CDK consensus:	S/T-P-x-K/R
Cdc7 consensus:	S/T-D/E
CDK priming site:	S/T-S/T-P

Protein	Peptides	Array	Protein	Peptides	Array
Sld3	A1-B21	1	Orc2	A1-B17	5
Orc1	B23-E2		Orc3	B19-D5	
Orc4	E4-F13		Cdc7	D7-E14	
Orc5	F15-G20		Clb6	E16-F14	
Orc6	G22-H24		Cdc28	F16-G7	
Cdc6	H26-J4		Sld2	G9-H12	
Cdt1	J6-K21		Cdc45	H14-J2	
Mcm2	K23-M30		Dpb11	J4-L1	
Mcm3	N2-P15		Sld5	L3-L24	
Mcm4	P17-R27		Psf1	L26-M10	
Mcm5	R29-T27		Psf2	M12-M27	
Mcm6	A1-C18		Psf3	M29-N12	
Mcm7	C20-G15		Pol12	N14-P7	
Mcm10	G17-H29	Pri1	P9-Q9		
Dbf4	I1-J24	Pri2	Q11-R21		
Pol1	J26-N17	Pol31	R23-S29		
Pol2	N19-S25	Pol30	T1-T19		
Clb5	S27-T30	Rfa3	T21-T29		
Dna2	A1-D27	Pol3	A1-C24	6	
Mrc1	D29-G22	Rfa1	C26-E12		
Tof1	G24-J28	Rfa2	E14-F3		
Top1	J30-L28	Pol32	F5-F30		
Top2	L30-P18	Rfc1	G2-I7		
Top3	P20-R9	Rfc2	I9-J5		
Dpb2	R11-T3	Rfc4	J7-J30		
Rfc3	T5-T30	Rfc5	K2-K28		
Sgs1	A1-D21	Fen1	K30-L28		
Srs2	D23-G22	Csm3	L30-M23		
Smc5	G24-J17	Cdc9	M25-O22		
Smc6	J19-M13	Nse1	O24-P18		
Ctf4	M15-O25	Nse3	P20-Q12		
Ctf18	O27-Q22				
Elg1	Q24-S23				
Dpb3	S25-T9				
Dpb4	T11-T25				

Membrane 1

A 1 Sld3 M-E-T-W-E-V-I-A-S-V-K-E-A-T-K-G-L-D-L-S-L-D-H-P-L-I
 A 2 T-K-G-L-D-L-S-L-D-H-P-L-I-I-K-S-E-D-V-P-S-N-I-L-Q-L
 A 3 I-K-S-E-D-V-P-S-N-I-L-Q-L-L-Q-Q-K-N-R-R-Q-L-K-H-I-C
 A 4 L-Q-Q-K-N-R-R-Q-L-K-H-I-C-M-K-S-R-K-E-Y-F-L-L-E-E-Y
 A 5 M-K-S-R-K-E-Y-F-L-L-E-E-Y-G-P-G-F-W-V-K-W-P-Y-N-Y-F
 A 6 G-P-G-F-W-V-K-W-P-Y-N-Y-F-N-G-Y-S-L-P-E-R-R-T-E-V-V
 A 7 N-G-Y-S-L-P-E-R-R-T-E-V-V-T-T-V-E-R-E-R-A-K-R-E-T-L
 A 8 T-T-V-E-R-E-R-A-K-R-E-T-L-K-T-W-D-E-L-K-F-K-E-L-L-H
 A 9 K-T-W-D-E-L-K-F-K-E-L-L-H-L-W-S-E-E-P-K-G-S-C-K-L-E
 A10 L-W-S-E-E-P-K-G-S-C-K-L-E-K-D-K-D-L-K-L-D-M-N-P-P-D
 A11 K-D-K-D-L-K-L-D-M-N-P-P-D-M-K-G-E-S-K-I-N-D-Y-Y-S-D
 A12 M-K-G-E-S-K-I-N-D-Y-Y-S-D-P-K-E-Y-I-E-S-K-Y-Y-D-A-L
 A13 P-K-E-Y-I-E-S-K-Y-Y-D-A-L-F-S-I-H-T-P-L-A-Y-F-V-K-S
 A14 F-S-I-H-T-P-L-A-Y-F-V-K-S-N-L-V-R-L-K-N-T-C-R-T-K-Y
 A15 N-L-V-R-L-K-N-T-C-R-T-K-Y-G-S-D-S-Y-K-I-A-Y-Q-A-M-L
 A16 G-S-D-S-Y-K-I-A-Y-Q-A-M-L-Q-K-F-L-L-S-I-V-Q-F-K-D-R
 A17 Q-K-F-L-L-S-I-V-Q-F-K-D-R-H-D-N-R-L-L-L-E-P-F-S-S-P
 A18 H-D-N-R-L-L-L-E-P-F-S-S-P-I-A-D-E-K-R-K-N-C-L-T-K-F
 A19 I-A-D-E-K-R-K-N-C-L-T-K-F-V-I-Q-D-E-N-K-N-S-S-T-I-A
 A20 V-I-Q-D-E-N-K-N-S-S-T-I-A-D-L-C-V-V-L-K-S-R-E-I-K-L
 A21 D-L-C-V-V-L-K-S-R-E-I-K-L-Q-I-L-L-L-L-E-I-I-G-L-N-D
 A22 Q-I-L-L-L-L-E-I-I-G-L-N-D-L-D-W-N-F-R-D-F-E-K-K-Y-K
 A23 L-D-W-N-F-R-D-F-E-K-K-Y-K-L-K-L-K-K-R-S-L-N-L-T-K-K
 A24 L-K-L-K-K-R-S-L-N-L-T-K-K-G-L-V-R-R-R-S-K-K-K-T-S-E
 A25 G-L-V-R-R-R-S-K-K-K-T-S-E-K-D-K-G-I-E-R-I-T-T-S-L-D
 A26 K-D-K-G-I-E-R-I-T-T-S-L-D-Y-C-E-Q-L-D-L-Y-L-D-R-A-C
 A27 Y-C-E-Q-L-D-L-Y-L-D-R-A-C-I-L-D-I-L-L-S-S-E-T-P-N-P
 A28 I-L-D-I-L-L-S-S-E-T-P-N-P-D-A-I-E-A-S-N-G-T-I-Q-E-H
 A29 D-A-I-E-A-S-N-G-T-I-Q-E-H-K-K-N-I-L-D-K-S-K-E-A-S-L
 A30 K-K-N-I-L-D-K-S-K-E-A-S-L-V-G-F-I-N-Y-V-L-I-P-Y-F-N
 B 1 V-G-F-I-N-Y-V-L-I-P-Y-F-N-K-K-V-P-H-A-V-E-F-I-I-Q-K
 B 2 K-K-V-P-H-A-V-E-F-I-I-Q-K-L-K-G-P-S-M-R-P-K-R-A-L-K
 B 3 L-K-G-P-S-M-R-P-K-R-A-L-K-K-V-N-D-S-T-N-V-S-S-P-N-T
 B 4 K-V-N-D-S-T-N-V-S-S-P-N-T-V-E-T-Y-N-R-L-S-T-S-Q-R-A
 B 5 V-E-T-Y-N-R-L-S-T-S-Q-R-A-S-R-S-S-I-I-N-S-V-P-S-S-P
 B 6 S-R-S-S-I-I-N-S-V-P-S-S-P-A-L-R-R-V-D-A-N-L-F-S-R-K
 B 7 A-L-R-R-V-D-A-N-L-F-S-R-K-S-I-A-S-P-T-P-E-L-L-N-S-R
 B 8 S-I-A-S-P-T-P-E-L-L-N-S-R-T-N-S-N-L-N-E-F-L-E-S-E-T
 B 9 T-N-S-N-L-N-E-F-L-E-S-E-T-R-S-L-K-R-P-S-Q-L-G-R-T-K
 B10 R-S-L-K-R-P-S-Q-L-G-R-T-K-S-D-L-T-M-N-H-L-Q-K-R-Q-F
 B11 S-D-L-T-M-N-H-L-Q-K-R-Q-F-S-V-S-D-L-S-T-T-R-V-P-N-S
 B12 S-V-S-D-L-S-T-T-R-V-P-N-S-S-T-I-T-L-K-T-P-F-S-H-S-T
 B13 S-T-I-T-L-K-T-P-F-S-H-S-T-I-N-A-Y-K-T-M-N-N-S-F-R-R
 B14 I-N-A-Y-K-T-M-N-N-S-F-R-R-V-G-K-R-K-D-I-N-E-T-I-R-L
 B15 V-G-K-R-K-D-I-N-E-T-I-R-L-H-E-R-V-D-S-E-E-N-V-Q-V-Q
 B16 H-E-R-V-D-S-E-E-N-V-Q-V-Q-A-T-P-A-V-K-K-R-T-V-T-P-N
 B17 A-T-P-A-V-K-K-R-T-V-T-P-N-K-K-A-Q-L-Q-S-I-I-E-S-P-L

B18 K-K-A-Q-L-Q-S-I-I-E-S-P-L-N-F-K-D-D-D-T-H-E-G-R-K-N
 B19 N-F-K-D-D-D-T-H-E-G-R-K-N-T-S-N-I-T-S-T-P-T-N-K-P-P
 B20 T-S-N-I-T-S-T-P-T-N-K-P-P-E-N-S-S-K-R-R-V-R-R-R-L-F
 B21 S-T-P-T-N-K-P-P-E-N-S-S-K-R-R-V-R-R-R-L-F-A-P-E-S-T
 B22
 B23 Orc1 M-A-K-T-L-K-D-L-Q-G-W-E-I-I-T-T-D-E-Q-G-N-I-I-D-G-G
 B24 I-T-T-D-E-Q-G-N-I-I-D-G-G-Q-K-R-L-R-R-R-G-A-K-T-E-H
 B25 Q-K-R-L-R-R-R-G-A-K-T-E-H-Y-L-K-R-S-S-D-G-I-K-L-G-R
 B26 Y-L-K-R-S-S-D-G-I-K-L-G-R-G-D-S-V-V-M-H-N-E-A-A-G-T
 B27 G-D-S-V-V-M-H-N-E-A-A-G-T-Y-S-V-Y-M-I-Q-E-L-R-L-N-T
 B28 Y-S-V-Y-M-I-Q-E-L-R-L-N-T-L-N-N-V-V-E-L-W-A-L-T-Y-L
 B29 L-N-N-V-V-E-L-W-A-L-T-Y-L-R-W-F-E-V-N-P-L-A-H-Y-R-Q
 B30 R-W-F-E-V-N-P-L-A-H-Y-R-Q-F-N-P-D-A-N-I-L-N-R-P-L-N
 C 1 F-N-P-D-A-N-I-L-N-R-P-L-N-Y-Y-N-K-L-F-S-E-T-A-N-K-N
 C 2 Y-Y-N-K-L-F-S-E-T-A-N-K-N-E-L-Y-L-T-A-E-L-A-E-L-Q-L
 C 3 E-L-Y-L-T-A-E-L-A-E-L-Q-L-F-N-F-I-R-V-A-N-V-M-D-G-S
 C 4 F-N-F-I-R-V-A-N-V-M-D-G-S-K-W-E-V-L-K-G-N-V-D-P-E-R
 C 5 K-W-E-V-L-K-G-N-V-D-P-E-R-D-F-T-V-R-Y-I-C-E-P-T-G-E
 C 6 D-F-T-V-R-Y-I-C-E-P-T-G-E-K-F-V-D-I-N-I-E-D-V-K-A-Y
 C 7 K-F-V-D-I-N-I-E-D-V-K-A-Y-I-K-K-V-E-P-R-E-A-Q-E-Y-L
 C 8 I-K-K-V-E-P-R-E-A-Q-E-Y-L-K-D-L-T-L-P-S-K-K-K-E-I-K
 C 9 K-D-L-T-L-P-S-K-K-K-E-I-K-R-G-P-Q-K-K-D-K-A-T-Q-T-A
 C10 R-G-P-Q-K-K-D-K-A-T-Q-T-A-Q-I-S-D-A-E-T-R-A-T-D-I-T
 C11 Q-I-S-D-A-E-T-R-A-T-D-I-T-D-N-E-D-G-N-E-D-E-S-S-D-Y
 C12 D-N-E-D-G-N-E-D-E-S-S-D-Y-E-S-P-S-D-I-D-V-S-E-D-M-D
 C13 E-S-P-S-D-I-D-V-S-E-D-M-D-S-G-E-I-S-A-D-E-L-E-E-E-E
 C14 S-G-E-I-S-A-D-E-L-E-E-E-E-D-E-E-E-D-E-D-E-E-E-K-E-A
 C15 D-E-E-E-D-E-D-E-E-E-K-E-A-R-H-T-N-S-P-R-K-R-G-R-K-I
 C16 R-H-T-N-S-P-R-K-R-G-R-K-I-K-L-G-K-D-D-I-D-A-S-V-Q-P
 C17 K-L-G-K-D-D-I-D-A-S-V-Q-P-P-P-K-K-R-G-R-K-P-K-D-P-S
 C18 P-P-K-K-R-G-R-K-P-K-D-P-S-K-P-R-Q-M-L-L-I-S-S-C-R-A
 C19 K-P-R-Q-M-L-L-I-S-S-C-R-A-N-N-T-P-V-I-R-K-F-T-K-K-N
 C20 N-N-T-P-V-I-R-K-F-T-K-K-N-V-A-R-A-K-K-K-Y-T-P-F-S-K
 C21 V-A-R-A-K-K-K-Y-T-P-F-S-K-R-F-K-S-I-A-A-I-P-D-L-T-S
 C22 R-F-K-S-I-A-A-I-P-D-L-T-S-L-P-E-F-Y-G-N-S-S-E-L-M-A
 C23 L-P-E-F-Y-G-N-S-S-E-L-M-A-S-R-F-E-N-K-L-K-T-T-Q-K-H
 C24 S-R-F-E-N-K-L-K-T-T-Q-K-H-Q-I-V-E-T-I-F-S-K-V-K-K-Q
 C25 Q-I-V-E-T-I-F-S-K-V-K-K-Q-L-N-S-S-Y-V-K-E-E-I-L-K-S
 C26 L-N-S-S-Y-V-K-E-E-I-L-K-S-A-N-F-Q-D-Y-L-P-A-R-E-N-E
 C27 A-N-F-Q-D-Y-L-P-A-R-E-N-E-F-A-S-I-Y-L-S-A-Y-S-A-I-E
 C28 F-A-S-I-Y-L-S-A-Y-S-A-I-E-S-D-S-A-T-T-I-Y-V-A-G-T-P
 C29 S-D-S-A-T-T-I-Y-V-A-G-T-P-G-V-G-K-T-L-T-V-R-E-V-V-K
 C30 G-V-G-K-T-L-T-V-R-E-V-V-K-E-L-L-S-S-S-A-Q-R-E-I-P-D
 D 1 E-L-L-S-S-S-A-Q-R-E-I-P-D-F-L-Y-V-E-I-N-G-L-K-M-V-K
 D 2 F-L-Y-V-E-I-N-G-L-K-M-V-K-P-T-D-C-Y-E-T-L-W-N-K-V-S
 D 3 P-T-D-C-Y-E-T-L-W-N-K-V-S-G-E-R-L-T-W-A-A-S-M-E-S-L
 D 4 G-E-R-L-T-W-A-A-S-M-E-S-L-E-F-Y-F-K-R-V-P-K-N-K-K-K
 D 5 E-F-Y-F-K-R-V-P-K-N-K-K-K-T-I-V-V-L-L-D-E-L-D-A-M-V
 D 6 T-I-V-V-L-L-D-E-L-D-A-M-V-T-K-S-Q-D-I-M-Y-N-F-F-N-W
 D 7 T-K-S-Q-D-I-M-Y-N-F-F-N-W-T-T-Y-E-N-A-K-L-I-V-I-A-V
 D 8 T-T-Y-E-N-A-K-L-I-V-I-A-V-A-N-T-M-D-L-P-E-R-Q-L-G-N

D 9 A-N-T-M-D-L-P-E-R-Q-L-G-N-K-I-T-S-R-I-G-F-T-R-I-M-F
D10 K-I-T-S-R-I-G-F-T-R-I-M-F-T-G-Y-T-H-E-E-L-K-N-I-I-D
D11 T-G-Y-T-H-E-E-L-K-N-I-I-D-L-R-L-K-G-L-N-D-S-F-F-Y-V
D12 L-R-L-K-G-L-N-D-S-F-F-Y-V-D-T-K-T-G-N-A-I-L-I-D-A-A
D13 D-T-K-T-G-N-A-I-L-I-D-A-A-G-N-D-T-T-V-K-Q-T-L-P-E-D
D14 G-N-D-T-T-V-K-Q-T-L-P-E-D-V-R-K-V-R-L-R-M-S-A-D-A-I
D15 V-R-K-V-R-L-R-M-S-A-D-A-I-E-I-A-S-R-K-V-A-S-V-S-G-D
D16 E-I-A-S-R-K-V-A-S-V-S-G-D-A-R-R-A-L-K-V-C-K-R-A-A-E
D17 A-R-R-A-L-K-V-C-K-R-A-A-E-I-A-E-K-H-Y-M-A-K-H-G-Y-G
D18 I-A-E-K-H-Y-M-A-K-H-G-Y-G-Y-D-G-K-T-V-I-E-D-E-N-E-E
D19 Y-D-G-K-T-V-I-E-D-E-N-E-E-Q-I-Y-D-D-E-D-K-D-L-I-E-S
D20 Q-I-Y-D-D-E-D-K-D-L-I-E-S-N-K-A-K-D-D-N-D-D-D-D-N
D21 N-K-A-K-D-D-N-D-D-D-D-N-D-G-V-Q-T-V-H-I-T-H-V-M-K
D22 D-G-V-Q-T-V-H-I-T-H-V-M-K-A-L-N-E-T-L-N-S-H-V-I-T-F
D23 A-L-N-E-T-L-N-S-H-V-I-T-F-M-T-R-L-S-F-T-A-K-L-F-I-Y
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D26 Q-E-L-G-D-I-V-D-E-I-K-L-L-I-E-V-N-G-S-N-K-F-V-M-E-I
D27 I-E-V-N-G-S-N-K-F-V-M-E-I-A-K-T-L-F-Q-Q-G-S-D-N-I-S
D28 A-K-T-L-F-Q-Q-G-S-D-N-I-S-E-Q-L-R-I-I-S-W-D-F-V-L-N
D29 E-Q-L-R-I-I-S-W-D-F-V-L-N-Q-L-L-D-A-G-I-L-F-K-Q-T-M
D30 Q-L-L-D-A-G-I-L-F-K-Q-T-M-K-N-D-R-I-C-C-V-K-L-N-I-S
E 1 K-N-D-R-I-C-C-V-K-L-N-I-S-V-E-E-A-K-R-A-M-N-E-D-E-T
E 2 I-C-C-V-K-L-N-I-S-V-E-E-A-K-R-A-M-N-E-D-E-T-L-R-N-L
E 3
E 4 Orc4 M-T-I-S-E-A-R-L-S-P-Q-V-N-L-L-P-I-K-R-H-S-N-E-E-V-E
E 5 L-L-P-I-K-R-H-S-N-E-E-V-E-E-T-A-A-I-L-K-K-R-T-I-D-N
E 6 E-T-A-A-I-L-K-K-R-T-I-D-N-E-K-C-K-D-S-D-P-G-F-G-S-L
E 7 E-K-C-K-D-S-D-P-G-F-G-S-L-Q-R-R-L-L-Q-Q-L-Y-G-T-L-P
E 8 Q-R-R-L-L-Q-Q-L-Y-G-T-L-P-T-D-E-K-I-I-F-T-Y-L-Q-D-C
E 9 T-D-E-K-I-I-F-T-Y-L-Q-D-C-Q-Q-E-I-D-R-I-I-K-Q-S-I-I
E10 Q-Q-E-I-D-R-I-I-K-Q-S-I-I-Q-K-E-S-H-S-V-I-L-V-G-P-R
E11 Q-K-E-S-H-S-V-I-L-V-G-P-R-Q-S-Y-K-T-Y-L-L-D-Y-E-L-S
E12 Q-S-Y-K-T-Y-L-L-D-Y-E-L-S-L-L-Q-Q-S-Y-K-E-Q-F-I-T-I
E13 L-L-Q-Q-S-Y-K-E-Q-F-I-T-I-R-L-N-G-F-I-H-S-E-Q-T-A-I
E14 R-L-N-G-F-I-H-S-E-Q-T-A-I-N-G-I-A-T-Q-L-E-Q-Q-L-Q-K
E15 N-G-I-A-T-Q-L-E-Q-Q-L-Q-K-I-H-G-S-E-E-K-I-D-D-T-S-L
E16 I-H-G-S-E-E-K-I-D-D-T-S-L-E-T-I-S-S-G-S-L-T-E-V-F-E
E17 E-T-I-S-S-G-S-L-T-E-V-F-E-K-I-L-L-L-L-D-S-T-T-K-T-R
E18 K-I-L-L-L-L-D-S-T-T-K-T-R-N-E-D-S-G-E-V-D-R-E-S-I-T
E19 N-E-D-S-G-E-V-D-R-E-S-I-T-K-I-T-V-V-F-I-F-D-E-I-D-T
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E21 F-A-G-P-V-R-Q-T-L-L-Y-N-L-F-D-M-V-E-H-S-R-V-P-V-C-I
E22 F-D-M-V-E-H-S-R-V-P-V-C-I-F-G-C-T-T-K-L-N-I-L-E-Y-L
E23 F-G-C-T-T-K-L-N-I-L-E-Y-L-E-K-R-V-K-S-R-F-S-Q-R-V-I
E24 E-K-R-V-K-S-R-F-S-Q-R-V-I-Y-M-P-Q-I-Q-N-L-D-D-M-V-D
E25 Y-M-P-Q-I-Q-N-L-D-D-M-V-D-A-V-R-N-L-L-T-V-R-S-E-I-S
E26 A-V-R-N-L-L-T-V-R-S-E-I-S-P-W-V-S-Q-W-N-E-T-L-E-K-E
E27 P-W-V-S-Q-W-N-E-T-L-E-K-E-L-S-D-P-R-S-N-L-N-R-H-I-R
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F 1 G-S-L-C-T-A-I-K-S-C-S-F-L-D-I-Y-N-K-N-Q-L-S-N-N-L-T
F 2 D-I-Y-N-K-N-Q-L-S-N-N-L-T-G-R-L-Q-S-L-S-D-L-E-L-A-I
F 3 G-R-L-Q-S-L-S-D-L-E-L-A-I-L-I-S-A-A-R-V-A-L-R-A-K-D
F 4 L-I-S-A-A-R-V-A-L-R-A-K-D-G-S-F-N-F-N-L-A-Y-A-E-Y-E
F 5 G-S-F-N-F-N-L-A-Y-A-E-Y-E-K-M-I-K-A-I-N-S-R-I-P-T-V
F 6 K-M-I-K-A-I-N-S-R-I-P-T-V-A-P-T-T-N-V-G-T-G-Q-S-T-F
F 7 A-P-T-T-N-V-G-T-G-Q-S-T-F-S-I-D-N-T-I-K-L-W-L-K-K-D
F 8 S-I-D-N-T-I-K-L-W-L-K-K-D-V-K-N-V-W-E-N-L-V-Q-L-D-F
F 9 V-K-N-V-W-E-N-L-V-Q-L-D-F-F-T-E-K-S-A-V-G-L-R-D-N-A
F10 F-T-E-K-S-A-V-G-L-R-D-N-A-T-A-A-F-Y-A-S-N-Y-Q-F-Q-G
F11 T-A-A-F-Y-A-S-N-Y-Q-F-Q-G-T-M-I-P-F-D-L-R-S-Y-Q-M-Q
F12 T-M-I-P-F-D-L-R-S-Y-Q-M-Q-I-I-L-Q-E-L-R-R-I-I-P-K-S
F13 Y-Q-M-Q-I-I-L-Q-E-L-R-R-I-I-P-K-S-N-M-Y-Y-S-W-T-Q-L
F14
F15 Orc5 M-N-V-T-T-P-E-V-A-F-R-E-Y-Q-T-N-C-L-A-S-Y-I-S-A-D-P
F16 Q-T-N-C-L-A-S-Y-I-S-A-D-P-D-I-T-P-S-N-L-I-L-Q-G-Y-S
F17 D-I-T-P-S-N-L-I-L-Q-G-Y-S-G-T-G-K-T-Y-T-L-K-K-Y-F-N
F18 G-T-G-K-T-Y-T-L-K-K-Y-F-N-A-N-P-N-L-H-A-V-W-L-E-P-V
F19 A-N-P-N-L-H-A-V-W-L-E-P-V-E-L-V-S-W-K-P-L-L-Q-A-I-A
F20 E-L-V-S-W-K-P-L-L-Q-A-I-A-R-T-V-Q-Y-K-L-K-T-L-Y-P-N
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F26 A-L-F-N-K-Y-I-K-L-N-E-L-L-P-K-D-S-K-I-N-I-K-F-I-Y-T
F27 P-K-D-S-K-I-N-I-K-F-I-Y-T-M-L-E-T-S-F-L-Q-R-Y-S-T-H
F28 M-L-E-T-S-F-L-Q-R-Y-S-T-H-C-I-P-T-V-M-F-P-R-Y-N-V-D
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G 1 L-M-E-D-S-C-L-R-K-R-I-I-E-E-Q-I-T-D-C-T-D-D-Q-F-Q-N
G 2 E-Q-I-T-D-C-T-D-D-Q-F-Q-N-V-A-A-N-F-I-H-L-I-V-Q-A-F
G 3 V-A-A-N-F-I-H-L-I-V-Q-A-F-H-S-Y-T-G-N-D-I-F-A-L-N-D
G 4 H-S-Y-T-G-N-D-I-F-A-L-N-D-L-I-D-F-K-W-P-K-Y-V-S-R-I
G 5 L-I-D-F-K-W-P-K-Y-V-S-R-I-T-K-E-N-I-F-E-P-L-A-L-Y-K
G 6 T-K-E-N-I-F-E-P-L-A-L-Y-K-S-A-I-K-L-F-L-S-T-D-D-N-L
G 7 S-A-I-K-L-F-L-S-T-D-D-N-L-S-E-N-G-Q-G-E-S-A-I-T-T-N
G 8 S-E-N-G-Q-G-E-S-A-I-T-T-N-R-D-D-L-E-N-S-Q-T-Y-D-L-S
G 9 R-D-D-L-E-N-S-Q-T-Y-D-L-S-I-I-S-K-Y-L-L-I-A-S-Y-I-C
G10 I-I-S-K-Y-L-L-I-A-S-Y-I-C-S-Y-L-E-P-R-Y-D-A-S-I-F-S
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G14 P-S-L-F-A-I-E-R-L-L-A-I-F-Q-A-I-F-P-I-Q-G-K-A-E-S-G
G15 Q-A-I-F-P-I-Q-G-K-A-E-S-G-S-L-S-A-L-R-E-E-S-L-M-K-A
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G18 L-K-L-I-A-T-T-M-N-K-N-I-D-Y-L-S-P-K-V-R-W-K-V-N-V-P
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G21
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H 1 K-K-N-K-R-S-P-V-K-N-G-G-R-F-T-S-S-D-P-K-E-L-R-N-Q-L
H 2 F-T-S-S-D-P-K-E-L-R-N-Q-L-F-G-T-P-T-K-V-R-K-S-Q-N-N
H 3 F-G-T-P-T-K-V-R-K-S-Q-N-N-D-S-F-V-I-P-E-L-P-P-M-Q-T
H 4 D-S-F-V-I-P-E-L-P-P-M-Q-T-N-E-S-P-S-I-T-R-R-K-L-A-F
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H 6 E-E-D-E-D-E-D-E-E-E-P-G-N-D-G-L-S-L-K-S-H-S-N-K-S-I
H 7 D-G-L-S-L-K-S-H-S-N-K-S-I-T-G-T-R-N-V-D-S-D-E-Y-E-N
H 8 T-G-T-R-N-V-D-S-D-E-Y-E-N-H-E-S-D-P-T-S-E-E-E-P-L-G
H 9 H-E-S-D-P-T-S-E-E-E-P-L-G-V-Q-E-S-R-S-G-R-T-K-Q-N-K
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H14 R-L-C-N-D-F-E-L-P-R-E-V-A-Y-K-I-V-D-E-Y-N-I-N-A-S-R
H15 Y-K-I-V-D-E-Y-N-I-N-A-S-R-L-V-C-P-W-Q-L-V-C-G-L-V-L
H16 L-V-C-P-W-Q-L-V-C-G-L-V-L-N-C-T-F-I-V-F-N-E-R-R-R-K
H17 N-C-T-F-I-V-F-N-E-R-R-R-K-D-P-R-I-D-H-F-I-V-S-K-M-C
H18 D-P-R-I-D-H-F-I-V-S-K-M-C-S-L-M-L-T-S-K-V-D-D-V-I-E
H19 S-L-M-L-T-S-K-V-D-D-V-I-E-C-V-K-L-V-K-E-L-I-I-G-E-K
H20 C-V-K-L-V-K-E-L-I-I-G-E-K-W-F-R-D-L-Q-I-R-Y-D-D-F-D
H21 W-F-R-D-L-Q-I-R-Y-D-D-F-D-G-I-R-Y-D-E-I-I-F-R-K-L-G
H22 G-I-R-Y-D-E-I-I-F-R-K-L-G-S-M-L-Q-T-T-N-I-L-V-T-D-D
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H25
H26 Cdc6 M-S-A-I-P-I-T-P-T-K-R-I-R-R-N-L-F-D-D-A-P-A-T-P-P-R
H27 R-N-L-F-D-D-A-P-A-T-P-P-R-P-L-K-R-K-K-L-Q-F-T-D-V-T
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 J 2 G-L-V-S-I-K-K-T-K-C-K-G-K-T-K-R-F-V-D-K-I-D-V-D-L-D
 J3 T-K-R-F-V-D-K-I-D-V-D-L-D-M-R-E-F-Y-D-E-M-T-K-I-S-I
 J 4 K-I-D-V-D-L-D-M-R-E-F-Y-D-E-M-T-K-I-S-I-L-K-P-F-L-H
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 J 7 R-V-P-V-I-D-L-N-R-V-S-D-E-E-Q-L-L-P-V-V-R-A-I-L-L-Q
 J 8 E-Q-L-L-P-V-V-R-A-I-L-L-Q-H-D-T-F-L-L-K-N-Y-A-N-K-A
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 K 1 S-L-N-A-L-S-R-S-T-G-V-P-P-E-L-H-V-L-L-P-Q-I-S-S-M-M
 K 2 E-L-H-V-L-L-P-Q-I-S-S-M-M-K-R-K-I-V-Q-D-D-I-L-K-L-L

K 3 K-R-K-I-V-Q-D-D-I-L-K-L-L-T-I-W-S-D-A-Y-V-V-E-L-N-S
 K 4 T-I-W-S-D-A-Y-V-V-E-L-N-S-R-G-E-L-T-M-N-L-P-K-R-D-N
 K 5 R-G-E-L-T-M-N-L-P-K-R-D-N-L-T-T-L-T-N-K-S-R-T-L-A-F
 K 6 L-T-T-L-T-N-K-S-R-T-L-A-F-V-E-R-A-E-S-W-Y-Q-Q-V-I-A
 K 7 V-E-R-A-E-S-W-Y-Q-Q-V-I-A-S-K-D-E-I-M-T-D-V-P-A-F-K
 K 8 S-K-D-E-I-M-T-D-V-P-A-F-K-I-N-K-R-R-S-S-S-N-S-K-T-V
 K 9 I-N-K-R-R-S-S-S-N-S-K-T-V-L-S-S-K-V-Q-T-K-S-S-N-A-N
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 L 3 D-E-D-E-E-Q-E-G-A-A-Q-L-D-E-M-G-L-P-V-Q-R-R-R-R-R-R
 L 4 E-M-G-L-P-V-Q-R-R-R-R-R-R-Q-Y-E-D-L-E-N-T-D-D-D-L-L
 L 5 Q-Y-E-D-L-E-N-T-D-D-D-L-L-S-D-I-H-I-D-P-L-R-E-E-L-T
 L 6 S-D-I-H-I-D-P-L-R-E-E-L-T-L-E-S-L-S-N-V-K-A-N-S-Y-S
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 M 1 D-K-I-I-S-S-M-A-P-S-I-Y-G-H-R-D-I-K-T-A-V-A-C-S-L-F
 M 2 H-R-D-I-K-T-A-V-A-C-S-L-F-G-G-V-P-K-N-V-N-P-K-H-S-I
 M 3 G-G-V-P-K-N-V-N-P-K-H-S-I-R-G-D-I-N-V-L-L-L-G-D-P-G
 M 4 R-G-D-I-N-V-L-L-L-G-D-P-G-T-A-K-S-Q-I-L-K-Y-V-E-K-T
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 M16 E-A-D-E-R-L-A-T-F-V-V-D-S-H-V-R-S-H-P-E-N-D-Q-D-R-E
 M17 H-V-R-S-H-P-E-N-D-Q-D-R-E-G-E-E-L-K-N-N-G-E-S-A-I-E
 M18 G-E-E-L-K-N-N-G-E-S-A-I-E-Q-G-E-D-Q-R-K-K-E-E-E-I-S
 M19 Q-G-E-D-Q-R-K-K-E-E-E-I-S-P-I-P-Q-E-L-L-M-K-Y-I-H-Y
 M20 P-I-P-Q-E-L-L-M-K-Y-I-H-Y-A-R-T-K-I-Y-P-K-L-H-Q-M-D
 M21 A-R-T-K-I-Y-P-K-L-H-Q-M-D-M-D-K-V-S-R-V-Y-A-D-L-R-R
 M22 M-D-K-V-S-R-V-Y-A-D-L-R-R-E-S-I-S-T-G-S-F-P-I-T-V-R
 M23 E-S-I-S-T-G-S-F-P-I-T-V-R-H-L-E-S-I-L-R-I-A-E-S-F-A
 M24 H-L-E-S-I-L-R-I-A-E-S-F-A-K-M-R-L-S-E-F-V-S-S-Y-D-L
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 M29 L-R-I-S-G-Y-E-D-K-K-F-S-V-S-I-H-V-L-A-I-L-F-S-I-Y-K
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 N 1
 N 2 Mcm3 M-E-G-S-T-G-F-D-G-D-A-T-T-F-F-A-P-D-A-V-F-G-D-R-V-R
 N 3 F-F-A-P-D-A-V-F-G-D-R-V-R-R-F-Q-E-F-L-D-T-F-T-S-Y-R
 N 4 R-F-Q-E-F-L-D-T-F-T-S-Y-R-D-S-V-R-S-I-Q-V-Y-N-S-N-N
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 N23 I-D-V-I-L-D-D-D-L-V-D-K-T-K-P-G-D-R-V-N-V-V-G-V-F-K
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P16
P17 Mcm4 M-S-Q-Q-S-S-S-P-T-K-E-D-N-N-S-S-S-P-V-V-P-N-P-D-S-V
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P22 I-G-S-S-P-L-N-F-P-S-S-S-Q-R-Q-N-S-D-V-F-Q-S-Q-G-R-Q
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P26 T-S-S-S-S-L-G-R-N-G-Q-N-R-V-H-M-R-R-N-D-I-H-T-S-D-L
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Q 1 T-N-V-S-I-Q-E-C-T-T-N-F-R-N-F-L-M-S-F-K-Y-K-F-R-K-I
Q 2 N-F-L-M-S-F-K-Y-K-F-R-K-I-L-D-E-R-E-E-F-I-N-N-T-T-D
Q 3 L-D-E-R-E-E-F-I-N-N-T-T-D-E-E-L-Y-Y-I-K-Q-L-N-E-M-R
Q 4 E-E-L-Y-Y-I-K-Q-L-N-E-M-R-E-L-G-T-S-N-L-N-L-D-A-R-N
Q 5 E-L-G-T-S-N-L-N-L-D-A-R-N-L-L-A-Y-K-Q-T-E-D-L-Y-H-Q
Q 6 L-L-A-Y-K-Q-T-E-D-L-Y-H-Q-L-L-N-Y-P-Q-E-V-I-S-I-M-D
Q 7 L-L-N-Y-P-Q-E-V-I-S-I-M-D-Q-T-I-K-D-C-M-V-S-L-I-V-D
Q 8 Q-T-I-K-D-C-M-V-S-L-I-V-D-N-N-L-D-Y-D-L-D-E-I-E-T-K
Q 9 N-N-L-D-Y-D-L-D-E-I-E-T-K-F-Y-K-V-R-P-Y-N-V-G-S-C-K
Q10 F-Y-K-V-R-P-Y-N-V-G-S-C-K-G-M-R-E-L-N-P-N-D-I-D-K-L
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 R 2 P-R-G-V-Y-T-S-G-K-G-S-S-A-V-G-L-T-A-Y-I-T-R-D-V-D-T
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 R12 N-D-R-E-L-A-K-H-L-T-N-L-Y-L-E-D-K-P-E-H-I-S-Q-D-D-V
 R13 L-E-D-K-P-E-H-I-S-Q-D-D-V-L-P-V-E-F-L-T-M-Y-I-S-Y-A
 R14 L-P-V-E-F-L-T-M-Y-I-S-Y-A-K-E-H-I-H-P-I-I-T-E-A-A-K
 R15 K-E-H-I-H-P-I-I-T-E-A-A-K-T-E-L-V-R-A-Y-V-G-M-R-K-M
 R16 T-E-L-V-R-A-Y-V-G-M-R-K-M-G-D-D-S-R-S-D-E-K-R-I-T-A
 R17 G-D-D-S-R-S-D-E-K-R-I-T-A-T-T-R-Q-L-E-S-M-I-R-L-A-E
 R18 T-T-R-Q-L-E-S-M-I-R-L-A-E-A-H-A-K-M-K-L-K-N-V-V-E-L
 R19 A-H-A-K-M-K-L-K-N-V-V-E-L-E-D-V-Q-E-A-V-R-L-I-R-S-A
 R20 E-D-V-Q-E-A-V-R-L-I-R-S-A-I-K-D-Y-A-T-D-P-K-T-G-K-I
 R21 I-K-D-Y-A-T-D-P-K-T-G-K-I-D-M-N-L-V-Q-T-G-K-S-V-I-Q
 R22 D-M-N-L-V-Q-T-G-K-S-V-I-Q-R-K-L-Q-E-D-L-S-R-E-I-M-N
 R23 R-K-L-Q-E-D-L-S-R-E-I-M-N-V-L-K-D-Q-A-S-D-S-M-S-F-N
 R24 V-L-K-D-Q-A-S-D-S-M-S-F-N-E-L-I-K-Q-I-N-E-H-S-Q-D-R
 R25 E-L-I-K-Q-I-N-E-H-S-Q-D-R-V-E-S-S-D-I-Q-E-A-L-S-R-L
 R26 V-E-S-S-D-I-Q-E-A-L-S-R-L-Q-Q-E-D-K-V-I-V-L-G-E-G-V
 R27 S-R-L-Q-Q-E-D-K-V-I-V-L-G-E-G-V-R-R-S-V-R-L-N-N-R-V
 R28
 R29 Mcm5 M-S-F-D-R-P-E-I-Y-S-A-P-V-L-Q-G-E-S-P-N-D-D-D-N-T-E
 R30 L-Q-G-E-S-P-N-D-D-D-N-T-E-I-I-K-S-F-K-N-F-I-L-E-F-R
 S 1 I-I-K-S-F-K-N-F-I-L-E-F-R-L-D-S-Q-F-I-Y-R-D-Q-L-R-N
 S 2 L-D-S-Q-F-I-Y-R-D-Q-L-R-N-N-I-L-V-K-N-Y-S-L-T-V-N-M
 S 3 N-I-L-V-K-N-Y-S-L-T-V-N-M-E-H-L-I-G-Y-N-E-D-I-Y-K-K
 S 4 E-H-L-I-G-Y-N-E-D-I-Y-K-K-L-S-D-E-P-S-D-T-I-P-L-F-E
 S 5 L-S-D-E-P-S-D-T-I-P-L-F-E-T-A-I-T-Q-V-A-K-R-I-S-I-L
 S 6 T-A-I-T-Q-V-A-K-R-I-S-I-L-S-R-A-Q-S-A-N-N-N-D-K-D-P
 S 7 S-R-A-Q-S-A-N-N-N-D-K-D-P-E-N-T-S-M-D-T-D-S-L-L-L-N
 S 8 E-N-T-S-M-D-T-D-S-L-L-L-N-S-L-P-T-F-Q-L-I-L-N-S-N-A
 S 9 S-L-P-T-F-Q-L-I-L-N-S-N-A-N-Q-I-P-L-R-D-L-D-S-E-H-V
 S10 N-Q-I-P-L-R-D-L-D-S-E-H-V-S-K-I-V-R-L-S-G-I-I-I-S-T
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 S12 S-V-L-S-S-R-A-T-Y-L-S-I-M-C-R-N-C-R-H-T-T-S-I-T-I-N
 S13 C-R-N-C-R-H-T-T-S-I-T-I-N-N-F-N-S-I-T-G-N-T-V-S-L-P
 S14 N-F-N-S-I-T-G-N-T-V-S-L-P-R-S-C-L-S-T-I-E-S-E-S-S-M
 S15 R-S-C-L-S-T-I-E-S-E-S-S-M-A-N-E-S-N-I-G-D-E-S-T-K-K
 S16 A-N-E-S-N-I-G-D-E-S-T-K-K-N-C-G-P-D-P-Y-I-I-I-H-E-S

S17 N-C-G-P-D-P-Y-I-I-I-H-E-S-S-K-F-I-D-Q-Q-F-L-K-L-Q-E
 S18 S-K-F-I-D-Q-Q-F-L-K-L-Q-E-I-P-E-L-V-P-V-G-E-M-P-R-N
 S19 I-P-E-L-V-P-V-G-E-M-P-R-N-L-T-M-T-C-D-R-Y-L-T-N-K-V
 S20 L-T-M-T-C-D-R-Y-L-T-N-K-V-I-P-G-T-R-V-T-I-V-G-I-Y-S
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 S23 G-G-G-N-G-G-S-G-V-A-I-R-T-P-Y-I-K-I-L-G-I-Q-S-D-V-E
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 S25 T-S-S-I-W-N-S-V-T-M-F-T-E-E-E-E-E-E-F-L-Q-L-S-R-N-P
 S26 E-E-E-E-E-F-L-Q-L-S-R-N-P-K-L-Y-E-I-L-T-N-S-I-A-P-S
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 S28 I-F-G-N-E-D-I-K-K-A-I-V-C-L-L-M-G-G-S-K-K-I-L-P-D-G
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 T 1 D-P-G-T-A-K-S-Q-L-L-K-F-V-E-K-V-S-P-I-A-V-Y-T-S-G-K
 T 2 E-K-V-S-P-I-A-V-Y-T-S-G-K-G-S-S-A-A-G-L-T-A-S-V-Q-R
 T 3 G-S-S-A-A-G-L-T-A-S-V-Q-R-D-P-M-T-R-E-F-Y-L-E-G-G-A
 T 4 D-P-M-T-R-E-F-Y-L-E-G-G-A-M-V-L-A-D-G-G-V-V-C-I-D-E
 T 5 M-V-L-A-D-G-G-V-V-C-I-D-E-F-D-K-M-R-D-E-D-R-V-A-I-H
 T 6 F-D-K-M-R-D-E-D-R-V-A-I-H-E-A-M-E-Q-Q-T-I-S-I-A-K-A
 T 7 E-A-M-E-Q-Q-T-I-S-I-A-K-A-G-I-T-T-V-L-N-S-R-T-S-V-L
 T 8 G-I-T-T-V-L-N-S-R-T-S-V-L-A-A-A-N-P-I-Y-G-R-Y-D-D-L
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 T12 H-N-E-E-R-D-I-S-I-A-N-H-V-I-N-I-H-T-G-N-A-N-A-M-Q-N
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 T14 Q-Q-E-E-N-G-S-E-I-S-I-E-K-M-K-R-Y-I-I-T-Y-C-R-L-K-C
 T15 M-K-R-Y-I-I-T-Y-C-R-L-K-C-A-P-R-L-S-P-Q-A-A-E-K-L-S
 T16 A-P-R-L-S-P-Q-A-A-E-K-L-S-S-N-F-V-T-I-R-K-Q-L-L-I-N
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 T19 I-R-Q-L-E-A-I-I-R-I-T-E-S-L-A-K-L-E-L-S-P-I-A-Q-E-R
 T20 L-A-K-L-E-L-S-P-I-A-Q-E-R-H-V-D-E-A-I-R-L-F-Q-A-S-T
 T21 H-V-D-E-A-I-R-L-F-Q-A-S-T-M-D-A-A-S-Q-D-P-I-G-G-L-N
 T22 M-D-A-A-S-Q-D-P-I-G-G-L-N-Q-A-S-G-T-S-L-S-E-I-R-R-F
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 T24 E-Q-E-L-K-R-R-L-P-I-G-W-S-T-S-Y-Q-T-L-R-R-E-F-V-D-T
 T25 T-S-Y-Q-T-L-R-R-E-F-V-D-T-H-R-F-S-Q-L-A-L-D-K-A-L-Y
 T26 H-R-F-S-Q-L-A-L-D-K-A-L-Y-A-L-E-K-H-E-T-I-Q-L-R-H-Q
 T27 K-A-L-Y-A-L-E-K-H-E-T-I-Q-L-R-H-Q-G-Q-N-I-Y-R-S-G-V
 T28
 T29
 T30

Membrane 2

A 1 Mcm6 M-S-S-P-F-P-A-D-T-P-S-S-N-R-P-S-N-S-S-P-P-P-S-S-I-G
A 2 R-P-S-N-S-S-P-P-P-S-S-I-G-A-G-F-G-S-S-S-G-L-D-S-Q-I
A 3 A-G-F-G-S-S-S-G-L-D-S-Q-I-G-S-R-L-H-F-P-S-S-S-Q-P-H
A 4 G-S-R-L-H-F-P-S-S-S-Q-P-H-V-S-N-S-Q-T-G-P-F-V-N-D-S
A 5 V-S-N-S-Q-T-G-P-F-V-N-D-S-T-Q-F-S-S-Q-R-L-Q-T-D-G-S
A 6 T-Q-F-S-S-Q-R-L-Q-T-D-G-S-A-T-N-D-M-E-G-N-E-P-A-R-S
A 7 A-T-N-D-M-E-G-N-E-P-A-R-S-F-K-S-R-A-L-N-H-V-K-K-V-D
A 8 F-K-S-R-A-L-N-H-V-K-K-V-D-D-V-T-G-E-K-V-R-E-A-F-E-Q
A 9 D-V-T-G-E-K-V-R-E-A-F-E-Q-F-L-E-D-F-S-V-Q-S-T-D-T-G
A10 F-L-E-D-F-S-V-Q-S-T-D-T-G-E-V-E-K-V-Y-R-A-Q-I-E-F-M
A11 E-V-E-K-V-Y-R-A-Q-I-E-F-M-K-I-Y-D-L-N-T-I-Y-I-D-Y-Q
A12 K-I-Y-D-L-N-T-I-Y-I-D-Y-Q-H-L-S-M-R-E-N-G-A-L-A-M-A
A13 H-L-S-M-R-E-N-G-A-L-A-M-A-I-S-E-Q-Y-Y-R-F-L-A-F-L-Q
A14 I-S-E-Q-Y-Y-R-F-L-A-F-L-Q-K-G-L-R-R-V-V-R-K-Y-A-P-E
A15 K-G-L-R-R-V-V-R-K-Y-A-P-E-L-L-N-T-S-D-S-L-K-R-S-E-G
A16 L-L-N-T-S-D-S-L-K-R-S-E-G-D-E-G-Q-A-D-E-D-E-Q-Q-D-D
A17 D-E-G-Q-A-D-E-D-E-Q-Q-D-D-D-M-N-G-S-S-L-P-R-D-S-G-S
A18 D-M-N-G-S-S-L-P-R-D-S-G-S-S-A-A-P-G-N-G-T-S-A-M-A-T
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A20 R-S-I-T-T-S-T-S-P-E-Q-T-E-R-V-F-Q-I-S-F-F-N-L-P-T-V
A21 R-V-F-Q-I-S-F-F-N-L-P-T-V-H-R-I-R-D-I-R-S-E-K-I-G-S
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A27 E-N-R-A-F-W-T-L-N-V-T-R-S-R-F-L-D-W-Q-K-V-R-I-Q-E-N
A28 R-F-L-D-W-Q-K-V-R-I-Q-E-N-A-N-E-I-P-T-G-S-M-P-R-T-L
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A30 D-V-I-L-R-G-D-S-V-E-R-A-K-P-G-D-R-C-K-F-T-G-V-E-I-V
B 1 P-G-D-R-C-K-F-T-G-V-E-I-V-V-P-D-V-T-Q-L-G-L-P-G-V-K
B 2 V-P-D-V-T-Q-L-G-L-P-G-V-K-P-S-S-T-L-D-T-R-G-I-S-K-T
B 3 P-S-S-T-L-D-T-R-G-I-S-K-T-T-E-G-L-N-S-G-V-T-G-L-R-S
B 4 T-E-G-L-N-S-G-V-T-G-L-R-S-L-G-V-R-D-L-T-Y-K-I-S-F-L
B 5 L-G-V-R-D-L-T-Y-K-I-S-F-L-A-C-H-V-I-S-I-G-S-N-I-G-A
B 6 A-C-H-V-I-S-I-G-S-N-I-G-A-S-S-P-D-A-N-S-N-N-R-E-T-E
B 7 S-S-P-D-A-N-S-N-N-R-E-T-E-L-Q-M-A-A-N-L-Q-A-N-N-V-Y
B 8 L-Q-M-A-A-N-L-Q-A-N-N-V-Y-Q-D-N-E-R-D-Q-E-V-F-L-N-S
B 9 Q-D-N-E-R-D-Q-E-V-F-L-N-S-L-S-S-D-E-I-N-E-L-K-E-M-V
B10 L-S-S-D-E-I-N-E-L-K-E-M-V-K-D-E-H-I-Y-D-K-L-V-R-S-I
B11 K-D-E-H-I-Y-D-K-L-V-R-S-I-A-P-A-V-F-G-H-E-A-V-K-K-G
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B14 V-E-G-I-K-L-R-G-D-I-N-I-C-V-V-G-D-P-S-T-S-K-S-Q-F-L
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B17 S-G-K-A-S-S-A-A-G-L-T-A-A-V-V-R-D-E-E-G-G-D-Y-T-I-E
B18 V-V-R-D-E-E-G-G-D-Y-T-I-E-A-G-A-L-M-L-A-D-N-G-I-C-C

B19 A-G-A-L-M-L-A-D-N-G-I-C-C-I-D-E-F-D-K-M-D-I-S-D-Q-V
 B20 I-D-E-F-D-K-M-D-I-S-D-Q-V-A-I-H-E-A-M-E-Q-Q-T-I-S-I
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 C 1 R-K-D-D-A-Q-G-F-S-R-S-S-Y-R-I-T-V-R-Q-L-E-S-M-I-R-L
 C 2 R-I-T-V-R-Q-L-E-S-M-I-R-L-S-E-A-I-A-R-A-N-C-V-D-E-I
 C 3 S-E-A-I-A-R-A-N-C-V-D-E-I-T-P-S-F-I-A-E-A-Y-D-L-L-R
 C 4 T-P-S-F-I-A-E-A-Y-D-L-L-R-Q-S-I-I-R-V-D-V-D-D-V-E-M
 C 5 Q-S-I-I-R-V-D-V-D-D-V-E-M-D-E-E-F-D-N-I-E-S-Q-S-H-A
 C 6 D-E-E-F-D-N-I-E-S-Q-S-H-A-A-S-G-N-N-D-D-N-D-D-G-T-G
 C 7 A-S-G-N-N-D-D-N-D-D-G-T-G-S-G-V-I-T-S-E-P-P-A-D-I-E
 C 8 S-G-V-I-T-S-E-P-P-A-D-I-E-E-G-Q-S-E-A-T-A-R-P-G-T-S
 C 9 E-G-Q-S-E-A-T-A-R-P-G-T-S-E-K-K-K-T-T-V-T-Y-D-K-Y-V
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 C11 S-M-M-N-M-I-V-R-K-I-A-E-V-D-R-E-G-A-E-E-L-T-A-V-D-I
 C12 D-R-E-G-A-E-E-L-T-A-V-D-I-V-D-W-Y-L-L-Q-K-E-N-D-L-G
 C13 V-D-W-Y-L-L-Q-K-E-N-D-L-G-S-L-A-E-Y-W-E-E-R-R-L-A-F
 C14 S-L-A-E-Y-W-E-E-R-R-L-A-F-K-V-I-K-R-L-V-K-D-R-I-L-M
 C15 K-V-I-K-R-L-V-K-D-R-I-L-M-E-I-H-G-T-R-H-N-L-R-D-L-E
 C16 E-I-H-G-T-R-H-N-L-R-D-L-E-N-E-E-N-E-N-N-K-T-V-Y-V-I
 C17 N-E-E-N-E-N-N-K-T-V-Y-V-I-H-P-N-C-E-V-L-D-Q-L-E-P-Q
 C18 N-E-N-N-K-T-V-Y-V-I-H-P-N-C-E-V-L-D-Q-L-E-P-Q-D-S-S
 C19
 C20 Mcm7 M-S-A-A-L-P-S-I-Q-L-P-V-D-Y-N-N-L-F-N-E-I-T-D-F-L-V
 C21 Y-N-N-L-F-N-E-I-T-D-F-L-V-T-F-K-Q-D-T-L-S-S-D-A-T-R
 C22 T-F-K-Q-D-T-L-S-S-D-A-T-R-N-E-N-E-D-E-N-L-D-A-E-N-I
 C23 N-E-N-E-D-E-N-L-D-A-E-N-I-E-Q-H-L-L-E-K-G-P-K-Y-M-A
 C24 E-Q-H-L-L-E-K-G-P-K-Y-M-A-M-L-Q-K-V-A-N-R-E-L-N-S-V
 C25 M-L-Q-K-V-A-N-R-E-L-N-S-V-I-I-D-L-D-D-I-L-Q-Y-Q-N-E
 C26 I-I-D-L-D-D-I-L-Q-Y-Q-N-E-K-F-L-Q-G-T-Q-A-D-D-L-V-S
 C27 K-F-L-Q-G-T-Q-A-D-D-L-V-S-A-I-Q-Q-N-A-N-H-F-T-E-L-F
 C28 A-I-Q-Q-N-A-N-H-F-T-E-L-F-C-R-A-I-D-N-N-M-P-L-P-T-K
 C29 C-R-A-I-D-N-N-M-P-L-P-T-K-E-I-D-Y-K-D-D-V-L-D-V-I-L
 C30 E-I-D-Y-K-D-D-V-L-D-V-I-L-N-Q-R-R-L-R-N-E-R-M-L-S-D
 D 1 N-Q-R-R-L-R-N-E-R-M-L-S-D-R-T-N-E-I-R-S-E-N-L-M-D-T
 D 2 R-T-N-E-I-R-S-E-N-L-M-D-T-T-M-D-P-P-S-S-M-N-D-A-L-R
 D 3 T-M-D-P-P-S-S-M-N-D-A-L-R-E-V-V-E-D-E-T-E-L-F-P-P-N
 D 4 E-V-V-E-D-E-T-E-L-F-P-P-N-L-T-R-R-Y-F-L-Y-F-K-P-L-S
 D 5 L-T-R-R-Y-F-L-Y-F-K-P-L-S-Q-N-C-A-R-R-Y-R-K-K-A-I-S
 D 6 Q-N-C-A-R-R-Y-R-K-K-A-I-S-S-K-P-L-S-V-R-Q-I-K-G-D-F
 D 7 S-K-P-L-S-V-R-Q-I-K-G-D-F-L-G-Q-L-I-T-V-R-G-I-I-T-R
 D 8 L-G-Q-L-I-T-V-R-G-I-I-T-R-V-S-D-V-K-P-A-V-E-V-I-A-Y
 D 9 V-S-D-V-K-P-A-V-E-V-I-A-Y-T-C-D-Q-C-G-Y-E-V-F-Q-E-V

D10 T-C-D-Q-C-G-Y-E-V-F-Q-E-V-N-S-R-T-F-T-P-L-S-E-C-T-S
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D20 F-S-L-T-S-D-V-E-E-R-V-M-E-L-I-T-S-G-D-V-Y-N-R-L-A-K
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D22 S-I-A-P-E-I-Y-G-N-L-D-V-K-K-A-L-L-L-L-L-V-G-G-V-D-K
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D24 R-V-G-D-G-M-K-I-R-G-D-I-N-V-C-L-M-G-D-P-G-V-A-K-S-Q
D25 V-C-L-M-G-D-P-G-V-A-K-S-Q-L-L-K-A-I-C-K-I-S-P-R-G-V
D26 L-L-K-A-I-C-K-I-S-P-R-G-V-Y-T-T-G-K-G-S-S-G-V-G-L-T
D27 Y-T-T-G-K-G-S-S-G-V-G-L-T-A-A-V-M-K-D-P-V-T-D-E-M-I
D28 A-A-V-M-K-D-P-V-T-D-E-M-I-L-E-G-G-A-L-V-L-A-D-N-G-I
D29 L-E-G-G-A-L-V-L-A-D-N-G-I-C-C-I-D-E-F-D-K-M-D-E-S-D
D30 C-C-I-D-E-F-D-K-M-D-E-S-D-R-T-A-I-H-E-V-M-E-Q-Q-T-I
E 1 R-T-A-I-H-E-V-M-E-Q-Q-T-I-S-I-S-K-A-G-I-N-T-T-L-N-A
E 2 S-I-S-K-A-G-I-N-T-T-L-N-A-R-T-S-I-L-A-A-A-N-P-L-Y-G
E 3 R-T-S-I-L-A-A-A-N-P-L-Y-G-R-Y-N-P-R-L-S-P-L-D-N-I-N
E 4 R-Y-N-P-R-L-S-P-L-D-N-I-N-L-P-A-A-L-L-S-R-F-D-I-L-F
E 5 L-P-A-A-L-L-S-R-F-D-I-L-F-L-M-L-D-I-P-S-R-D-D-D-E-K
E 6 L-M-L-D-I-P-S-R-D-D-D-E-K-L-A-E-H-V-T-Y-V-H-M-H-N-K
E 7 L-A-E-H-V-T-Y-V-H-M-H-N-K-Q-P-D-L-D-F-T-P-V-E-P-S-K
E 8 Q-P-D-L-D-F-T-P-V-E-P-S-K-M-R-E-Y-I-A-Y-A-K-T-K-R-P
E 9 M-R-E-Y-I-A-Y-A-K-T-K-R-P-V-M-S-E-A-V-N-D-Y-V-V-Q-A
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E14 R-L-A-D-M-V-D-I-D-D-V-E-E-A-L-R-L-V-R-V-S-K-E-S-L-Y
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E25 L-R-E-V-V-E-D-E-T-E-L-F-P-P-N-L-T-R-R-Y-F-L-Y-F-K-P
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E29 D-F-L-G-Q-L-I-T-V-R-G-I-I-T-R-V-S-D-V-K-P-A-V-E-V-I
E30 T-R-V-S-D-V-K-P-A-V-E-V-I-A-Y-T-C-D-Q-C-G-Y-E-V-F-Q

F 1 A-Y-T-C-D-Q-C-G-Y-E-V-F-Q-E-V-N-S-R-T-F-T-P-L-S-E-C
 F 2 E-V-N-S-R-T-F-T-P-L-S-E-C-T-S-E-E-C-S-Q-N-Q-T-K-G-Q
 F 3 T-S-E-E-C-S-Q-N-Q-T-K-G-Q-L-F-M-S-T-R-A-S-K-F-S-A-F
 F 4 L-F-M-S-T-R-A-S-K-F-S-A-F-Q-E-C-K-I-Q-E-L-S-Q-Q-V-P
 F 5 Q-E-C-K-I-Q-E-L-S-Q-Q-V-P-V-G-H-I-P-R-S-L-N-I-H-V-N
 F 6 V-G-H-I-P-R-S-L-N-I-H-V-N-G-T-L-V-R-S-L-S-P-G-D-I-V
 F 7 G-T-L-V-R-S-L-S-P-G-D-I-V-D-V-T-G-I-F-L-P-A-P-Y-T-G
 F 8 D-V-T-G-I-F-L-P-A-P-Y-T-G-F-K-A-L-K-A-G-L-L-T-E-T-Y
 F 9 F-K-A-L-K-A-G-L-L-T-E-T-Y-L-E-A-Q-F-V-R-Q-H-K-K-K-F
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 F11 A-S-F-S-L-T-S-D-V-E-E-R-V-M-E-L-I-T-S-G-D-V-Y-N-R-L
 F12 M-E-L-I-T-S-G-D-V-Y-N-R-L-A-K-S-I-A-P-E-I-Y-G-N-L-D
 F13 A-K-S-I-A-P-E-I-Y-G-N-L-D-V-K-K-A-L-L-L-L-L-V-G-G-V
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 G 2 Q-A-Y-I-R-L-R-Q-D-S-K-R-E-M-D-S-K-F-S-F-G-Q-A-T-P-R
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 G 8 P-T-T-K-I-F-T-I-I-K-K-M-L-Q-E-T-G-K-N-T-L-S-Y-E-N-I
 G 9 Q-E-T-G-K-N-T-L-S-Y-E-N-I-V-K-T-V-R-L-R-G-F-T-M-L-Q
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 G19 A-R-E-L-E-F-M-E-R-K-R-Q-A-L-V-E-R-L-K-R-K-Q-E-F-K-K
 G20 L-V-E-R-L-K-R-K-Q-E-F-K-K-P-Q-D-P-N-F-E-A-I-E-V-P-Q
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G27 F-E-S-M-M-N-A-R-V-H-T-F-S-T-D-E-K-K-Y-V-P-I-I-T-N-E
G28 T-D-E-K-K-Y-V-P-I-I-T-N-E-L-E-S-F-S-N-L-W-V-K-K-R-Y
G29 L-E-S-F-S-N-L-W-V-K-K-R-Y-I-P-E-D-D-L-K-R-A-L-H-E-I
G30 I-P-E-D-D-L-K-R-A-L-H-E-I-K-I-L-R-L-G-K-L-F-A-K-I-R
H 1 K-I-L-R-L-G-K-L-F-A-K-I-R-P-P-K-F-Q-E-P-E-Y-A-N-W-A
H 2 P-P-K-F-Q-E-P-E-Y-A-N-W-A-T-V-G-L-I-S-H-K-S-D-I-K-F
H 3 T-V-G-L-I-S-H-K-S-D-I-K-F-T-S-S-E-K-P-V-K-F-F-M-F-T
H 4 T-S-S-E-K-P-V-K-F-F-M-F-T-I-T-D-F-Q-H-T-L-D-V-Y-I-F
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H 6 G-K-K-G-V-E-R-Y-Y-N-L-R-L-G-D-V-I-A-I-L-N-P-E-V-L-P
H 7 G-D-V-I-A-I-L-N-P-E-V-L-P-W-R-P-S-G-R-G-N-F-I-K-S-F
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H 9 N-L-R-I-S-H-D-F-K-C-I-L-E-I-G-S-S-R-D-L-G-W-C-P-I-V
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H17 E-E-R-L-K-K-S-S-H-N-F-T-N-S-N-S-A-K-A-F-F-D-E-K-F-Q
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H30
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I 2 K-E-T-D-T-N-L-K-H-N-N-G-I-A-A-S-T-T-A-A-G-H-L-N-V-F
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I 9 T-P-K-E-L-L-E-W-Q-T-N-W-K-K-I-M-K-R-D-S-R-I-Y-F-D-I
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 J 2 D-V-R-N-E-H-A-D-Q-T-D-E-K-N-S-I-L-L-N-E-T-E-T-K-E-P
 J 3 N-S-I-L-L-N-E-T-E-T-K-E-P-P-L-K-E-E-K-E-N-K-Q-S-I-A
 J 4 P-L-K-E-E-K-E-N-K-Q-S-I-A-E-E-S-N-K-Y-P-Q-R-K-E-L-A
 J 5 E-E-S-N-K-Y-P-Q-R-K-E-L-A-A-T-P-K-L-N-H-P-V-L-A-T-F
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 K 1 G-Y-V-D-R-G-V-E-E-D-W-R-E-V-D-N-S-S-S-D-E-D-T-G-N-L
 K 2 V-D-N-S-S-S-D-E-D-T-G-N-L-A-S-K-D-S-K-R-K-K-N-I-K-R
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K 4 E-K-D-H-Q-I-T-D-M-L-R-T-Q-H-S-K-S-T-L-L-A-H-A-K-K-S
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M 3 I-I-Q-E-F-N-I-C-F-T-T-V-D-R-N-K-E-D-I-D-E-L-P-S-V-P
M 4 R-N-K-E-D-I-D-E-L-P-S-V-P-P-S-E-V-D-Q-G-V-L-P-R-L-L
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M 7 M-K-T-E-T-D-P-H-K-R-V-Q-C-D-I-R-Q-Q-A-L-K-L-T-A-N-S
M 8 D-I-R-Q-Q-A-L-K-L-T-A-N-S-M-Y-G-C-L-G-Y-V-N-S-R-F-Y
M 9 M-Y-G-C-L-G-Y-V-N-S-R-F-Y-A-K-P-L-A-M-L-V-T-N-K-G-R
M10 A-K-P-L-A-M-L-V-T-N-K-G-R-E-I-L-M-N-T-R-Q-L-A-E-S-M
M11 E-I-L-M-N-T-R-Q-L-A-E-S-M-N-L-L-V-V-Y-G-D-T-D-S-V-M
M12 N-L-L-V-V-Y-G-D-T-D-S-V-M-I-D-T-G-C-D-N-Y-A-D-A-I-K
M13 I-D-T-G-C-D-N-Y-A-D-A-I-K-I-G-L-G-F-K-R-L-V-N-E-R-Y
M14 I-G-L-G-F-K-R-L-V-N-E-R-Y-R-L-L-E-I-D-I-D-N-V-F-K-K
M15 R-L-L-E-I-D-I-D-N-V-F-K-K-L-L-L-H-A-K-K-K-Y-A-A-L-T
M16 L-L-L-H-A-K-K-K-Y-A-A-L-T-V-N-L-D-K-N-G-N-G-T-T-V-L
M17 V-N-L-D-K-N-G-N-G-T-T-V-L-E-V-K-G-L-D-M-K-R-R-E-F-C
M18 E-V-K-G-L-D-M-K-R-R-E-F-C-P-L-S-R-D-V-S-I-H-V-L-N-T
M19 P-L-S-R-D-V-S-I-H-V-L-N-T-I-L-S-D-K-D-P-E-E-A-L-Q-E
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M26 V-I-T-K-Q-D-E-I-D-N-A-A-D-T-P-A-L-S-V-A-E-R-A-H-A-L
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M28 N-E-V-M-I-K-S-N-N-L-I-P-D-P-Q-Y-Y-L-E-K-Q-I-F-A-P-V
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N 2 F-R-R-E-G-G-N-N-N-G-E-D-I-N-N-L-Q-P-L-E-T-T-I-T-D-V
N 3 N-N-L-Q-P-L-E-T-T-I-T-D-V-E-R-F-K-D-T-V-T-L-E-L-S-C
N 4 E-R-F-K-D-T-V-T-L-E-L-S-C-P-S-C-D-K-R-F-P-F-G-G-I-V
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N 7 C-K-H-C-E-Q-L-F-T-P-L-Q-L-T-S-Q-I-E-H-S-I-R-A-H-I-S
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 O 4 S-N-Q-L-F-E-A-R-K-L-L-R-P-I-L-Q-D-N-A-N-N-N-V-Q-R-N
 O 5 I-L-Q-D-N-A-N-N-N-V-Q-R-N-I-Y-N-V-A-A-N-G-S-E-K-V-D
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 P 4 Q-E-L-P-E-A-L-K-F-S-V-E-V-E-N-K-S-S-V-D-K-V-T-N-F-E
 P 5 E-N-K-S-S-V-D-K-V-T-N-F-E-E-I-K-N-Q-I-T-Q-K-L-L-E-L
 P 6 E-I-K-N-Q-I-T-Q-K-L-L-E-L-K-E-N-N-I-R-N-E-L-P-L-I-Y

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 Q 3 K-G-I-K-K-R-Y-A-V-F-N-E-D-G-S-L-A-E-L-K-G-F-E-L-K-R
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 R 4 M-P-L-Q-K-I-K-N-C-L-I-E-K-S-S-A-S-L-P-N-N-P-K-T-S-N
 R 5 S-S-A-S-L-P-N-N-P-K-T-S-N-P-A-G-G-Q-L-F-K-I-T-L-P-E
 R 6 P-A-G-G-Q-L-F-K-I-T-L-P-E-S-V-F-L-E-E-K-E-N-C-T-S-I
 R 7 S-V-F-L-E-E-K-E-N-C-T-S-I-F-N-D-E-N-V-L-G-V-F-E-G-T
 R 8 F-N-D-E-N-V-L-G-V-F-E-G-T-I-T-P-H-Q-R-A-I-M-D-L-G-A
 R 9 I-T-P-H-Q-R-A-I-M-D-L-G-A-S-V-T-F-R-S-K-A-M-G-A-L-G
 R10 S-V-T-F-R-S-K-A-M-G-A-L-G-K-G-I-Q-Q-G-F-E-M-K-D-L-S
 R11 K-G-I-Q-Q-G-F-E-M-K-D-L-S-M-A-E-N-E-R-Y-L-S-G-F-S-M
 R12 M-A-E-N-E-R-Y-L-S-G-F-S-M-D-I-G-Y-L-L-H-F-P-T-S-I-G
 R13 D-I-G-Y-L-L-H-F-P-T-S-I-G-Y-E-F-F-S-L-F-K-S-W-G-D-T
 R14 Y-E-F-F-S-L-F-K-S-W-G-D-T-I-T-I-L-V-L-K-P-S-N-Q-A-Q
 R15 I-T-I-L-V-L-K-P-S-N-Q-A-Q-E-I-N-A-S-S-L-G-Q-I-Y-K-Q
 R16 E-I-N-A-S-S-L-G-Q-I-Y-K-Q-M-F-E-K-K-K-G-K-I-E-T-Y-S
 R17 M-F-E-K-K-K-G-K-I-E-T-Y-S-Y-L-V-D-I-K-E-D-I-N-F-E-F
 R18 Y-L-V-D-I-K-E-D-I-N-F-E-F-V-Y-F-T-D-I-S-K-L-Y-R-R-L
 R19 V-Y-F-T-D-I-S-K-L-Y-R-R-L-S-Q-E-T-T-K-L-K-E-E-R-G-L
 R20 S-Q-E-T-T-K-L-K-E-E-R-G-L-Q-F-L-L-L-L-Q-S-P-F-I-T-K
 R21 Q-F-L-L-L-L-Q-S-P-F-I-T-K-L-L-G-T-I-R-L-L-N-Q-M-P-I
 R22 L-L-G-T-I-R-L-L-N-Q-M-P-I-V-K-L-S-L-N-E-V-L-L-P-Q-L
 R23 V-K-L-S-L-N-E-V-L-L-P-Q-L-N-W-Q-P-T-L-L-K-K-L-V-N-H
 R24 N-W-Q-P-T-L-L-K-K-L-V-N-H-V-L-S-S-G-S-W-I-S-H-L-I-K
 R25 V-L-S-S-G-S-W-I-S-H-L-I-K-L-S-Q-Y-S-N-I-P-I-C-N-L-R
 R26 L-S-Q-Y-S-N-I-P-I-C-N-L-R-L-D-S-M-D-Y-I-I-D-V-L-Y-A
 R27 L-D-S-M-D-Y-I-I-D-V-L-Y-A-R-K-L-K-K-E-N-I-V-L-W-W-N
 R28 R-K-L-K-K-E-N-I-V-L-W-W-N-E-K-A-P-L-P-D-H-G-G-I-Q-N
 R29 E-K-A-P-L-P-D-H-G-G-I-Q-N-D-F-D-L-N-T-S-W-I-M-N-D-S
 R30 D-F-D-L-N-T-S-W-I-M-N-D-S-E-F-P-K-I-N-N-S-G-V-Y-D-N
 S 1 E-F-P-K-I-N-N-S-G-V-Y-D-N-V-V-L-D-V-G-V-D-N-L-T-V-N
 S 2 V-V-L-D-V-G-V-D-N-L-T-V-N-T-I-L-T-S-A-L-I-N-D-A-E-G
 S 3 T-I-L-T-S-A-L-I-N-D-A-E-G-S-D-L-V-N-N-N-M-G-I-D-D-K
 S 4 S-D-L-V-N-N-N-M-G-I-D-D-K-D-A-V-I-N-S-P-S-E-F-V-H-D
 S 5 D-A-V-I-N-S-P-S-E-F-V-H-D-A-F-S-N-D-A-L-N-V-L-R-G-M
 S 6 A-F-S-N-D-A-L-N-V-L-R-G-M-L-K-E-W-W-D-E-A-L-K-E-N-S
 S 7 L-K-E-W-W-D-E-A-L-K-E-N-S-T-A-D-L-L-V-N-S-L-A-S-W-V
 S 8 T-A-D-L-L-V-N-S-L-A-S-W-V-Q-N-P-N-A-K-L-F-D-G-L-L-R
 S 9 Q-N-P-N-A-K-L-F-D-G-L-L-R-Y-H-V-H-N-L-T-K-K-A-L-L-Q
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 S15 D-L-N-I-K-R-Y-W-D-L-L-I-W-M-D-K-F-N-F-S-G-L-A-C-I-E
 S16 M-D-K-F-N-F-S-G-L-A-C-I-E-I-E-E-K-E-N-Q-D-Y-T-A-V-S
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 S18 Q-W-Q-L-K-K-F-L-S-P-I-Y-Q-P-E-F-E-D-W-M-M-I-I-L-D-S

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S25 Q-E-F-I-L-D-P-Q-Y-E-A-D-Y-V-I-P-V-L-P-G-S-H-L-N-V-K
S26
S27 Clb5 M-G-E-N-H-D-H-E-Q-S-I-K-R-N-S-M-I-Y-N-E-N-E-R-Q-L-C
S28 N-S-M-I-Y-N-E-N-E-R-Q-L-C-N-S-N-L-K-I-L-Q-N-K-R-A-L
S29 N-S-N-L-K-I-L-Q-N-K-R-A-L-S-K-N-D-S-S-F-T-S-K-Q-Q-V
S30 S-K-N-D-S-S-F-T-S-K-Q-Q-V-Q-D-S-K-P-R-R-A-L-T-D-V-P
T 1 Q-D-S-K-P-R-R-A-L-T-D-V-P-V-N-N-N-P-L-S-Q-N-K-R-I-V
T 2 V-N-N-N-P-L-S-Q-N-K-R-I-V-A-G-S-K-A-A-K-V-R-R-E-E-N
T 3 A-G-S-K-A-A-K-V-R-R-E-E-N-I-R-P-I-V-S-A-V-Q-K-R-Q-I
T 4 I-R-P-I-V-S-A-V-Q-K-R-Q-I-Y-N-F-T-D-R-T-A-A-E-Q-E-E
T 5 Y-N-F-T-D-R-T-A-A-E-Q-E-E-E-E-E-E-E-G-E-D-D-D-A-A-S
T 6 E-E-E-E-E-G-E-D-D-D-A-A-S-I-V-N-K-K-R-R-I-D-A-E-G-V
T 7 I-V-N-K-K-R-R-I-D-A-E-G-V-S-E-I-V-G-W-Q-D-L-D-Y-V-E
T 8 S-E-I-V-G-W-Q-D-L-D-Y-V-E-K-D-D-T-A-M-V-A-E-Y-S-F-T
T 9 K-D-D-T-A-M-V-A-E-Y-S-F-T-A-E-I-F-A-F-L-Y-R-R-E-L-E
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T13 D-W-L-V-E-V-H-E-K-F-Q-C-Y-P-E-T-L-F-L-S-F-T-I-N-L-M
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T15 D-R-F-L-A-K-N-K-V-T-M-N-K-L-Q-L-L-A-V-T-S-L-F-I-A-A
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T17 K-F-E-E-V-N-L-P-K-L-A-E-Y-A-Y-I-T-D-G-A-A-S-K-N-D-I
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T19 K-N-A-F-T-E-M-F-M-L-T-S-L-E-F-N-I-G-W-P-N-P-L-N-F-L
T20 E-F-N-I-G-W-P-N-P-L-N-F-L-R-R-I-S-K-A-D-D-Y-D-P-V-N
T21 R-R-I-S-K-A-D-D-Y-D-P-V-N-R-N-I-G-K-F-I-L-E-Y-A-Y-C
T22 R-N-I-G-K-F-I-L-E-Y-A-Y-C-C-H-Q-F-I-H-L-P-P-S-T-V-F
T23 C-H-Q-F-I-H-L-P-P-S-T-V-F-T-S-A-M-A-M-Y-I-A-R-R-M-T
T24 T-S-A-M-A-M-Y-I-A-R-R-M-T-N-R-N-K-N-E-L-W-N-G-T-L-Q
T25 N-R-N-K-N-E-L-W-N-G-T-L-Q-H-Y-S-G-G-I-D-P-I-H-D-E-A
T26 H-Y-S-G-G-I-D-P-I-H-D-E-A-F-Q-S-L-C-I-D-L-V-K-D-I-A
T27 F-Q-S-L-C-I-D-L-V-K-D-I-A-S-S-K-T-H-L-D-S-F-T-L-I-L
T28 S-S-K-T-H-L-D-S-F-T-L-I-L-K-Y-K-K-P-R-Y-G-S-V-Y-F-Q
T29 K-Y-K-K-P-R-Y-G-S-V-Y-F-Q-T-F-K-W-C-T-S-E-M-H-S-N-F
T30 G-S-V-Y-F-Q-T-F-K-W-C-T-S-E-M-H-S-N-F-Q-N-L-F-N-L-K

Membrane 3

A 1 Dna2 M-P-G-T-P-Q-K-N-K-R-S-A-S-I-S-V-S-P-A-K-K-T-E-E-K-E
A 2 I-S-V-S-P-A-K-K-T-E-E-K-E-I-I-Q-N-D-S-K-A-I-L-S-K-Q
A 3 I-I-Q-N-D-S-K-A-I-L-S-K-Q-T-K-R-K-K-K-Y-A-F-A-P-I-N
A 4 T-K-R-K-K-K-Y-A-F-A-P-I-N-N-L-N-G-K-N-T-K-V-S-N-A-S
A 5 N-L-N-G-K-N-T-K-V-S-N-A-S-V-L-K-S-I-A-V-S-Q-V-R-N-T
A 6 V-L-K-S-I-A-V-S-Q-V-R-N-T-S-R-T-K-D-I-N-K-A-V-S-K-S
A 7 S-R-T-K-D-I-N-K-A-V-S-K-S-V-K-Q-L-P-N-S-Q-V-K-P-K-R
A 8 V-K-Q-L-P-N-S-Q-V-K-P-K-R-E-M-S-N-L-S-R-H-H-D-F-T-Q
A 9 E-M-S-N-L-S-R-H-H-D-F-T-Q-D-E-D-G-P-M-E-E-V-I-W-K-Y
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A12 A-A-E-Y-S-D-D-Y-E-D-V-Q-N-P-S-S-T-P-I-V-P-N-R-L-K-T
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A17 R-N-I-D-D-I-L-D-D-I-E-G-D-L-T-I-K-P-T-I-T-K-F-S-D-L
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A24 G-A-Q-E-S-C-G-K-N-D-N-T-K-S-R-G-E-I-E-D-H-E-N-V-D-N
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B 1 V-S-D-D-L-R-I-A-T-D-S-T-L-S-A-Y-A-L-R-A-K-S-G-A-P-R
B 2 S-A-Y-A-L-R-A-K-S-G-A-P-R-D-G-V-V-R-L-V-I-V-S-L-R-S
B 3 D-G-V-V-R-L-V-I-V-S-L-R-S-V-E-L-P-K-I-G-T-Q-K-I-L-E
B 4 V-E-L-P-K-I-G-T-Q-K-I-L-E-C-I-D-G-K-G-E-Q-S-S-V-V-V
B 5 C-I-D-G-K-G-E-Q-S-S-V-V-V-R-H-P-W-V-Y-L-E-F-E-V-G-D
B 6 R-H-P-W-V-Y-L-E-F-E-V-G-D-V-I-H-I-I-E-G-K-N-I-E-N-K
B 7 V-I-H-I-I-E-G-K-N-I-E-N-K-R-L-L-S-D-D-K-N-P-K-T-Q-L
B 8 R-L-L-S-D-D-K-N-P-K-T-Q-L-A-N-D-N-L-L-V-L-N-P-D-V-L
B 9 A-N-D-N-L-L-V-L-N-P-D-V-L-F-S-A-T-S-V-G-S-S-V-G-C-L
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B16 C-N-E-E-I-Q-Y-V-K-E-L-V-M-K-E-H-A-E-N-I-L-Y-F-V-N-K
B17 K-E-H-A-E-N-I-L-Y-F-V-N-K-F-V-S-K-S-N-Y-G-C-Y-T-S-I
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 B22 V-E-N-N-K-K-H-I-V-P-L-E-V-K-T-G-K-S-R-S-V-S-Y-E-V-Q
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 B28 F-K-H-Q-L-Q-E-V-F-G-Q-A-Q-S-R-F-E-L-P-P-L-L-R-D-S-S
 B29 S-R-F-E-L-P-P-L-L-R-D-S-S-C-D-S-C-F-I-K-E-S-C-M-V-L
 B30 C-D-S-C-F-I-K-E-S-C-M-V-L-N-K-L-L-E-D-G-T-P-E-E-S-G
 C 1 N-K-L-L-E-D-G-T-P-E-E-S-G-L-V-E-G-E-F-E-I-L-T-N-H-L
 C 2 L-V-E-G-E-F-E-I-L-T-N-H-L-S-Q-N-L-A-N-Y-K-E-F-F-T-K
 C 3 S-Q-N-L-A-N-Y-K-E-F-F-T-K-Y-N-D-L-I-T-K-E-E-S-S-I-T
 C 4 Y-N-D-L-I-T-K-E-E-S-S-I-T-C-V-N-K-E-L-F-L-L-D-G-S-T
 C 5 C-V-N-K-E-L-F-L-L-D-G-S-T-R-E-S-R-S-G-R-C-L-S-G-L-V
 C 6 R-E-S-R-S-G-R-C-L-S-G-L-V-V-S-E-V-V-E-H-E-K-T-E-G-A
 C 7 V-S-E-V-V-E-H-E-K-T-E-G-A-Y-I-Y-C-F-S-R-R-R-N-D-N-N
 C 8 Y-I-Y-C-F-S-R-R-R-N-D-N-N-S-Q-S-M-L-S-S-Q-I-A-A-N-D
 C 9 S-Q-S-M-L-S-S-Q-I-A-A-N-D-F-V-I-I-S-D-E-E-G-H-F-C-L
 C10 F-V-I-I-S-D-E-E-G-H-F-C-L-C-Q-G-R-V-Q-F-I-N-P-A-K-I
 C11 C-Q-G-R-V-Q-F-I-N-P-A-K-I-G-I-S-V-K-R-K-L-L-N-N-R-L
 C12 G-I-S-V-K-R-K-L-L-N-N-R-L-L-D-K-E-K-G-V-T-T-I-Q-S-V
 C13 L-D-K-E-K-G-V-T-T-I-Q-S-V-V-E-S-E-L-E-Q-S-S-L-I-A-T
 C14 V-E-S-E-L-E-Q-S-S-L-I-A-T-Q-N-L-V-T-Y-R-I-D-K-N-D-I
 C15 Q-N-L-V-T-Y-R-I-D-K-N-D-I-Q-Q-S-L-S-L-A-R-F-N-L-L-S
 C16 Q-Q-S-L-S-L-A-R-F-N-L-L-S-L-F-L-P-A-V-S-P-G-V-D-I-V
 C17 L-F-L-P-A-V-S-P-G-V-D-I-V-D-E-R-S-K-L-C-R-K-T-K-R-S
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 C19 D-G-G-N-E-I-L-R-S-L-L-V-D-N-R-A-P-K-F-R-D-A-N-D-D-P
 C20 N-R-A-P-K-F-R-D-A-N-D-D-P-V-I-P-Y-K-L-S-K-D-T-T-L-N
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 C22 L-N-Q-K-E-A-I-D-K-V-M-R-A-E-D-Y-A-L-I-L-G-M-P-G-T-G
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 D 1 I-L-F-T-L-N-E-K-D-F-D-Y-V-I-L-D-E-A-S-Q-I-S-M-P-V-A
 D 2 I-L-D-E-A-S-Q-I-S-M-P-V-A-L-G-P-L-R-Y-G-N-R-F-I-M-V
 D 3 L-G-P-L-R-Y-G-N-R-F-I-M-V-G-D-H-Y-Q-L-P-P-L-V-K-N-D
 D 4 G-D-H-Y-Q-L-P-P-L-V-K-N-D-A-A-R-L-G-G-L-E-E-S-L-F-K
 D 5 A-A-R-L-G-G-L-E-E-S-L-F-K-T-F-C-E-K-H-P-E-S-V-A-E-L
 D 6 T-F-C-E-K-H-P-E-S-V-A-E-L-T-L-Q-Y-R-M-C-G-D-I-V-T-L
 D 7 T-L-Q-Y-R-M-C-G-D-I-V-T-L-S-N-F-L-I-Y-D-N-K-L-K-C-G
 D 8 S-N-F-L-I-Y-D-N-K-L-K-C-G-N-N-E-V-F-A-Q-S-L-E-L-P-M
 D 9 N-N-E-V-F-A-Q-S-L-E-L-P-M-P-E-A-L-S-R-Y-R-N-E-S-A-N

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D17 N-K-N-V-Y-D-G-L-E-I-L-T-A-D-Q-F-Q-G-R-D-K-K-C-I-I-I
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D22 T-I-G-S-V-P-E-I-K-S-F-V-N-L-L-E-E-R-N-W-V-Y-T-M-C-K
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D26 G-A-K-P-I-T-S-K-S-K-F-V-S-D-K-P-I-I-K-E-I-L-Q-E-Y-E
D27 A-K-P-I-T-S-K-S-K-F-V-S-D-K-P-I-I-K-E-I-L-Q-E-Y-E-S
D28
D29 Mrc1 M-D-D-A-L-H-A-L-S-S-L-T-A-K-K-R-T-T-T-Y-K-K-V-A-V-P
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E 1 I-L-D-E-N-D-N-T-N-G-N-G-P-N-D-I-D-N-P-P-E-L-T-G-N-G
E 2 N-D-I-D-N-P-P-E-L-T-G-N-G-F-L-F-A-N-A-T-L-N-R-V-K-N
E 3 F-L-F-A-N-A-T-L-N-R-V-K-N-R-L-E-G-K-K-A-P-E-Q-N-H-N
E 4 R-L-E-G-K-K-A-P-E-Q-N-H-N-N-G-K-D-R-S-E-N-S-L-P-T-Q
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E 6 L-I-S-N-L-Y-D-G-G-E-E-L-E-K-S-E-V-K-D-N-S-Y-S-E-K-N
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E 8 V-S-S-S-F-T-Q-T-Q-R-I-P-V-S-I-Q-Q-D-K-V-F-N-V-P-I-H
E 9 S-I-Q-Q-D-K-V-F-N-V-P-I-H-S-V-N-D-G-K-P-T-Q-L-I-K-E
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E19 G-I-P-Q-A-T-H-Q-E-Q-K-T-Q-I-D-T-V-A-Q-T-L-Q-D-E-V-P
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F 1 D-D-M-D-S-I-K-L-S-K-D-E-S-V-L-P-I-S-Q-L-S-K-A-T-I-L
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F 6 K-Q-I-L-D-H-Q-K-E-V-I-E-T-K-G-L-K-L-E-D-M-A-K-E-K-E
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H 1 L-I-D-W-E-N-K-C-S-L-A-A-K-L-A-K-N-N-P-D-H-E-E-F-R-N
H 2 L-A-K-N-N-P-D-H-E-E-F-R-N-K-A-Y-Y-D-K-I-A-L-N-C-L-Q
H 3 K-A-Y-Y-D-K-I-A-L-N-C-L-Q-L-L-V-L-M-T-W-P-L-I-V-T-E
H 4 L-L-V-L-M-T-W-P-L-I-V-T-E-Q-S-S-S-N-Q-I-T-L-Y-G-E-L
H 5 Q-S-S-S-N-Q-I-T-L-Y-G-E-L-K-K-H-Q-L-V-Y-K-K-T-I-L-S
H 6 K-K-H-Q-L-V-Y-K-K-T-I-L-S-M-E-S-G-K-V-L-R-A-A-I-R-L
H 7 M-E-S-G-K-V-L-R-A-A-I-R-L-A-L-D-V-I-K-I-D-R-L-S-R-T
H 8 A-L-D-V-I-K-I-D-R-L-S-R-T-P-R-D-N-M-V-L-K-L-V-L-N-F
H 9 P-R-D-N-M-V-L-K-L-V-L-N-F-F-R-N-V-I-A-I-E-P-G-E-F-T
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H15 S-K-E-F-D-Q-D-F-I-N-I-P-L-L-E-I-M-F-Y-F-T-K-D-V-N-Q
H16 L-E-I-M-F-Y-F-T-K-D-V-N-Q-E-L-L-F-P-R-Q-F-E-T-G-T-H
H17 E-L-L-F-P-R-Q-F-E-T-G-T-H-S-K-V-V-N-K-N-E-S-S-S-A-N
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H30 T-L-E-Q-V-E-Y-L-L-F-F-A-W-F-V-K-Y-Q-L-L-R-S-K-I-D-N
I 1 F-V-K-Y-Q-L-L-R-S-K-I-D-N-S-A-D-I-K-Q-V-S-E-A-L-K-E
I 2 S-A-D-I-K-Q-V-S-E-A-L-K-E-V-T-F-I-L-V-S-S-L-L-R-S-A
I 3 V-T-F-I-L-V-S-S-L-L-R-S-A-Y-D-L-K-N-W-T-V-T-H-A-G-M
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I 9 Q-F-M-K-S-C-I-E-L-T-H-S-V-L-K-V-L-E-Q-Y-S-D-D-K-T-L
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 I14 S-L-R-S-I-E-V-N-F-Q-K-V-Q-A-N-Y-M-T-E-P-V-I-E-T-Y-I
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 J 4 V-E-V-S-D-L-T-V-S-C-E-L-V-K-K-Y-L-S-T-P-F-E-T-P-N-G
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 J16 P-I-F-F-E-N-E-T-Y-M-R-W-L-L-D-K-N-N-G-Q-L-T-E-D-R-Y
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 J27 A-D-E-S-D-E-D-E-E-A-I-R-L-F-G-K-K-S-R-V-V-L-S-Q-G-D
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 J29
 J30 Top1 M-T-I-A-D-A-S-K-V-N-H-E-L-S-S-D-D-D-D-V-P-L-S-Q-T
 K 1 S-S-D-D-D-D-V-P-L-S-Q-T-L-K-K-R-K-V-A-S-M-N-S-A-S
 K 2 L-K-K-R-K-V-A-S-M-N-S-A-S-L-Q-D-E-A-E-P-Y-D-S-D-E-A
 K 3 L-Q-D-E-A-E-P-Y-D-S-D-E-A-I-S-K-I-S-K-K-K-T-K-K-I-K

K 4 I-S-K-I-S-K-K-K-T-K-K-I-K-T-E-P-V-Q-S-S-S-L-P-S-P-P
 K 5 T-E-P-V-Q-S-S-S-L-P-S-P-P-A-K-K-S-A-T-S-K-P-K-K-I-K
 K 6 A-K-K-S-A-T-S-K-P-K-K-I-K-K-E-D-G-D-V-K-V-K-T-T-K-K
 K 7 K-E-D-G-D-V-K-V-K-T-T-K-K-E-E-Q-E-N-E-K-K-K-R-E-E-E
 K 8 E-E-Q-E-N-E-K-K-K-R-E-E-E-E-E-E-D-K-K-A-K-E-E-E-E-E
 K 9 E-E-E-D-K-K-A-K-E-E-E-E-E-Y-K-W-W-E-K-E-N-E-D-D-T-I
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 K15 H-A-K-N-P-V-F-Q-K-N-F-F-N-D-F-L-Q-V-L-K-E-S-G-G-P-L
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 L 2 F-A-L-R-A-G-G-E-K-S-E-D-E-A-D-T-V-G-C-C-S-L-R-Y-E-H
 L 3 A-D-T-V-G-C-C-S-L-R-Y-E-H-V-T-L-K-P-P-N-T-V-I-F-D-F
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 L14 A-N-N-R-I-Q-E-L-E-W-Q-K-I-R-C-K-R-A-I-L-Q-L-D-K-D-L
 L15 R-C-K-R-A-I-L-Q-L-D-K-D-L-L-K-K-E-P-K-Y-F-E-E-I-D-D
 L16 L-K-K-E-P-K-Y-F-E-E-I-D-D-L-T-K-E-D-E-A-T-I-H-K-R-I
 L17 L-T-K-E-D-E-A-T-I-H-K-R-I-I-D-R-E-I-E-K-Y-Q-R-K-F-V
 L18 I-D-R-E-I-E-K-Y-Q-R-K-F-V-R-E-N-D-K-R-K-F-E-K-E-E-L
 L19 R-E-N-D-K-R-K-F-E-K-E-E-L-L-P-E-S-Q-L-K-E-W-L-E-K-V
 L20 L-P-E-S-Q-L-K-E-W-L-E-K-V-D-E-K-K-Q-E-F-E-K-E-L-K-T
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 L26 D-P-R-L-S-V-V-F-C-K-K-Y-D-V-P-I-E-K-I-F-T-K-T-L-R-E
 L27 V-P-I-E-K-I-F-T-K-T-L-R-E-K-F-K-W-A-I-E-S-V-D-E-N-W
 L28 I-E-K-I-F-T-K-T-L-R-E-K-F-K-W-A-I-E-S-V-D-E-N-W-R-F
 L29
 L30 Top2 M-S-T-E-P-V-S-A-S-D-K-Y-Q-K-I-S-Q-L-E-H-I-L-K-R-P-D
 M 1 K-I-S-Q-L-E-H-I-L-K-R-P-D-T-Y-I-G-S-V-E-T-Q-E-Q-L-Q
 M 2 T-Y-I-G-S-V-E-T-Q-E-Q-L-Q-W-I-Y-D-E-E-T-D-C-M-I-E-K
 M 3 W-I-Y-D-E-E-T-D-C-M-I-E-K-N-V-T-I-V-P-G-L-F-K-I-F-D
 M 4 N-V-T-I-V-P-G-L-F-K-I-F-D-E-I-L-V-N-A-A-D-N-K-V-R-D
 M 5 E-I-L-V-N-A-A-D-N-K-V-R-D-P-S-M-K-R-I-D-V-N-I-H-A-E
 M 6 P-S-M-K-R-I-D-V-N-I-H-A-E-E-H-T-I-E-V-K-N-D-G-K-G-I
 M 7 E-H-T-I-E-V-K-N-D-G-K-G-I-P-I-E-I-H-N-K-E-N-I-Y-I-P
 M 8 P-I-E-I-H-N-K-E-N-I-Y-I-P-E-M-I-F-G-H-L-L-T-S-S-N-Y
 M 9 E-M-I-F-G-H-L-L-T-S-S-N-Y-D-D-D-E-K-K-V-T-G-G-R-N-G
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 M16 K-E-L-D-N-D-I-L-G-V-M-R-R-R-V-Y-D-I-N-G-S-V-R-D-I-N
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 M20 K-R-Q-L-D-N-G-E-D-G-A-A-K-S-D-I-P-T-I-L-Y-E-R-I-N-N
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 M24 G-G-T-H-V-N-Y-I-T-D-Q-I-V-K-K-I-S-E-I-L-K-K-K-K-K-K
 M25 K-K-I-S-E-I-L-K-K-K-K-K-S-V-K-S-F-Q-I-K-N-N-M-F-I
 M26 S-V-K-S-F-Q-I-K-N-N-M-F-I-F-I-N-C-L-I-E-N-P-A-F-T-S
 M27 F-I-N-C-L-I-E-N-P-A-F-T-S-Q-T-K-E-Q-L-T-T-R-V-K-D-F
 M28 Q-T-K-E-Q-L-T-T-R-V-K-D-F-G-S-R-C-E-I-P-L-E-Y-I-N-K
 M29 G-S-R-C-E-I-P-L-E-Y-I-N-K-I-M-K-T-D-L-A-T-R-M-F-E-I
 M30 I-M-K-T-D-L-A-T-R-M-F-E-I-A-D-A-N-E-E-N-A-L-K-K-S-D
 N 1 A-D-A-N-E-E-N-A-L-K-K-S-D-G-T-R-K-S-R-I-T-N-Y-P-K-L
 N 2 G-T-R-K-S-R-I-T-N-Y-P-K-L-E-D-A-N-K-A-G-T-K-E-G-Y-K
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 N13 I-T-P-I-I-K-V-S-I-T-K-P-T-K-N-T-I-A-F-Y-N-M-P-D-Y-E
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 N22 N-K-E-L-I-L-F-S-L-A-D-N-I-R-S-I-P-N-V-L-D-G-F-K-P-G
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 N28 S-N-N-I-Y-L-L-L-P-N-G-A-F-G-T-R-A-T-G-G-K-D-A-A-A-A
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 O 2 Q-E-D-E-K-T-V-E-P-E-W-Y-L-P-I-L-P-M-I-L-V-N-G-A-E-G
 O 3 P-I-L-P-M-I-L-V-N-G-A-E-G-I-G-T-G-W-S-T-Y-I-P-P-F-N
 O 4 I-G-T-G-W-S-T-Y-I-P-P-F-N-P-L-E-I-I-K-N-I-R-H-L-M-N
 O 5 P-L-E-I-I-K-N-I-R-H-L-M-N-D-E-E-L-E-Q-M-H-P-W-F-R-G
 O 6 D-E-E-L-E-Q-M-H-P-W-F-R-G-W-T-G-T-I-E-E-I-E-P-L-R-Y
 O 7 W-T-G-T-I-E-E-I-E-P-L-R-Y-R-M-Y-G-R-I-E-Q-I-G-D-N-V
 O 8 R-M-Y-G-R-I-E-Q-I-G-D-N-V-L-E-I-T-E-L-P-A-R-T-W-T-S
 O 9 L-E-I-T-E-L-P-A-R-T-W-T-S-T-I-K-E-Y-L-L-L-G-L-S-G-N
 O10 T-I-K-E-Y-L-L-L-G-L-S-G-N-D-K-I-K-P-W-I-K-D-M-E-E-Q
 O11 D-K-I-K-P-W-I-K-D-M-E-E-Q-H-D-D-N-I-K-F-I-I-T-L-S-P
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Q 4 I-E-I-D-L-R-A-G-V-T-F-T-R-L-L-T-E-T-L-R-N-K-L-R-N-Q
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Q 8 Q-F-P-T-L-G-F-V-V-D-R-F-E-R-I-R-N-F-V-P-E-E-F-W-Y-I
Q 9 R-I-R-N-F-V-P-E-E-F-W-Y-I-Q-L-V-V-E-N-K-D-N-G-G-T-T
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Q24 R-R-V-Y-E-Y-V-A-R-H-F-L-A-C-C-S-E-D-A-K-G-Q-S-M-T-L
Q25 C-C-S-E-D-A-K-G-Q-S-M-T-L-V-L-D-W-A-V-E-R-F-S-A-S-G
Q26 V-L-D-W-A-V-E-R-F-S-A-S-G-L-V-V-L-E-R-N-F-L-D-V-Y-P
Q27 L-V-V-L-E-R-N-F-L-D-V-Y-P-W-A-R-W-E-T-T-K-Q-L-P-R-L

Q28 W-A-R-W-E-T-T-K-Q-L-P-R-L-E-M-N-A-L-V-D-I-A-K-A-E-M
 Q29 E-M-N-A-L-V-D-I-A-K-A-E-M-K-A-G-T-T-A-P-P-K-P-M-T-E
 Q30 K-A-G-T-T-A-P-P-K-P-M-T-E-S-E-L-I-L-L-M-D-T-N-G-I-G
 R 1 S-E-L-I-L-L-M-D-T-N-G-I-G-T-D-A-T-I-A-E-H-I-D-K-I-Q
 R 2 T-D-A-T-I-A-E-H-I-D-K-I-Q-V-R-N-Y-V-R-S-E-K-V-G-K-E
 R 3 V-R-N-Y-V-R-S-E-K-V-G-K-E-T-Y-L-Q-P-T-T-L-G-V-S-L-V
 R 4 T-Y-L-Q-P-T-T-L-G-V-S-L-V-H-G-F-E-A-I-G-L-E-D-S-F-A
 R 5 H-G-F-E-A-I-G-L-E-D-S-F-A-K-P-F-Q-R-R-E-M-E-Q-D-L-K
 R 6 K-P-F-Q-R-R-E-M-E-Q-D-L-K-K-I-C-E-G-H-A-S-K-T-D-V-V
 R 7 K-I-C-E-G-H-A-S-K-T-D-V-V-K-D-I-V-E-K-Y-R-K-Y-W-H-K
 R 8 K-D-I-V-E-K-Y-R-K-Y-W-H-K-T-N-A-C-K-N-T-L-L-Q-V-Y-D
 R 9 Y-R-K-Y-W-H-K-T-N-A-C-K-N-T-L-L-Q-V-Y-D-R-V-K-A-S-M
 R10
 R11 Dpb2 M-C-E-M-F-G-S-G-N-V-L-P-V-K-I-Q-P-P-L-L-R-P-L-A-Y-R
 R12 K-I-Q-P-P-L-L-R-P-L-A-Y-R-V-L-S-R-K-Y-G-L-S-I-K-S-D
 R13 V-L-S-R-K-Y-G-L-S-I-K-S-D-G-L-S-A-L-A-E-F-V-G-T-N-I
 R14 G-L-S-A-L-A-E-F-V-G-T-N-I-G-A-N-W-R-Q-G-P-A-T-I-K-F
 R15 G-A-N-W-R-Q-G-P-A-T-I-K-F-L-E-Q-F-A-A-V-W-K-Q-Q-E-R
 R16 L-E-Q-F-A-A-V-W-K-Q-Q-E-R-G-L-F-I-D-Q-S-G-V-K-E-V-I
 R17 G-L-F-I-D-Q-S-G-V-K-E-V-I-Q-E-M-K-E-R-E-K-V-E-W-S-H
 R18 Q-E-M-K-E-R-E-K-V-E-W-S-H-E-H-P-I-Q-H-E-E-N-I-L-G-R
 R19 E-H-P-I-Q-H-E-E-N-I-L-G-R-T-D-D-D-E-N-N-S-D-D-E-M-P
 R20 T-D-D-D-E-N-N-S-D-D-E-M-P-I-A-A-D-S-S-L-Q-N-V-S-L-S
 R21 I-A-A-D-S-S-L-Q-N-V-S-L-S-S-P-M-R-Q-P-T-E-R-D-E-Y-K
 R22 S-P-M-R-Q-P-T-E-R-D-E-Y-K-Q-P-F-K-P-E-S-S-K-A-L-D-W
 R23 Q-P-F-K-P-E-S-S-K-A-L-D-W-R-D-Y-F-K-V-I-N-A-S-Q-Q-Q
 R24 R-D-Y-F-K-V-I-N-A-S-Q-Q-Q-R-F-S-Y-N-P-H-K-M-Q-F-I-F
 R25 R-F-S-Y-N-P-H-K-M-Q-F-I-F-V-P-N-K-K-Q-N-G-L-G-G-I-A
 R26 V-P-N-K-K-Q-N-G-L-G-G-I-A-G-F-L-P-D-I-E-D-K-V-Q-M-F
 R27 G-F-L-P-D-I-E-D-K-V-Q-M-F-L-T-R-Y-Y-L-T-N-D-R-V-M-R
 R28 L-T-R-Y-Y-L-T-N-D-R-V-M-R-N-E-N-F-Q-N-S-D-M-F-N-P-L
 R29 N-E-N-F-Q-N-S-D-M-F-N-P-L-S-S-M-V-S-L-Q-N-E-L-S-N-T
 R30 S-S-M-V-S-L-Q-N-E-L-S-N-T-N-R-Q-Q-Q-S-S-S-M-S-I-T-P
 S 1 N-R-Q-Q-Q-S-S-S-M-S-I-T-P-I-K-N-L-L-G-R-D-A-Q-N-F-L
 S 2 I-K-N-L-L-G-R-D-A-Q-N-F-L-L-L-G-L-L-N-K-N-F-K-G-N-W
 S 3 L-L-G-L-L-N-K-N-F-K-G-N-W-S-L-E-D-P-S-G-S-V-E-I-D-I
 S 4 S-L-E-D-P-S-G-S-V-E-I-D-I-S-Q-T-I-P-T-Q-G-H-Y-Y-V-P
 S 5 S-Q-T-I-P-T-Q-G-H-Y-Y-V-P-G-C-M-V-L-V-E-G-I-Y-Y-S-V
 S 6 G-C-M-V-L-V-E-G-I-Y-Y-S-V-G-N-K-F-H-V-T-S-M-T-L-P-P
 S 7 G-N-K-F-H-V-T-S-M-T-L-P-P-G-E-R-R-E-I-T-L-E-T-I-G-N
 S 8 G-E-R-R-E-I-T-L-E-T-I-G-N-L-D-L-L-G-I-H-G-I-S-N-N-N
 S 9 L-D-L-L-G-I-H-G-I-S-N-N-N-F-I-A-R-L-D-K-D-L-K-I-R-L
 S10 F-I-A-R-L-D-K-D-L-K-I-R-L-H-L-L-E-K-E-L-T-D-H-K-F-V
 S11 H-L-L-E-K-E-L-T-D-H-K-F-V-I-L-G-A-N-L-F-L-D-D-L-K-I
 S12 I-L-G-A-N-L-F-L-D-D-L-K-I-M-T-A-L-S-K-I-L-Q-K-L-N-D
 S13 M-T-A-L-S-K-I-L-Q-K-L-N-D-D-P-P-T-L-L-I-W-Q-G-S-F-T
 S14 D-P-P-T-L-L-I-W-Q-G-S-F-T-S-V-P-V-F-A-S-M-S-S-R-N-I
 S15 S-V-P-V-F-A-S-M-S-S-R-N-I-S-S-S-T-Q-F-K-N-N-F-D-A-L
 S16 S-S-S-T-Q-F-K-N-N-F-D-A-L-A-T-L-L-S-R-F-D-N-L-T-E-N
 S17 A-T-L-L-S-R-F-D-N-L-T-E-N-T-T-M-I-F-I-P-G-P-N-D-L-W
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S19 G-S-M-V-S-L-G-A-S-G-T-L-P-Q-D-P-I-P-S-A-F-T-K-K-I-N
 S20 Q-D-P-I-P-S-A-F-T-K-K-I-N-K-V-C-K-N-V-V-W-S-S-N-P-T
 S21 K-V-C-K-N-V-V-W-S-S-N-P-T-R-I-A-Y-L-S-Q-E-I-V-I-F-R
 S22 R-I-A-Y-L-S-Q-E-I-V-I-F-R-D-D-L-S-G-R-F-K-R-H-R-L-E
 S23 D-D-L-S-G-R-F-K-R-H-R-L-E-F-P-F-N-E-S-E-D-V-Y-T-E-N
 S24 F-P-F-N-E-S-E-D-V-Y-T-E-N-D-N-M-M-S-K-D-T-D-I-V-P-I
 S25 D-N-M-M-S-K-D-T-D-I-V-P-I-D-E-L-V-K-E-P-D-Q-L-P-Q-K
 S26 D-E-L-V-K-E-P-D-Q-L-P-Q-K-V-Q-E-T-R-K-L-V-K-T-I-L-D
 S27 V-Q-E-T-R-K-L-V-K-T-I-L-D-Q-G-H-L-S-P-F-L-D-S-L-R-P
 S28 Q-G-H-L-S-P-F-L-D-S-L-R-P-I-S-W-D-L-D-H-T-L-T-L-C-P
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 S30 I-P-S-T-M-V-L-C-D-T-T-S-A-Q-F-D-L-T-Y-N-G-C-K-V-I-N
 T 1 Q-F-D-L-T-Y-N-G-C-K-V-I-N-P-G-S-F-I-H-N-R-R-A-R-Y-M
 T 2 P-G-S-F-I-H-N-R-R-A-R-Y-M-E-Y-V-P-S-S-K-K-T-I-Q-E-E
 T 3 F-I-H-N-R-R-A-R-Y-M-E-Y-V-P-S-S-K-K-T-I-Q-E-E-I-Y-I
 T 4
 T 5 Rfc3 M-S-T-S-T-E-K-R-S-K-E-N-L-P-W-V-E-K-Y-R-P-E-T-L-D-E
 T 6 P-W-V-E-K-Y-R-P-E-T-L-D-E-V-Y-G-Q-N-E-V-I-T-T-V-R-K
 T 7 V-Y-G-Q-N-E-V-I-T-T-V-R-K-F-V-D-E-G-K-L-P-H-L-L-F-Y
 T 8 F-V-D-E-G-K-L-P-H-L-L-F-Y-G-P-P-G-T-G-K-T-S-T-I-V-A
 T 9 G-P-P-G-T-G-K-T-S-T-I-V-A-L-A-R-E-I-Y-G-K-N-Y-S-N-M
 T10 L-A-R-E-I-Y-G-K-N-Y-S-N-M-V-L-E-L-N-A-S-D-D-R-G-I-D
 T11 V-L-E-L-N-A-S-D-D-R-G-I-D-V-V-R-N-Q-I-K-D-F-A-S-T-R
 T12 V-V-R-N-Q-I-K-D-F-A-S-T-R-Q-I-F-S-K-G-F-K-L-I-I-L-D
 T13 Q-I-F-S-K-G-F-K-L-I-I-L-D-E-A-D-A-M-T-N-A-A-Q-N-A-L
 T14 E-A-D-A-M-T-N-A-A-Q-N-A-L-R-R-V-I-E-R-Y-T-K-N-T-R-F
 T15 R-R-V-I-E-R-Y-T-K-N-T-R-F-C-V-L-A-N-Y-A-H-K-L-T-P-A
 T16 C-V-L-A-N-Y-A-H-K-L-T-P-A-L-L-S-R-C-T-R-F-R-F-Q-P-L
 T17 L-L-S-R-C-T-R-F-R-F-Q-P-L-P-Q-E-A-I-E-R-R-I-A-N-V-L
 T18 P-Q-E-A-I-E-R-R-I-A-N-V-L-V-H-E-K-L-K-L-S-P-N-A-E-K
 T19 V-H-E-K-L-K-L-S-P-N-A-E-K-A-L-I-E-L-S-N-G-D-M-R-R-V
 T20 A-L-I-E-L-S-N-G-D-M-R-R-V-L-N-V-L-Q-S-C-K-A-T-L-D-N
 T21 L-N-V-L-Q-S-C-K-A-T-L-D-N-P-D-E-D-E-I-S-D-D-V-I-Y-E
 T22 P-D-E-D-E-I-S-D-D-V-I-Y-E-C-C-G-A-P-R-P-S-D-L-K-A-V
 T23 C-C-G-A-P-R-P-S-D-L-K-A-V-L-K-S-I-L-E-D-D-W-G-T-A-H
 T24 L-K-S-I-L-E-D-D-W-G-T-A-H-Y-T-L-N-K-V-R-S-A-K-G-L-A
 T25 Y-T-L-N-K-V-R-S-A-K-G-L-A-L-I-D-L-I-E-G-I-V-K-I-L-E
 T26 L-I-D-L-I-E-G-I-V-K-I-L-E-D-Y-E-L-Q-N-E-E-T-R-V-H-L
 T27 D-Y-E-L-Q-N-E-E-T-R-V-H-L-L-T-K-L-A-D-I-E-Y-S-I-S-K
 T28 L-T-K-L-A-D-I-E-Y-S-I-S-K-G-G-N-D-Q-I-Q-G-S-A-V-I-G
 T29 G-G-N-D-Q-I-Q-G-S-A-V-I-G-A-I-K-A-S-F-E-N-E-T-V-K-A
 T30 N-D-Q-I-Q-G-S-A-V-I-G-A-I-K-A-S-F-E-N-E-T-V-K-A-N-V

Membrane 4

A 1 Sgs1 M-V-T-K-P-S-H-N-L-R-R-E-H-K-W-L-K-E-T-A-T-L-Q-E-D-K
A 2 K-W-L-K-E-T-A-T-L-Q-E-D-K-D-F-V-F-Q-A-I-Q-K-H-I-A-N
A 3 D-F-V-F-Q-A-I-Q-K-H-I-A-N-K-R-P-K-T-N-S-P-P-T-T-P-S
A 4 K-R-P-K-T-N-S-P-P-T-T-P-S-K-D-E-C-G-P-G-T-T-N-F-I-T
A 5 K-D-E-C-G-P-G-T-T-N-F-I-T-S-I-P-A-S-G-P-T-N-T-A-T-K
A 6 S-I-P-A-S-G-P-T-N-T-A-T-K-Q-H-E-V-M-Q-T-L-S-N-D-T-E
A 7 Q-H-E-V-M-Q-T-L-S-N-D-T-E-W-L-S-Y-T-A-T-S-N-Q-Y-A-D
A 8 W-L-S-Y-T-A-T-S-N-Q-Y-A-D-V-P-M-V-D-I-P-A-S-T-S-V-V
A 9 V-P-M-V-D-I-P-A-S-T-S-V-V-S-N-P-R-T-P-N-G-S-K-T-H-N
A10 S-N-P-R-T-P-N-G-S-K-T-H-N-F-N-T-F-R-P-H-M-A-S-S-L-V
A11 F-N-T-F-R-P-H-M-A-S-S-L-V-E-N-D-S-S-R-N-L-G-S-R-N-N
A12 E-N-D-S-S-R-N-L-G-S-R-N-N-N-K-S-V-I-D-N-S-S-I-G-K-Q
A13 N-K-S-V-I-D-N-S-S-I-G-K-Q-L-E-N-D-I-K-L-E-V-I-R-L-Q
A14 L-E-N-D-I-K-L-E-V-I-R-L-Q-G-S-L-I-M-A-L-K-E-Q-S-K-L
A15 G-S-L-I-M-A-L-K-E-Q-S-K-L-L-L-Q-K-C-S-I-I-E-S-T-S-L
A16 L-L-Q-K-C-S-I-I-E-S-T-S-L-S-E-D-A-K-R-L-Q-L-S-R-D-I
A17 S-E-D-A-K-R-L-Q-L-S-R-D-I-R-P-Q-L-S-N-M-S-I-R-I-D-S
A18 R-P-Q-L-S-N-M-S-I-R-I-D-S-L-E-K-E-I-I-K-A-K-K-D-G-M
A19 L-E-K-E-I-I-K-A-K-K-D-G-M-S-K-D-Q-S-K-G-R-S-Q-V-S-S
A20 S-K-D-Q-S-K-G-R-S-Q-V-S-S-Q-D-D-N-I-I-S-S-I-L-P-S-P
A21 Q-D-D-N-I-I-S-S-I-L-P-S-P-L-E-Y-N-T-S-S-R-N-S-N-L-T
A22 L-E-Y-N-T-S-S-R-N-S-N-L-T-S-T-T-A-T-T-V-T-K-A-L-A-I
A23 S-T-T-A-T-T-V-T-K-A-L-A-I-T-G-A-K-Q-N-I-T-N-N-T-G-K
A24 T-G-A-K-Q-N-I-T-N-N-T-G-K-N-S-N-N-D-S-N-N-D-D-L-I-Q
A25 N-S-N-N-D-S-N-N-D-D-L-I-Q-V-L-D-D-E-D-D-I-D-C-D-P-P
A26 V-L-D-D-E-D-D-I-D-C-D-P-P-V-I-L-K-E-G-A-P-H-S-P-A-F
A27 V-I-L-K-E-G-A-P-H-S-P-A-F-P-H-L-H-M-T-S-E-E-Q-D-E-L
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A29 T-R-R-R-N-M-R-S-R-E-P-V-N-Y-R-I-P-D-R-D-D-P-F-D-Y-V
A30 Y-R-I-P-D-R-D-D-P-F-D-Y-V-M-G-K-S-L-R-D-D-Y-P-D-V-E
B 1 M-G-K-S-L-R-D-D-Y-P-D-V-E-R-E-E-D-E-L-T-M-E-A-E-D-D
B 2 R-E-E-D-E-L-T-M-E-A-E-D-D-A-H-S-S-Y-M-T-T-R-D-E-E-K
B 3 A-H-S-S-Y-M-T-T-R-D-E-E-K-E-E-N-E-L-L-N-Q-S-D-F-D-F
B 4 E-E-N-E-L-L-N-Q-S-D-F-D-F-V-V-N-D-D-L-D-P-T-Q-D-T-D
B 5 V-V-N-D-D-L-D-P-T-Q-D-T-D-Y-H-D-N-M-D-V-S-A-N-I-Q-E
B 6 Y-H-D-N-M-D-V-S-A-N-I-Q-E-S-S-Q-E-G-D-T-R-S-T-I-T-L
B 7 S-S-Q-E-G-D-T-R-S-T-I-T-L-S-Q-N-K-N-V-Q-V-I-L-S-S-P
B 8 S-Q-N-K-N-V-Q-V-I-L-S-S-P-T-A-Q-S-V-P-S-N-G-Q-N-Q-I
B 9 T-A-Q-S-V-P-S-N-G-Q-N-Q-I-G-V-E-H-I-D-L-L-E-D-D-L-E
B10 G-V-E-H-I-D-L-L-E-D-D-L-E-K-D-A-I-L-D-D-S-M-S-F-S-F
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B12 G-R-Q-H-M-P-M-S-H-S-D-L-E-L-I-D-S-E-K-E-N-E-D-F-E-E
B13 L-I-D-S-E-K-E-N-E-D-F-E-E-D-N-N-N-N-G-I-E-Y-L-S-D-S
B14 D-N-N-N-N-G-I-E-Y-L-S-D-S-D-L-E-R-F-D-E-E-R-E-N-R-T
B15 D-L-E-R-F-D-E-E-R-E-N-R-T-Q-V-A-D-I-Q-E-L-D-N-D-L-K
B16 Q-V-A-D-I-Q-E-L-D-N-D-L-K-I-I-T-E-R-K-L-T-G-D-N-E-H
B17 I-I-T-E-R-K-L-T-G-D-N-E-H-P-P-P-S-W-S-P-K-I-K-R-E-K
B18 P-P-P-S-W-S-P-K-I-K-R-E-K-S-S-V-S-Q-K-D-E-E-D-D-F-D

B19 S-S-V-S-Q-K-D-E-E-D-D-F-D-D-D-F-S-L-S-D-I-V-S-K-S-N
 B20 D-D-F-S-L-S-D-I-V-S-K-S-N-L-S-S-K-T-N-G-P-T-Y-P-W-S
 B21 L-S-S-K-T-N-G-P-T-Y-P-W-S-D-E-V-L-Y-R-L-H-E-V-F-K-L
 B22 D-E-V-L-Y-R-L-H-E-V-F-K-L-P-G-F-R-P-N-Q-L-E-A-V-N-A
 B23 P-G-F-R-P-N-Q-L-E-A-V-N-A-T-L-Q-G-K-D-V-F-V-L-M-P-T
 B24 T-L-Q-G-K-D-V-F-V-L-M-P-T-G-G-G-K-S-L-C-Y-Q-L-P-A-V
 B25 G-G-G-K-S-L-C-Y-Q-L-P-A-V-V-K-S-G-K-T-H-G-T-T-I-V-I
 B26 V-K-S-G-K-T-H-G-T-T-I-V-I-S-P-L-I-S-L-M-Q-D-Q-V-E-H
 B27 S-P-L-I-S-L-M-Q-D-Q-V-E-H-L-L-N-K-N-I-K-A-S-M-F-S-S
 B28 L-L-N-K-N-I-K-A-S-M-F-S-S-R-G-T-A-E-Q-R-R-Q-T-F-N-L
 B29 R-G-T-A-E-Q-R-R-Q-T-F-N-L-F-I-N-G-L-L-D-L-V-Y-I-S-P
 B30 F-I-N-G-L-L-D-L-V-Y-I-S-P-E-M-I-S-A-S-E-Q-C-K-R-A-I
 C 1 E-M-I-S-A-S-E-Q-C-K-R-A-I-S-R-L-Y-A-D-G-K-L-A-R-I-V
 C 2 S-R-L-Y-A-D-G-K-L-A-R-I-V-V-D-E-A-H-C-V-S-N-W-G-H-D
 C 3 V-D-E-A-H-C-V-S-N-W-G-H-D-F-R-P-D-Y-K-E-L-K-F-F-K-R
 C 4 F-R-P-D-Y-K-E-L-K-F-F-K-R-E-Y-P-D-I-P-M-I-A-L-T-A-T
 C 5 E-Y-P-D-I-P-M-I-A-L-T-A-T-A-S-E-Q-V-R-M-D-I-I-H-N-L
 C 6 A-S-E-Q-V-R-M-D-I-I-H-N-L-E-L-K-E-P-V-F-L-K-Q-S-F-N
 C 7 E-L-K-E-P-V-F-L-K-Q-S-F-N-R-T-N-L-Y-Y-E-V-N-K-K-T-K
 C 8 R-T-N-L-Y-Y-E-V-N-K-K-T-K-N-T-I-F-E-I-C-D-A-V-K-S-R
 C 9 N-T-I-F-E-I-C-D-A-V-K-S-R-F-K-N-Q-T-G-I-I-Y-C-H-S-K
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 C14 E-I-Q-V-I-C-A-T-V-A-F-G-M-G-I-D-K-P-D-V-R-F-V-Y-H-F
 C15 G-I-D-K-P-D-V-R-F-V-Y-H-F-T-V-P-R-T-L-E-G-Y-Y-Q-E-T
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D22
D23 Srs2 M-S-S-N-N-D-L-W-L-H-L-V-S-Q-L-N-T-Q-Q-R-A-A-A-L-F-D
D24 Q-L-N-T-Q-Q-R-A-A-A-L-F-D-Y-T-R-G-L-Q-V-I-A-G-P-G-T
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E 1 K-I-L-Y-R-F-G-H-L-V-D-L-Q-K-D-W-R-I-I-D-E-K-E-I-D-V
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E 5 C-M-P-S-K-N-G-D-E-W-T-I-H-P-K-L-T-Q-E-T-D-I-K-A-K-I
E 6 P-K-L-T-Q-E-T-D-I-K-A-K-I-N-A-I-L-P-E-E-Y-I-L-D-S-N
E 7 N-A-I-L-P-E-E-Y-I-L-D-S-N-H-D-A-A-L-G-Y-F-Y-Q-I-Y-Q
E 8 H-D-A-A-L-G-Y-F-Y-Q-I-Y-Q-S-E-L-S-K-K-N-T-L-D-F-D-D
E 9 S-E-L-S-K-K-N-T-L-D-F-D-D-L-L-M-Y-T-F-R-L-L-T-R-V-R
E10 L-L-M-Y-T-F-R-L-L-T-R-V-R-V-L-S-N-I-K-H-V-L-V-D-E-F
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F 1 S-V-I-A-D-F-I-S-M-I-E-N-C-Q-L-L-L-Q-S-T-L-L-G-G-L-S
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 F10 N-F-F-N-S-L-S-L-H-S-D-A-A-E-E-E-E-S-E-S-N-K-D-A-K-I
 F11 E-E-E-E-S-E-S-N-K-D-A-K-I-K-R-E-K-N-G-F-V-T-I-S-T-I
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 G21 E-P-A-S-S-Q-M-D-I-F-S-Q-L-S-R-A-K-K-K-S-K-L-N-N-G-E

G22 Q-M-D-I-F-S-Q-L-S-R-A-K-K-K-S-K-L-N-N-G-E-I-I-V-I-D
G23
G24 Smc5 M-T-S-L-I-D-L-G-R-Y-V-E-R-T-H-H-G-E-D-T-E-P-R-S-K-R
G25 T-H-H-G-E-D-T-E-P-R-S-K-R-V-K-I-A-K-P-D-L-S-S-F-Q-P
G26 V-K-I-A-K-P-D-L-S-S-F-Q-P-G-S-I-I-K-I-R-L-Q-D-F-V-T
G27 G-S-I-I-K-I-R-L-Q-D-F-V-T-Y-T-L-T-E-F-N-L-S-P-S-L-N
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G30 V-C-A-V-C-L-G-L-A-G-K-P-E-Y-I-G-R-S-K-K-V-E-D-F-I-K
H 1 Y-I-G-R-S-K-K-V-E-D-F-I-K-N-G-Q-D-V-S-K-I-E-I-T-L-K
H 2 N-G-Q-D-V-S-K-I-E-I-T-L-K-N-S-P-N-V-T-D-I-E-Y-I-D-A
H 3 N-S-P-N-V-T-D-I-E-Y-I-D-A-R-D-E-T-I-K-I-T-R-I-I-T-R
H 4 R-D-E-T-I-K-I-T-R-I-I-T-R-S-K-R-R-S-D-Y-L-I-N-D-Y-Q
H 5 S-K-R-R-S-D-Y-L-I-N-D-Y-Q-V-S-E-S-V-V-K-T-L-V-A-Q-L
H 6 V-S-E-S-V-V-K-T-L-V-A-Q-L-N-I-Q-L-D-N-L-C-Q-F-L-S-Q
H 7 N-I-Q-L-D-N-L-C-Q-F-L-S-Q-E-R-V-E-E-F-A-R-L-K-S-V-K
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H11 E-Q-S-L-Q-K-D-L-D-F-K-K-A-K-I-V-H-L-R-Q-E-S-D-K-L-R
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 J 8 E-W-K-I-E-I-M-V-K-F-R-D-N-A-P-L-K-K-L-D-S-H-T-Q-S-G
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 J19 Smc6 M-I-S-T-T-I-S-G-K-R-P-I-E-Q-V-D-D-E-L-L-S-L-T-A-Q-Q
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 L 8 L-I-E-D-K-N-E-A-R-N-F-L-Q-R-N-P-V-N-V-N-M-A-L-S-L-R
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 L19 I-A-S-Y-E-A-A-K-E-E-L-G-L-K-I-E-Q-I-A-Q-E-A-Q-P-I-K
 L20 K-I-E-Q-I-A-Q-E-A-Q-P-I-K-E-Q-Y-D-S-T-K-L-A-L-V-E-A
 L21 E-Q-Y-D-S-T-K-L-A-L-V-E-A-Q-D-E-L-Q-Q-L-K-E-D-I-N-S
 L22 Q-D-E-L-Q-Q-L-K-E-D-I-N-S-R-Q-S-K-I-Q-K-Y-K-D-D-T-I
 L23 R-Q-S-K-I-Q-K-Y-K-D-D-T-I-Y-Y-E-D-K-K-K-V-Y-L-E-N-I
 L24 Y-Y-E-D-K-K-K-V-Y-L-E-N-I-K-K-I-E-V-N-V-A-A-L-K-E-G

L25 K-K-I-E-V-N-V-A-A-L-K-E-G-I-Q-R-Q-I-Q-N-A-C-A-F-C-S
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L27 K-E-R-I-E-N-V-D-L-P-D-T-Q-E-E-I-K-R-E-L-D-K-V-S-R-M
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 Q 5 F-G-D-I-A-N-K-D-D-I-R-M-K-N-S-E-Y-E-Q-R-E-L-K-R-A-N
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 Q15 Q-H-K-R-A-N-N-L-S-S-L-L-A-K-I-E-E-N-R-A-K-K-R-H-I-D
 Q16 K-I-E-E-N-R-A-K-K-R-H-I-D-Q-V-T-E-D-R-L-Q-S-Q-E-M-H
 Q17 Q-V-T-E-D-R-L-Q-S-Q-E-M-H-S-K-K-V-K-T-G-L-N-S-S-S-S
 Q18 S-K-K-V-K-T-G-L-N-S-S-S-S-T-I-D-F-F-K-N-Q-Y-G-L-L-K
 Q19 T-I-D-F-F-K-N-Q-Y-G-L-L-K-Q-T-Q-E-L-E-E-T-Q-K-T-I-G
 Q20 Q-T-Q-E-L-E-E-T-Q-K-T-I-G-S-D-E-T-N-Q-A-D-D-C-N-Q-T
 Q21 S-D-E-T-N-Q-A-D-D-C-N-Q-T-V-K-I-W-V-K-Y-N-E-G-F-S-N
 Q22 V-K-I-W-V-K-Y-N-E-G-F-S-N-A-V-R-K-N-V-T-W-N-N-L-W-E
 Q23
 Q24 Elg1 M-K-R-H-V-S-L-S-D-I-L-T-G-N-K-R-K-V-R-R-Q-D-A-L-Q-I
 Q25 N-K-R-K-V-R-R-Q-D-A-L-Q-I-T-I-D-D-E-N-D-T-E-S-G-T-F
 Q26 T-I-D-D-E-N-D-T-E-S-G-T-F-D-A-R-T-A-K-H-D-D-S-S-V-I
 Q27 D-A-R-T-A-K-H-D-D-S-S-V-I-F-L-N-H-S-V-V-K-P-I-E-A-V

Q28 F-L-N-H-S-V-V-K-P-I-E-A-V-S-T-N-H-K-S-A-K-E-F-L-M-T
 Q29 S-T-N-H-K-S-A-K-E-F-L-M-T-K-R-T-K-E-K-C-D-D-D-D-D-D
 Q30 K-R-T-K-E-K-C-D-D-D-D-D-D-L-I-V-I-S-D-K-S-P-K-S-E-T
 R 1 L-I-V-I-S-D-K-S-P-K-S-E-T-N-C-S-K-I-A-L-S-Q-E-H-E-D
 R 2 N-C-S-K-I-A-L-S-Q-E-H-E-D-D-I-S-I-I-S-T-S-R-I-K-S-S
 R 3 D-I-S-I-I-S-T-S-R-I-K-S-S-L-L-N-E-R-A-S-K-I-K-N-F-L
 R 4 L-L-N-E-R-A-S-K-I-K-N-F-L-K-H-E-T-T-D-T-F-K-R-L-N-S
 R 5 K-H-E-T-T-D-T-F-K-R-L-N-S-I-S-K-L-N-E-I-E-P-P-L-P-L
 R 6 I-S-K-L-N-E-I-E-P-P-L-P-L-H-Q-S-I-F-P-V-G-D-K-E-L-S
 R 7 H-Q-S-I-F-P-V-G-D-K-E-L-S-D-R-S-V-D-I-P-L-P-F-R-T-I
 R 8 D-R-S-V-D-I-P-L-P-F-R-T-I-P-P-L-N-H-N-F-L-P-S-D-Y-E
 R 9 P-P-L-N-H-N-F-L-P-S-D-Y-E-S-L-K-D-K-N-S-A-S-C-I-P-V
 R10 S-L-K-D-K-N-S-A-S-C-I-P-V-R-Y-Q-A-P-V-L-L-G-T-N-I-K
 R11 R-Y-Q-A-P-V-L-L-G-T-N-I-K-R-N-T-T-L-T-W-P-Q-L-F-K-P
 R12 R-N-T-T-L-T-W-P-Q-L-F-K-P-V-T-L-K-Q-V-L-I-E-P-K-L-K
 R13 V-T-L-K-Q-V-L-I-E-P-K-L-K-L-R-I-K-N-W-I-E-T-S-F-H-T
 R14 L-R-I-K-N-W-I-E-T-S-F-H-T-L-E-K-P-T-L-R-N-R-L-L-N-R
 R15 L-E-K-P-T-L-R-N-R-L-L-N-R-I-N-P-N-K-Q-Q-G-S-G-D-E-L
 R16 I-N-P-N-K-Q-Q-G-S-G-D-E-L-A-N-F-I-V-P-D-L-E-E-D-E-N
 R17 A-N-F-I-V-P-D-L-E-E-D-E-N-L-R-P-D-F-Y-R-N-G-E-A-N-S
 R18 L-R-P-D-F-Y-R-N-G-E-A-N-S-S-L-S-E-F-V-P-L-M-I-L-H-G
 R19 S-L-S-E-F-V-P-L-M-I-L-H-G-N-S-I-G-K-K-T-L-I-Q-T-I-M
 R20 N-S-I-G-K-K-T-L-I-Q-T-I-M-R-E-I-A-G-D-D-N-S-Y-Q-I-Y
 R21 R-E-I-A-G-D-D-N-S-Y-Q-I-Y-E-V-N-S-N-M-N-R-S-K-K-D-L
 R22 E-V-N-S-N-M-N-R-S-K-K-D-L-L-D-I-L-L-D-F-T-T-T-H-Y-V
 R23 L-D-I-L-L-D-F-T-T-T-H-Y-V-K-D-S-S-K-R-K-S-D-Y-G-L-V
 R24 K-D-S-S-K-R-K-S-D-Y-G-L-V-L-F-N-D-V-D-V-L-F-K-E-H-D
 R25 L-F-N-D-V-D-V-L-F-K-E-H-D-R-G-Y-W-A-M-I-S-K-L-C-E-F
 R26 R-G-Y-W-A-M-I-S-K-L-C-E-F-S-R-R-P-L-V-L-T-C-K-D-L-S
 R27 S-R-R-P-L-V-L-T-C-K-D-L-S-L-V-P-S-E-L-I-A-L-A-S-E-Q
 R28 L-V-P-S-E-L-I-A-L-A-S-E-Q-N-S-L-F-H-T-K-K-I-S-T-S-T
 R29 N-S-L-F-H-T-K-K-I-S-T-S-T-V-Y-A-F-L-T-K-Y-L-K-S-L-E
 R30 V-Y-A-F-L-T-K-Y-L-K-S-L-E-I-E-V-C-D-D-W-L-R-D-V-V-K
 S 1 I-E-V-C-D-D-W-L-R-D-V-V-K-Q-N-N-A-D-I-R-K-C-L-M-H-L
 S 2 Q-N-N-A-D-I-R-K-C-L-M-H-L-Q-F-W-C-V-D-T-E-A-D-L-I-S
 S 3 Q-F-W-C-V-D-T-E-A-D-L-I-S-S-K-N-R-L-P-V-L-T-S-T-L-G
 S 4 S-K-N-R-L-P-V-L-T-S-T-L-G-S-S-V-K-D-I-S-Q-L-T-D-L-L
 S 5 S-S-V-K-D-I-S-Q-L-T-D-L-L-S-I-N-D-V-I-G-Q-A-T-L-N-R
 S 6 S-I-N-D-V-I-G-Q-A-T-L-N-R-S-M-V-R-Q-E-I-D-S-T-T-M-T
 S 7 S-M-V-R-Q-E-I-D-S-T-T-M-T-P-E-K-V-N-T-F-Q-D-Q-N-L-D
 S 8 P-E-K-V-N-T-F-Q-D-Q-N-L-D-D-E-M-K-L-K-F-D-Y-V-I-D-Y
 S 9 D-E-M-K-L-K-F-D-Y-V-I-D-Y-K-L-H-L-N-D-P-N-R-Q-P-L-L
 S10 K-L-H-L-N-D-P-N-R-Q-P-L-L-P-F-E-L-N-I-Y-Q-H-I-Q-E-Q
 S11 P-F-E-L-N-I-Y-Q-H-I-Q-E-Q-L-E-A-R-Y-S-Y-V-R-E-A-N-H
 S12 L-E-A-R-Y-S-Y-V-R-E-A-N-H-R-L-D-N-E-Y-L-V-N-R-F-K-K
 S13 R-L-D-N-E-Y-L-V-N-R-F-K-K-M-T-E-S-T-L-N-F-L-A-S-R-I
 S14 M-T-E-S-T-L-N-F-L-A-S-R-I-P-K-Y-D-H-L-Q-S-A-R-R-T-R
 S15 P-K-Y-D-H-L-Q-S-A-R-R-T-R-N-S-K-K-I-S-D-I-L-N-Q-F-K
 S16 N-S-K-K-I-S-D-I-L-N-Q-F-K-G-I-Y-N-D-E-T-L-N-E-N-A-E
 S17 G-I-Y-N-D-E-T-L-N-E-N-A-E-I-D-L-L-S-A-T-T-Q-Q-I-K-A
 S18 I-D-L-L-S-A-T-T-Q-Q-I-K-A-E-I-N-P-F-V-F-E-I-A-K-S-D

S19 E-I-N-P-F-V-F-E-I-A-K-S-D-A-N-V-K-N-E-N-K-Q-I-F-E-L
 S20 A-N-V-K-N-E-N-K-Q-I-F-E-L-H-S-E-N-V-S-E-R-R-Y-K-D-L
 S21 H-S-E-N-V-S-E-R-R-Y-K-D-L-V-Y-Q-L-S-Q-E-G-V-L-K-N-V
 S22 V-Y-Q-L-S-Q-E-G-V-L-K-N-V-W-F-N-A-D-P-S-I-V-V-R-K-W
 S23 N-V-W-F-N-A-D-P-S-I-V-V-R-K-W-E-H-L-H-S-G-F-S-K-N-K
 S24
 S25 Dpb3 M-S-N-L-V-K-E-K-A-P-V-F-P-I-S-K-V-K-K-I-A-K-C-D-P-E
 S26 I-S-K-V-K-K-I-A-K-C-D-P-E-Y-V-I-T-S-N-V-A-I-S-A-T-A
 S27 Y-V-I-T-S-N-V-A-I-S-A-T-A-F-A-A-E-L-F-V-Q-N-L-V-E-E
 S28 F-A-A-E-L-F-V-Q-N-L-V-E-E-S-L-V-L-A-Q-L-N-S-K-G-K-T
 S29 S-L-V-L-A-Q-L-N-S-K-G-K-T-S-L-R-L-S-L-N-S-I-E-E-C-V
 S30 S-L-R-L-S-L-N-S-I-E-E-C-V-E-K-R-D-N-F-R-F-L-E-D-A-I
 T 1 E-K-R-D-N-F-R-F-L-E-D-A-I-K-Q-L-K-K-N-S-A-L-D-K-K-R
 T 2 K-Q-L-K-K-N-S-A-L-D-K-K-R-E-L-N-M-Q-P-G-R-S-D-Q-E-V
 T 3 E-L-N-M-Q-P-G-R-S-D-Q-E-V-V-I-E-E-P-E-L-H-E-D-D-G-V
 T 4 V-I-E-E-P-E-L-H-E-D-D-G-V-E-E-E-E-E-E-D-E-V-S-E-E-E
 T 5 E-E-E-E-E-E-D-E-V-S-E-E-E-E-P-V-H-N-E-E-L-L-D-D-S-K
 T 6 E-P-V-H-N-E-E-L-L-D-D-S-K-D-Q-Q-N-D-K-S-T-R-S-V-A-S
 T 7 D-Q-Q-N-D-K-S-T-R-S-V-A-S-L-L-S-R-F-Q-Y-K-S-A-L-D-V
 T 8 L-L-S-R-F-Q-Y-K-S-A-L-D-V-G-E-H-S-D-S-S-D-I-E-V-D-H
 T 9 Y-K-S-A-L-D-V-G-E-H-S-D-S-S-D-I-E-V-D-H-T-K-S-T-D-P
 T10
 T11 Dpb4 M-P-P-K-G-W-R-K-D-A-Q-G-N-Y-P-T-T-S-Y-I-K-E-Q-E-N-I
 T12 Y-P-T-T-S-Y-I-K-E-Q-E-N-I-T-I-Q-D-L-L-F-P-K-S-T-I-V
 T13 T-I-Q-D-L-L-F-P-K-S-T-I-V-N-L-A-R-E-V-P-Q-Q-S-G-K-K
 T14 N-L-A-R-E-V-P-Q-Q-S-G-K-K-L-L-I-N-K-D-A-S-L-A-L-Q-R
 T15 L-L-I-N-K-D-A-S-L-A-L-Q-R-G-A-T-V-F-V-N-H-L-L-L-F-A
 T16 G-A-T-V-F-V-N-H-L-L-L-F-A-R-E-I-A-K-S-Q-D-K-K-S-C-S
 T17 R-E-I-A-K-S-Q-D-K-K-S-C-S-V-D-D-V-L-S-A-L-D-H-I-G-H
 T18 V-D-D-V-L-S-A-L-D-H-I-G-H-S-A-L-K-G-P-V-R-D-K-L-D-E
 T19 S-A-L-K-G-P-V-R-D-K-L-D-E-Y-Q-A-A-V-E-Q-R-K-K-E-K-L
 T20 Y-Q-A-A-V-E-Q-R-K-K-E-K-L-D-S-G-E-V-D-A-D-G-D-I-D-M
 T21 D-S-G-E-V-D-A-D-G-D-I-D-M-G-E-D-K-E-N-V-P-V-E-K-V-K
 T22 G-E-D-K-E-N-V-P-V-E-K-V-K-E-H-D-E-I-E-E-Q-G-D-A-L-Q
 T23 E-H-D-E-I-E-E-Q-G-D-A-L-Q-D-V-E-E-S-S-E-K-K-Q-K-T-E
 T24 D-V-E-E-S-S-E-K-K-Q-K-T-E-S-Q-D-V-E-T-R-V-Q-N-L-E-Q
 T25 V-E-E-S-S-E-K-K-Q-K-T-E-S-Q-D-V-E-T-R-V-Q-N-L-E-Q-T
 T26

Membrane 5

A 1 Orc2 M-L-N-G-E-D-F-V-E-H-N-D-I-L-S-S-P-A-K-S-R-N-V-T-P-K
A 2 L-S-S-P-A-K-S-R-N-V-T-P-K-R-V-D-P-H-G-E-R-Q-L-R-R-I
A 3 R-V-D-P-H-G-E-R-Q-L-R-R-I-H-S-S-K-K-N-L-L-E-R-I-S-L
A 4 H-S-S-K-K-N-L-L-E-R-I-S-L-V-G-N-E-R-K-N-T-S-P-D-P-A
A 5 V-G-N-E-R-K-N-T-S-P-D-P-A-L-K-P-K-T-P-S-K-A-P-R-K-R
A 6 L-K-P-K-T-P-S-K-A-P-R-K-R-G-R-P-R-K-I-Q-E-E-L-T-D-R
A 7 G-R-P-R-K-I-Q-E-E-L-T-D-R-I-K-K-D-E-K-D-T-I-S-S-K-K
A 8 I-K-K-D-E-K-D-T-I-S-S-K-K-K-R-K-L-D-K-D-T-S-G-N-V-N
A 9 K-R-K-L-D-K-D-T-S-G-N-V-N-E-E-S-K-T-S-N-N-K-Q-V-M-E
A10 E-E-S-K-T-S-N-N-K-Q-V-M-E-K-T-G-I-K-E-K-R-E-R-E-K-I
A11 K-T-G-I-K-E-K-R-E-R-E-K-I-Q-V-A-T-T-T-Y-E-D-N-V-T-P
A12 Q-V-A-T-T-T-Y-E-D-N-V-T-P-Q-T-D-D-N-F-V-S-N-S-P-E-P
A13 Q-T-D-D-N-F-V-S-N-S-P-E-P-P-E-P-A-T-P-S-K-K-S-L-T-T
A14 P-E-P-A-T-P-S-K-K-S-L-T-T-N-H-D-F-T-S-P-L-K-Q-I-I-M
A15 N-H-D-F-T-S-P-L-K-Q-I-I-M-N-N-L-K-E-Y-K-D-S-T-S-P-G
A16 N-N-L-K-E-Y-K-D-S-T-S-P-G-K-L-T-L-S-R-N-F-T-P-T-P-V
A17 K-L-T-L-S-R-N-F-T-P-T-P-V-P-K-N-K-K-L-Y-Q-T-S-E-T-K
A18 P-K-N-K-K-L-Y-Q-T-S-E-T-K-S-A-S-S-F-L-D-T-F-E-G-Y-F
A19 S-A-S-S-F-L-D-T-F-E-G-Y-F-D-Q-R-K-I-V-R-T-N-A-K-S-R
A20 D-Q-R-K-I-V-R-T-N-A-K-S-R-H-T-M-S-M-A-P-D-V-T-R-E-E
A21 H-T-M-S-M-A-P-D-V-T-R-E-E-F-S-L-V-S-N-F-F-N-E-N-F-Q
A22 F-S-L-V-S-N-F-F-N-E-N-F-Q-K-R-P-R-Q-K-L-F-E-I-Q-K-K
A23 K-R-P-R-Q-K-L-F-E-I-Q-K-K-M-F-P-Q-Y-W-F-E-L-T-Q-G-F
A24 M-F-P-Q-Y-W-F-E-L-T-Q-G-F-S-L-L-F-Y-G-V-G-S-K-R-N-F
A25 S-L-L-F-Y-G-V-G-S-K-R-N-F-L-E-E-F-A-I-D-Y-L-S-P-K-I
A26 L-E-E-F-A-I-D-Y-L-S-P-K-I-A-Y-S-Q-L-A-Y-E-N-E-L-Q-Q
A27 A-Y-S-Q-L-A-Y-E-N-E-L-Q-Q-N-K-P-V-N-S-I-P-C-L-I-L-N
A28 N-K-P-V-N-S-I-P-C-L-I-L-N-G-Y-N-P-S-C-N-Y-R-D-V-F-K
A29 G-Y-N-P-S-C-N-Y-R-D-V-F-K-E-I-T-D-L-L-V-P-A-E-L-T-R
A30 E-I-T-D-L-L-V-P-A-E-L-T-R-S-E-T-K-Y-W-G-N-H-V-I-L-Q
B 1 S-E-T-K-Y-W-G-N-H-V-I-L-Q-I-Q-K-M-I-D-F-Y-K-N-Q-P-L
B 2 I-Q-K-M-I-D-F-Y-K-N-Q-P-L-D-I-K-L-I-L-V-V-H-N-L-D-G
B 3 D-I-K-L-I-L-V-V-H-N-L-D-G-P-S-I-R-K-N-T-F-Q-T-M-L-S
B 4 P-S-I-R-K-N-T-F-Q-T-M-L-S-F-L-S-V-I-R-Q-I-A-I-V-A-S
B 5 F-L-S-V-I-R-Q-I-A-I-V-A-S-T-D-H-I-Y-A-P-L-L-W-D-N-M
B 6 T-D-H-I-Y-A-P-L-L-W-D-N-M-K-A-Q-N-Y-N-F-V-F-H-D-I-S
B 7 K-A-Q-N-Y-N-F-V-F-H-D-I-S-N-F-E-P-S-T-V-E-S-T-F-Q-D
B 8 N-F-E-P-S-T-V-E-S-T-F-Q-D-V-M-K-M-G-K-S-D-T-S-S-G-A
B 9 V-M-K-M-G-K-S-D-T-S-S-G-A-E-G-A-K-Y-V-L-Q-S-L-T-V-N
B10 E-G-A-K-Y-V-L-Q-S-L-T-V-N-S-K-K-M-Y-K-L-L-I-E-T-Q-M
B11 S-K-K-M-Y-K-L-L-I-E-T-Q-M-Q-N-M-G-N-L-S-A-N-T-G-P-K
B12 Q-N-M-G-N-L-S-A-N-T-G-P-K-R-G-T-Q-R-T-G-V-E-L-K-L-F
B13 R-G-T-Q-R-T-G-V-E-L-K-L-F-N-H-L-C-A-A-D-F-I-A-S-N-E
B14 N-H-L-C-A-A-D-F-I-A-S-N-E-I-A-L-R-S-M-L-R-E-F-I-E-H
B15 I-A-L-R-S-M-L-R-E-F-I-E-H-K-M-A-N-I-T-K-N-N-S-G-M-E
B16 K-M-A-N-I-T-K-N-N-S-G-M-E-I-I-W-V-P-Y-T-Y-A-E-L-E-K
B17 S-G-M-E-I-I-W-V-P-Y-T-Y-A-E-L-E-K-L-L-K-T-V-L-N-T-L
B18

B19 Orc3 M-S-D-L-N-Q-S-K-K-M-N-V-S-E-F-A-D-A-Q-R-S-H-Y-T-V-Y
 B20 E-F-A-D-A-Q-R-S-H-Y-T-V-Y-P-S-L-P-Q-S-N-K-N-D-K-H-I
 B21 P-S-L-P-Q-S-N-K-N-D-K-H-I-P-F-V-K-L-L-S-G-K-E-S-E-V
 B22 P-F-V-K-L-L-S-G-K-E-S-E-V-N-V-E-K-R-W-E-L-Y-H-Q-L-H
 B23 N-V-E-K-R-W-E-L-Y-H-Q-L-H-S-H-F-H-D-Q-V-D-H-I-I-D-N
 B24 S-H-F-H-D-Q-V-D-H-I-I-D-N-I-E-A-D-L-K-A-E-I-S-D-L-L
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 B26 Y-S-E-T-T-Q-K-R-R-C-F-N-T-I-F-L-L-G-S-D-S-T-T-K-I-E
 B27 I-F-L-L-G-S-D-S-T-T-K-I-E-L-K-D-E-S-S-R-Y-N-V-L-I-E
 B28 L-K-D-E-S-S-R-Y-N-V-L-I-E-L-T-P-K-E-S-P-N-V-R-M-M-L
 B29 L-T-P-K-E-S-P-N-V-R-M-M-L-R-R-S-M-Y-K-L-Y-S-A-A-D-A
 B30 R-R-S-M-Y-K-L-Y-S-A-A-D-A-E-E-H-P-T-I-K-Y-E-D-I-N-D
 C 1 E-E-H-P-T-I-K-Y-E-D-I-N-D-E-D-G-D-F-T-E-Q-N-N-D-V-S
 C 2 E-D-G-D-F-T-E-Q-N-N-D-V-S-Y-D-L-S-L-V-E-N-F-K-R-L-F
 C 3 Y-D-L-S-L-V-E-N-F-K-R-L-F-G-K-D-L-A-M-V-F-N-F-K-D-V
 C 4 G-K-D-L-A-M-V-F-N-F-K-D-V-D-S-I-N-F-N-T-L-D-N-F-I-I
 C 5 D-S-I-N-F-N-T-L-D-N-F-I-I-L-L-K-S-A-F-K-Y-D-H-V-K-I
 C 6 L-L-K-S-A-F-K-Y-D-H-V-K-I-S-L-I-F-N-I-N-T-N-L-S-N-I
 C 7 S-L-I-F-N-I-N-T-N-L-S-N-I-E-K-N-L-R-Q-S-T-I-R-L-L-K
 C 8 E-K-N-L-R-Q-S-T-I-R-L-L-K-R-N-Y-H-K-L-D-V-S-S-N-K-G
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 D 5 F-E-N-M-G-L-I-K-F-Q-S-T-K-S-Y-D-L-V-E-K-C-V-W-R-G-I
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 D 8 E-I-K-E-E-M-I-Q-L-Y-H-D-L-P-G-I-E-N-E-Y-K-L-I-D-K-I
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D15 P-L-C-D-A-K-R-V-R-D-Q-V-I-A-V-L-P-Y-Y-P-H-E-E-F-R-T
D16 A-V-L-P-Y-Y-P-H-E-E-F-R-T-F-Y-R-D-L-P-I-K-G-I-K-K-Y
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D20 L-F-N-L-E-L-G-R-G-V-L-V-D-F-G-L-A-E-A-Q-M-D-Y-K-S-M
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E 1 L-G-R-R-F-P-M-F-Q-S-L-D-D-A-D-S-L-L-E-L-C-T-I-F-G-W
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E 5 Y-S-N-G-L-K-E-F-V-Y-D-L-L-N-K-E-C-T-I-G-T-F-P-E-Y-S
E 6 N-K-E-C-T-I-G-T-F-P-E-Y-S-V-A-F-E-T-F-G-F-L-Q-Q-E-L
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E 8 H-D-R-M-S-I-E-P-Q-L-P-D-P-K-T-N-M-D-A-V-D-A-Y-E-L-K
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E16 Clb6 M-N-C-I-P-S-P-I-S-E-R-K-I-Q-I-N-N-E-D-C-I-G-K-E-N-A
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F 1 N-K-L-Q-L-L-C-I-T-C-L-F-I-A-C-K-F-E-E-V-K-L-P-K-I-T
 F 2 A-C-K-F-E-E-V-K-L-P-K-I-T-N-F-A-Y-V-T-D-G-A-A-T-V-E
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 G 9 Sld2 M-Y-S-F-E-L-D-K-L-K-I-E-L-K-T-W-E-H-D-F-I-D-K-N-K-R
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G22 T-S-S-P-C-K-N-R-N-G-K-L-V-E-I-K-K-C-S-P-T-I-N-P-P-L
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G29 R-P-L-T-K-S-L-I-E-L-A-R-E-H-T-E-I-V-K-E-F-G-V-L-Q-E
G30 H-T-E-I-V-K-E-F-G-V-L-Q-E-E-D-I-E-E-E-E-E-G-E-E-G-E
H 1 E-D-I-E-E-E-E-E-G-E-E-G-E-N-G-Y-D-E-K-N-H-E-D-D-F-G
H 2 N-G-Y-D-E-K-N-H-E-D-D-F-G-L-E-D-E-L-I-R-P-K-V-V-K-D
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H 6 K-V-I-R-R-L-R-D-N-D-P-E-T-E-T-A-G-F-E-R-D-V-H-K-E-L
H 7 E-T-A-G-F-E-R-D-V-H-K-E-L-V-K-L-K-R-R-K-V-A-E-F-L-G
H 8 V-K-L-K-R-R-K-V-A-E-F-L-G-S-T-S-Q-I-S-D-T-E-F-E-H-D
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H14 Cdc45 M-Y-Y-G-I-S-Q-F-S-E-A-Y-N-K-I-L-R-N-S-S-S-H-S-S-C-Q
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H20 N-S-L-L-L-V-G-F-G-G-V-I-D-L-E-A-F-L-E-I-D-P-Q-E-Y-V
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J 2 F-E-S-S-I-I-E-I-R-R-E-D-L-S-P-F-L-E-K-L-T-L-S-G-L-L
J 3
J 4 Dpb11 M-K-P-F-Q-G-I-T-F-C-P-T-A-I-N-N-E-I-L-A-K-K-I-S-K-K
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J 7 R-Q-V-N-V-L-V-V-G-S-T-T-N-T-N-K-F-K-F-A-V-K-H-R-F-D
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 L 2
 L 3 Sld5 M-D-I-N-I-D-D-I-L-A-E-L-D-K-E-T-T-A-V-D-S-T-K-I-T-Q
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 L21 P-P-D-G-K-W-N-E-D-P-L-L-Q-E-N-E-F-G-K-P-C-Y-T-V-T-I
 L22 E-N-E-F-G-K-P-C-Y-T-V-T-I-P-D-L-K-E-E-V-E-L-T-I-G-S
 L23 P-D-L-K-E-E-V-E-L-T-I-G-S-I-Y-V-M-R-Y-E-V-I-R-D-L-L
 L24 L-T-I-G-S-I-Y-V-M-R-Y-E-V-I-R-D-L-L-R-D-D-K-V-A-L-I

L25
L26 Psf1 M-Y-G-D-L-G-N-K-L-V-L-E-A-K-R-T-K-Q-L-Y-A-R-S-N-Q-D
L27 K-R-T-K-Q-L-Y-A-R-S-N-Q-D-V-N-L-P-M-Y-H-E-D-I-I-R-N
L28 V-N-L-P-M-Y-H-E-D-I-I-R-N-I-L-K-E-V-S-N-L-R-K-N-T-E
L29 I-L-K-E-V-S-N-L-R-K-N-T-E-Y-L-K-E-Q-Q-Q-L-G-M-L-D-D
L30 Y-L-K-E-Q-Q-Q-L-G-M-L-D-D-K-V-A-K-C-Q-Y-F-V-T-L-L-C
M 1 K-V-A-K-C-Q-Y-F-V-T-L-L-C-M-E-R-N-K-R-C-L-L-A-Y-Q-R
M 2 M-E-R-N-K-R-C-L-L-A-Y-Q-R-L-R-T-D-I-L-D-S-M-A-W-N-N
M 3 L-R-T-D-I-L-D-S-M-A-W-N-N-N-G-L-D-L-M-S-S-I-T-F-S-Q
M 4 N-G-L-D-L-M-S-S-I-T-F-S-Q-Q-D-T-N-N-L-S-H-Q-E-Q-E-Y
M 5 Q-D-T-N-N-L-S-H-Q-E-Q-E-Y-L-K-E-Y-C-D-L-I-T-D-L-K-S
M 6 L-K-E-Y-C-D-L-I-T-D-L-K-S-G-D-L-V-D-I-D-L-S-G-S-L-V
M 7 G-D-L-V-D-I-D-L-S-G-S-L-V-P-P-S-D-V-F-I-D-V-R-V-L-K
M 8 P-P-S-D-V-F-I-D-V-R-V-L-K-D-A-G-E-I-Q-T-E-Y-G-V-F-N
M 9 D-A-G-E-I-Q-T-E-Y-G-V-F-N-L-I-K-D-S-Q-F-F-V-R-Q-S-D
M10 L-I-K-D-S-Q-F-F-V-R-Q-S-D-V-E-R-L-I-Q-Q-G-Y-L-Q-K-I
M11
M12 Psf2 M-S-L-P-A-H-L-Q-Q-T-F-S-P-E-E-I-Q-F-I-V-E-N-E-P-I-K
M13 E-E-I-Q-F-I-V-E-N-E-P-I-K-I-F-P-R-I-T-T-R-Q-K-I-R-G
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M22 K-D-D-F-H-D-P-I-H-E-L-R-G-K-I-Q-D-L-R-E-I-R-Q-I-K-V
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N 3 I-L-A-I-V-G-G-D-E-A-L-V-D-E-E-P-V-P-F-V-E-L-L-P-P-D
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N 6 P-V-A-L-D-L-H-S-I-N-S-H-F-F-S-L-A-I-K-W-I-M-L-F-S-E
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N12 L-E-E-M-E-K-E-I-Y-K-K-S-H-E-S-Y-K-D-T-K-R-W-M-F-K-K
N13
N14 Pol12 M-S-G-S-I-D-V-I-T-H-F-G-P-D-A-D-K-P-E-I-I-T-A-L-E-N
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 O 6 T-I-Q-S-Q-S-E-I-Y-A-V-G-R-I-V-P-D-S-P-T-Y-D-K-F-L-N
 O 7 I-V-P-D-S-P-T-Y-D-K-F-L-N-P-E-S-L-S-L-E-T-S-R-M-G-G
 O 8 P-E-S-L-S-L-E-T-S-R-M-G-G-V-G-R-R-V-R-L-D-L-S-Q-V-N
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 P 6 D-G-K-L-T-L-V-E-G-E-E-P-V-Y-L-H-N-V-W-K-R-A-R-V-D-L

P 7 L-T-L-V-E-G-E-E-P-V-Y-L-H-N-V-W-K-R-A-R-V-D-L-I-A-S
P 8
P 9 Pri1 M-T-N-S-V-K-T-N-G-P-S-S-S-D-M-E-Y-Y-Y-K-S-L-Y-P-F-K
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Q 2 C-K-E-D-L-V-L-M-T-L-Y-P-K-L-D-V-E-V-T-K-Q-T-I-H-L-L
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Q 6 P-K-L-I-D-L-Q-T-E-M-E-K-N-N-D-V-S-L-T-A-L-Q-P-F-I-N
Q 7 N-D-V-S-L-T-A-L-Q-P-F-I-N-Q-F-Q-A-Y-V-S-S-L-L-K-N-E
Q 8 Q-F-Q-A-Y-V-S-S-L-L-K-N-E-L-G-S-V-K-R-E-R-E-D-D-D-E
Q 9 S-S-L-L-K-N-E-L-G-S-V-K-R-E-R-E-D-D-D-E-P-A-S-L-D-F
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Q21 D-Y-Y-S-H-F-I-L-R-L-C-F-C-R-S-K-E-L-R-E-K-F-V-R-A-E
Q22 R-S-K-E-L-R-E-K-F-V-R-A-E-T-F-L-F-K-I-R-F-N-M-L-F-T
Q23 T-F-L-F-K-I-R-F-N-M-L-F-T-T-S-T-D-Q-T-K-F-V-Q-S-L-D
Q24 T-S-T-D-Q-T-K-F-V-Q-S-L-D-L-P-L-L-Q-F-I-S-N-E-E-K-A
Q25 L-P-L-L-Q-F-I-S-N-E-E-K-A-E-L-S-H-Q-L-Y-Q-T-V-S-A-S
Q26 E-L-S-H-Q-L-Y-Q-T-V-S-A-S-L-Q-F-Q-L-N-L-N-E-E-H-Q-R
Q27 L-Q-F-Q-L-N-L-N-E-E-H-Q-R-K-Q-Y-F-Q-Q-E-F-T-K-F-I-K

Q28 K-Q-Y-F-Q-Q-E-F-T-K-F-I-K-L-P-F-E-N-V-I-E-L-V-G-N-R
 Q29 L-P-F-E-N-V-I-E-L-V-G-N-R-L-V-F-L-K-D-G-Y-A-Y-L-P-Q
 Q30 L-V-F-L-K-D-G-Y-A-Y-L-P-Q-F-Q-Q-L-N-L-L-S-N-E-F-A-S
 R 1 F-Q-Q-L-N-L-L-S-N-E-F-A-S-K-L-N-Q-E-L-I-K-T-Y-Q-Y-L
 R 2 K-L-N-Q-E-L-I-K-T-Y-Q-Y-L-P-R-L-F-T-N-E-D-D-R-L-L-P
 R 3 P-R-L-F-T-N-E-D-D-R-L-L-P-I-L-N-H-L-S-S-G-Y-T-I-A-D
 R 4 I-L-N-H-L-S-S-G-Y-T-I-A-D-F-N-Q-Q-K-A-N-Q-F-S-E-N-V
 R 5 F-N-Q-Q-K-A-N-Q-F-S-E-N-V-D-D-E-I-N-A-Q-S-V-W-S-E-E
 R 6 D-D-E-I-N-A-Q-S-V-W-S-E-E-I-S-S-N-Y-P-L-C-I-K-N-L-F
 R 7 I-S-S-N-Y-P-L-C-I-K-N-L-F-T-M-E-G-L-K-K-N-H-H-L-R-Y
 R 8 T-M-E-G-L-K-K-N-H-H-L-R-Y-Y-G-R-Q-Q-L-S-L-F-L-K-G-I
 R 9 Y-G-R-Q-Q-L-S-L-F-L-K-G-I-G-L-S-A-D-E-A-L-K-F-W-S-E
 R10 G-L-S-A-D-E-A-L-K-F-W-S-E-A-F-T-R-N-G-N-M-T-M-E-K-F
 R11 A-F-T-R-N-G-N-M-T-M-E-K-F-N-K-E-Y-R-Y-S-F-F-T-R-H-N
 R12 N-K-E-Y-R-Y-S-F-F-T-R-H-N-Y-G-L-E-G-N-R-I-N-Y-K-P-W
 R13 Y-G-L-E-G-N-R-I-N-Y-K-P-W-D-C-H-T-I-L-S-K-P-R-P-G-R
 R14 D-C-H-T-I-L-S-K-P-R-P-G-R-G-D-Y-H-G-C-P-F-R-D-W-S-H
 R15 G-D-Y-H-G-C-P-F-R-D-W-S-H-E-R-L-S-A-E-L-R-S-M-K-L-T
 R16 E-R-L-S-A-E-L-R-S-M-K-L-T-Q-A-Q-I-F-T-I-S-V-L-D-S-C
 R17 Q-A-Q-I-F-T-I-S-V-L-D-S-C-Q-K-G-E-Y-T-I-A-C-T-K-V-F
 R18 Q-K-G-E-Y-T-I-A-C-T-K-V-F-E-M-T-H-N-S-A-S-A-D-L-E-I
 R19 E-M-T-H-N-S-A-S-A-D-L-E-I-G-E-Q-T-H-I-A-H-P-N-L-Y-F
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 R21 E-R-S-R-Q-L-Q-K-K-Q-Q-K-L-F-T-E-K-E-K-L-F-N-N-G-N-H
 R22
 R23 Pol31 M-D-A-L-L-T-K-F-N-E-D-R-S-L-Q-D-E-N-L-S-Q-P-R-T-R-V
 R24 L-Q-D-E-N-L-S-Q-P-R-T-R-V-R-I-V-D-D-N-L-Y-N-K-S-N-P
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 R28 E-R-V-L-K-E-C-D-K-R-W-D-A-G-F-T-L-N-G-Q-L-V-L-K-K-D
 R29 G-F-T-L-N-G-Q-L-V-L-K-K-D-K-V-L-D-I-Q-G-N-Q-P-C-W-C
 R30 K-V-L-D-I-Q-G-N-Q-P-C-W-C-V-G-S-I-Y-C-E-M-K-Y-K-P-N
 S 1 V-G-S-I-Y-C-E-M-K-Y-K-P-N-V-L-D-E-V-I-N-D-T-Y-G-A-P
 S 2 V-L-D-E-V-I-N-D-T-Y-G-A-P-D-L-T-K-S-Y-T-D-K-E-G-G-S
 S 3 D-L-T-K-S-Y-T-D-K-E-G-G-S-D-E-I-M-L-E-D-E-S-G-R-V-L
 S 4 D-E-I-M-L-E-D-E-S-G-R-V-L-L-V-G-D-F-I-R-S-T-P-F-I-T
 S 5 L-V-G-D-F-I-R-S-T-P-F-I-T-G-V-V-V-G-I-L-G-M-E-A-E-A
 S 6 G-V-V-V-G-I-L-G-M-E-A-E-A-G-T-F-Q-V-L-D-I-C-Y-P-T-P
 S 7 G-T-F-Q-V-L-D-I-C-Y-P-T-P-L-P-Q-N-P-F-P-A-P-I-A-T-C
 S 8 L-P-Q-N-P-F-P-A-P-I-A-T-C-P-T-R-G-K-I-A-L-V-S-G-L-N
 S 9 P-T-R-G-K-I-A-L-V-S-G-L-N-L-N-N-T-S-P-D-R-L-L-R-L-E
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 S12 I-D-D-I-S-L-I-G-R-L-L-I-C-G-N-S-V-D-F-D-I-K-S-V-N-K
 S13 G-N-S-V-D-F-D-I-K-S-V-N-K-D-E-L-M-I-S-L-T-E-F-S-K-F
 S14 D-E-L-M-I-S-L-T-E-F-S-K-F-L-H-N-I-L-P-S-I-S-V-D-I-M
 S15 L-H-N-I-L-P-S-I-S-V-D-I-M-P-G-T-N-D-P-S-D-K-S-L-P-Q
 S16 P-G-T-N-D-P-S-D-K-S-L-P-Q-Q-P-F-H-K-S-L-F-D-K-S-L-E
 S17 Q-P-F-H-K-S-L-F-D-K-S-L-E-S-Y-F-N-G-S-N-K-E-I-L-N-L
 S18 S-Y-F-N-G-S-N-K-E-I-L-N-L-V-T-N-P-Y-E-F-S-Y-N-G-V-D

S19 V-T-N-P-Y-E-F-S-Y-N-G-V-D-V-L-A-V-S-G-K-N-I-N-D-I-C
S20 V-L-A-V-S-G-K-N-I-N-D-I-C-K-Y-V-I-P-S-N-D-N-G-E-S-E
S21 K-Y-V-I-P-S-N-D-N-G-E-S-E-N-K-V-E-E-G-E-S-N-D-F-K-D
S22 N-K-V-E-E-G-E-S-N-D-F-K-D-D-I-E-H-R-L-D-L-M-E-C-T-M
S23 D-I-E-H-R-L-D-L-M-E-C-T-M-K-W-Q-N-I-A-P-T-A-P-D-T-L
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S25 W-C-Y-P-Y-T-D-K-D-P-F-V-L-D-K-W-P-H-V-Y-I-V-A-N-Q-P
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S27 Y-F-G-T-R-V-V-E-I-G-G-K-N-I-K-I-I-S-V-P-E-F-S-S-T-G
S28 I-K-I-I-S-V-P-E-F-S-S-T-G-M-I-I-L-L-D-L-E-T-L-E-A-E
S29 P-E-F-S-S-T-G-M-I-I-L-L-D-L-E-T-L-E-A-E-T-V-K-I-D-I
S30
T 1 Pol130 M-L-E-A-K-F-E-E-A-S-L-F-K-R-I-I-D-G-F-K-D-C-V-Q-L-V
T 2 R-I-I-D-G-F-K-D-C-V-Q-L-V-N-F-Q-C-K-E-D-G-I-I-A-Q-A
T 3 N-F-Q-C-K-E-D-G-I-I-A-Q-A-V-D-D-S-R-V-L-L-V-S-L-E-I
T 4 V-D-D-S-R-V-L-L-V-S-L-E-I-G-V-E-A-F-Q-E-Y-R-C-D-H-P
T 5 G-V-E-A-F-Q-E-Y-R-C-D-H-P-V-T-L-G-M-D-L-T-S-L-S-K-I
T 6 V-T-L-G-M-D-L-T-S-L-S-K-I-L-R-C-G-N-N-T-D-T-L-T-L-I
T 7 L-R-C-G-N-N-T-D-T-L-T-L-I-A-D-N-T-P-D-S-I-I-L-L-F-E
T 8 A-D-N-T-P-D-S-I-I-L-L-F-E-D-T-K-K-D-R-I-A-E-Y-S-L-K
T 9 D-T-K-K-D-R-I-A-E-Y-S-L-K-L-M-D-I-D-A-D-F-L-K-I-E-E
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T12 F-S-K-I-V-R-D-L-S-Q-L-S-D-S-I-N-I-M-I-T-K-E-T-I-K-F
T13 S-I-N-I-M-I-T-K-E-T-I-K-F-V-A-D-G-D-I-G-S-G-S-V-I-I
T14 V-A-D-G-D-I-G-S-G-S-V-I-I-K-P-F-V-D-M-E-H-P-E-T-S-I
T15 K-P-F-V-D-M-E-H-P-E-T-S-I-K-L-E-M-D-Q-P-V-D-L-T-F-G
T16 K-L-E-M-D-Q-P-V-D-L-T-F-G-A-K-Y-L-L-D-I-I-K-G-S-S-L
T17 A-K-Y-L-L-D-I-I-K-G-S-S-L-S-D-R-V-G-I-R-L-S-S-E-A-P
T18 S-D-R-V-G-I-R-L-S-S-E-A-P-A-L-F-Q-F-D-L-K-S-G-F-L-Q
T19 A-P-A-L-F-Q-F-D-L-K-S-G-F-L-Q-F-F-L-A-P-K-F-N-D-E-E
T20
T21 Rfa3 M-A-S-E-T-P-R-V-D-P-T-E-I-S-N-V-N-A-P-V-F-R-I-I-A-Q
T22 S-N-V-N-A-P-V-F-R-I-I-A-Q-I-K-S-Q-P-T-E-S-Q-L-I-L-Q
T23 I-K-S-Q-P-T-E-S-Q-L-I-L-Q-S-P-T-I-S-S-K-N-G-S-E-V-E
T24 S-P-T-I-S-S-K-N-G-S-E-V-E-M-I-T-L-N-N-I-R-V-S-M-N-K
T25 M-I-T-L-N-N-I-R-V-S-M-N-K-T-F-E-I-D-S-W-Y-E-F-V-C-R
T26 T-F-E-I-D-S-W-Y-E-F-V-C-R-N-N-D-D-G-E-L-G-F-L-I-L-D
T27 N-N-D-D-G-E-L-G-F-L-I-L-D-A-V-L-C-K-F-K-E-N-E-D-L-S
T28 A-V-L-C-K-F-K-E-N-E-D-L-S-L-N-G-V-V-A-L-Q-R-L-C-K-K
T29 F-K-E-N-E-D-L-S-L-N-G-V-V-A-L-Q-R-L-C-K-K-Y-P-E-I-Y
T30

Membrane 6

A 1 Pol13 M-S-E-K-R-S-L-P-M-V-D-V-K-I-D-D-E-D-T-P-Q-L-E-K-K-I
A 2 I-D-D-E-D-T-P-Q-L-E-K-K-I-K-R-Q-S-I-D-H-G-V-G-S-E-P
A 3 K-R-Q-S-I-D-H-G-V-G-S-E-P-V-S-T-I-E-I-I-P-S-D-S-F-R
A 4 V-S-T-I-E-I-I-P-S-D-S-F-R-K-Y-N-S-Q-G-F-K-A-K-D-T-D
A 5 K-Y-N-S-Q-G-F-K-A-K-D-T-D-L-M-G-T-Q-L-E-S-T-F-E-Q-D
A 6 L-M-G-T-Q-L-E-S-T-F-E-Q-D-V-S-Q-M-E-H-D-M-A-D-Q-E-E
A 7 V-S-Q-M-E-H-D-M-A-D-Q-E-E-H-D-L-S-S-F-E-R-K-K-L-P-T
A 8 H-D-L-S-S-F-E-R-K-K-L-P-T-D-F-D-P-S-L-Y-D-I-S-F-Q-Q
A 9 D-F-D-P-S-L-Y-D-I-S-F-Q-Q-I-D-A-E-Q-S-V-L-N-G-I-K-D
A10 I-D-A-E-Q-S-V-L-N-G-I-K-D-E-N-T-S-T-V-V-R-F-F-G-V-T
A11 E-N-T-S-T-V-V-R-F-F-G-V-T-S-E-G-H-S-V-L-C-N-V-T-G-F
A12 S-E-G-H-S-V-L-C-N-V-T-G-F-K-N-Y-L-Y-V-P-A-P-N-S-S-D
A13 K-N-Y-L-Y-V-P-A-P-N-S-S-D-A-N-D-Q-E-Q-I-N-K-F-V-H-Y
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A25 A-P-L-R-I-M-S-F-D-I-E-C-A-G-R-I-G-V-F-P-E-P-E-Y-D-P
A26 G-R-I-G-V-F-P-E-P-E-Y-D-P-V-I-Q-I-A-N-V-V-S-I-A-G-A
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A30 T-E-E-E-M-L-S-N-W-R-N-F-I-I-K-V-D-P-D-V-I-I-G-Y-N-T
B 1 I-K-V-D-P-D-V-I-I-G-Y-N-T-T-N-F-D-I-P-Y-L-L-N-R-A-K
B 2 T-N-F-D-I-P-Y-L-L-N-R-A-K-A-L-K-V-N-D-F-P-Y-F-G-R-L
B 3 A-L-K-V-N-D-F-P-Y-F-G-R-L-K-T-V-K-Q-E-I-K-E-S-V-F-S
B 4 K-T-V-K-Q-E-I-K-E-S-V-F-S-S-K-A-Y-G-T-R-E-T-K-N-V-N
B 5 S-K-A-Y-G-T-R-E-T-K-N-V-N-I-D-G-R-L-Q-L-D-L-L-Q-F-I
B 6 I-D-G-R-L-Q-L-D-L-L-Q-F-I-Q-R-E-Y-K-L-R-S-Y-T-L-N-A
B 7 Q-R-E-Y-K-L-R-S-Y-T-L-N-A-V-S-A-H-F-L-G-E-Q-K-E-D-V
B 8 V-S-A-H-F-L-G-E-Q-K-E-D-V-H-Y-S-I-I-S-D-L-Q-N-G-D-S
B 9 H-Y-S-I-I-S-D-L-Q-N-G-D-S-E-T-R-R-R-L-A-V-Y-C-L-K-D
B10 E-T-R-R-R-L-A-V-Y-C-L-K-D-A-Y-L-P-L-R-L-M-E-K-L-M-A
B11 A-Y-L-P-L-R-L-M-E-K-L-M-A-L-V-N-Y-T-E-M-A-R-V-T-G-V
B12 L-V-N-Y-T-E-M-A-R-V-T-G-V-P-F-S-Y-L-L-A-R-G-Q-Q-I-K
B13 P-F-S-Y-L-L-A-R-G-Q-Q-I-K-V-V-S-Q-L-F-R-K-C-L-E-I-D
B14 V-V-S-Q-L-F-R-K-C-L-E-I-D-T-V-I-P-N-M-Q-S-Q-A-S-D-D
B15 T-V-I-P-N-M-Q-S-Q-A-S-D-D-Q-Y-E-G-A-T-V-I-E-P-I-R-G
B16 Q-Y-E-G-A-T-V-I-E-P-I-R-G-Y-Y-D-V-P-I-A-T-L-D-F-N-S
B17 Y-Y-D-V-P-I-A-T-L-D-F-N-S-L-Y-P-S-I-M-M-A-H-N-L-C-Y
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B19 T-T-L-C-N-K-A-T-V-E-R-L-N-L-K-I-D-E-D-Y-V-I-T-P-N-G
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 B21 D-Y-F-V-T-T-K-R-R-R-G-I-L-P-I-I-L-D-E-L-I-S-A-R-K-R
 B22 P-I-I-L-D-E-L-I-S-A-R-K-R-A-K-K-D-L-R-D-E-K-D-P-F-K
 B23 A-K-K-D-L-R-D-E-K-D-P-F-K-R-D-V-L-N-G-R-Q-L-A-L-K-I
 B24 R-D-V-L-N-G-R-Q-L-A-L-K-I-S-A-N-S-V-Y-G-F-T-G-A-T-V
 B25 S-A-N-S-V-Y-G-F-T-G-A-T-V-G-K-L-P-C-L-A-I-S-S-S-V-T
 B26 G-K-L-P-C-L-A-I-S-S-S-V-T-A-Y-G-R-T-M-I-L-K-T-K-T-A
 B27 A-Y-G-R-T-M-I-L-K-T-K-T-A-V-Q-E-K-Y-C-I-K-N-G-Y-K-H
 B28 V-Q-E-K-Y-C-I-K-N-G-Y-K-H-D-A-V-V-V-Y-G-D-T-D-S-V-M
 B29 D-A-V-V-V-Y-G-D-T-D-S-V-M-V-K-F-G-T-T-D-L-K-E-A-M-D
 B30 V-K-F-G-T-T-D-L-K-E-A-M-D-L-G-T-E-A-A-K-Y-V-S-T-L-F
 C 1 L-G-T-E-A-A-K-Y-V-S-T-L-F-K-H-P-I-N-L-E-F-E-K-A-Y-F
 C 2 K-H-P-I-N-L-E-F-E-K-A-Y-F-P-Y-L-L-I-N-K-K-R-Y-A-G-L
 C 3 P-Y-L-L-I-N-K-K-R-Y-A-G-L-F-W-T-N-P-D-K-F-D-K-L-D-Q
 C 4 F-W-T-N-P-D-K-F-D-K-L-D-Q-K-G-L-A-S-V-R-R-D-S-C-S-L
 C 5 K-G-L-A-S-V-R-R-D-S-C-S-L-V-S-I-V-M-N-K-V-L-K-K-I-L
 C 6 V-S-I-V-M-N-K-V-L-K-K-I-L-I-E-R-N-V-D-G-A-L-A-F-V-R
 C 7 I-E-R-N-V-D-G-A-L-A-F-V-R-E-T-I-N-D-I-L-H-N-R-V-D-I
 C 8 E-T-I-N-D-I-L-H-N-R-V-D-I-S-K-L-I-I-S-K-T-L-A-P-N-Y
 C 9 S-K-L-I-I-S-K-T-L-A-P-N-Y-T-N-P-Q-P-H-A-V-L-A-E-R-M
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 C13 N-R-A-E-D-P-L-F-V-L-E-N-N-I-Q-V-D-S-R-Y-Y-L-T-N-Q-L
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 D 1 A-S-K-F-Q-S-M-E-L-Q-R-G-D-I-I-R-V-I-I-A-E-P-A-I-V-R
 D 2 I-I-R-V-I-I-A-E-P-A-I-V-R-E-R-K-K-Y-V-L-L-V-D-D-F-E
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 D 4 L-V-Q-S-R-A-D-M-V-N-Q-T-S-T-F-L-D-N-Y-F-S-E-H-P-N-E
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 D 6 T-L-K-D-E-D-I-T-D-S-G-N-V-A-N-Q-T-N-A-S-N-A-G-V-P-D
 D 7 A-N-Q-T-N-A-S-N-A-G-V-P-D-M-L-H-S-N-S-N-L-N-A-N-E-R
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D16 K-L-Q-P-A-K-P-Q-F-T-N-L-T-H-P-Y-E-L-N-L-D-R-D-T-V-I
D17 H-P-Y-E-L-N-L-D-R-D-T-V-I-E-E-C-F-D-E-S-N-V-P-K-T-H
D18 E-E-C-F-D-E-S-N-V-P-K-T-H-F-N-F-I-K-L-D-A-I-Q-N-Q-E
D19 F-N-F-I-K-L-D-A-I-Q-N-Q-E-V-N-S-N-V-D-V-L-G-I-I-Q-T
D20 V-N-S-N-V-D-V-L-G-I-I-Q-T-I-N-P-H-F-E-L-T-S-R-A-G-K
D21 I-N-P-H-F-E-L-T-S-R-A-G-K-K-F-D-R-R-D-I-T-I-V-D-D-S
D22 K-F-D-R-R-D-I-T-I-V-D-D-S-G-F-S-I-S-V-G-L-W-N-Q-Q-A
D23 G-F-S-I-S-V-G-L-W-N-Q-Q-A-L-D-F-N-L-P-E-G-S-V-A-A-I
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D30 F-I-A-Q-R-I-T-I-A-R-A-Q-A-E-N-L-G-R-S-E-K-G-D-F-F-S
E 1 E-N-L-G-R-S-E-K-G-D-F-F-S-V-K-A-A-I-S-F-L-K-V-D-N-F
E 2 V-K-A-A-I-S-F-L-K-V-D-N-F-A-Y-P-A-C-S-N-E-N-C-N-K-K
E 3 A-Y-P-A-C-S-N-E-N-C-N-K-K-V-L-E-Q-P-D-G-T-W-R-C-E-K
E 4 V-L-E-Q-P-D-G-T-W-R-C-E-K-C-D-T-N-N-A-R-P-N-W-R-Y-I
E 5 C-D-T-N-N-A-R-P-N-W-R-Y-I-L-T-I-S-I-I-D-E-T-N-Q-L-W
E 6 L-T-I-S-I-I-D-E-T-N-Q-L-W-L-T-L-F-D-D-Q-A-K-Q-L-L-G
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E 8 V-D-A-N-T-L-M-S-L-K-E-E-D-P-N-E-F-T-K-I-T-Q-S-I-Q-M
E 9 P-N-E-F-T-K-I-T-Q-S-I-Q-M-N-E-Y-D-F-R-I-R-A-R-E-D-T
E10 N-E-Y-D-F-R-I-R-A-R-E-D-T-Y-N-D-Q-S-R-I-R-Y-T-V-A-N
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E13
E14 Rfa2 M-A-T-Y-Q-P-Y-N-E-Y-S-S-V-T-G-G-G-F-E-N-S-E-S-R-P-G
E15 T-G-G-G-F-E-N-S-E-S-R-P-G-S-G-E-S-E-T-N-T-R-V-N-T-L
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E20 T-D-H-T-A-N-I-F-L-T-I-E-D-G-T-G-Q-I-E-V-R-K-W-S-E-D
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F 1 C-E-G-K-D-A-N-S-F-A-V-P-I-P-L-I-S-Q-S-L-N-L-D-E-T-T
 F 2 P-L-I-S-Q-S-L-N-L-D-E-T-T-V-R-N-C-C-T-T-L-T-D-Q-G-F
 F 3 V-R-N-C-C-T-T-L-T-D-Q-G-F-I-Y-P-T-F-D-D-N-N-F-F-A-L
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 F 6 F-T-E-V-K-P-V-L-F-T-D-L-I-H-H-L-K-I-G-P-S-M-A-K-K-L
 F 7 H-H-L-K-I-G-P-S-M-A-K-K-L-M-F-D-Y-Y-K-Q-T-T-N-A-K-Y
 F 8 M-F-D-Y-Y-K-Q-T-T-N-A-K-Y-N-C-V-V-I-C-C-Y-K-D-Q-T-I
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 F10 K-I-I-H-D-L-S-N-I-P-Q-Q-D-S-I-I-D-C-F-I-Y-A-F-N-P-M
 F11 S-I-I-D-C-F-I-Y-A-F-N-P-M-D-S-F-I-P-Y-Y-D-I-I-D-Q-K
 F12 D-S-F-I-P-Y-Y-D-I-I-D-Q-K-D-C-L-T-I-K-N-S-Y-E-L-K-V
 F13 D-C-L-T-I-K-N-S-Y-E-L-K-V-S-E-S-S-K-I-I-E-R-T-K-T-L
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 G 2 Rfc1 M-V-N-I-S-D-F-F-G-K-N-K-K-S-V-R-S-S-T-S-R-P-T-R-Q-V
 G 3 S-V-R-S-S-T-S-R-P-T-R-Q-V-G-S-S-K-P-E-V-I-D-L-D-T-E
 G 4 G-S-S-K-P-E-V-I-D-L-D-T-E-S-D-Q-E-S-T-N-K-T-P-K-K-M
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 G12 F-K-S-A-N-S-N-A-D-P-D-E-I-V-S-E-I-G-S-F-P-E-G-K-P-N
 G13 V-S-E-I-G-S-F-P-E-G-K-P-N-C-L-L-G-L-T-I-V-F-T-G-V-L
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 G18 K-L-E-K-I-K-Q-L-K-I-K-A-I-D-E-E-G-F-K-Q-L-I-A-G-M-P
 G19 D-E-E-G-F-K-Q-L-I-A-G-M-P-A-E-G-G-D-G-E-A-A-E-K-A-R
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 G21 R-K-L-E-E-Q-H-N-I-A-T-K-E-A-E-L-L-V-K-K-E-E-E-R-S-K

G22 A-E-L-L-V-K-K-E-E-E-R-S-K-K-L-A-A-T-R-V-S-G-G-H-L-E
G23 K-L-A-A-T-R-V-S-G-G-H-L-E-R-D-N-V-V-R-E-E-D-K-L-W-T
G24 R-D-N-V-V-R-E-E-D-K-L-W-T-V-K-Y-A-P-T-N-L-Q-Q-V-C-G
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G29 Y-G-P-P-G-I-G-K-T-T-A-A-H-L-V-A-Q-E-L-G-Y-D-I-L-E-Q
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H 1 N-A-S-D-V-R-S-K-T-L-L-N-A-G-V-K-N-A-L-D-N-M-S-V-V-G
H 2 G-V-K-N-A-L-D-N-M-S-V-V-G-Y-F-K-H-N-E-E-A-Q-N-L-N-G
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H 4 K-H-F-V-I-I-M-D-E-V-D-G-M-S-G-G-D-R-G-G-V-G-Q-L-A-Q
H 5 S-G-G-D-R-G-G-V-G-Q-L-A-Q-F-C-R-K-T-S-T-P-L-I-L-I-C
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H 8 V-C-L-D-I-Q-F-R-R-P-D-A-N-S-I-K-S-R-L-M-T-I-A-I-R-E
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I 9 Rfc2 M-F-E-G-F-G-P-N-K-K-R-K-I-S-K-L-A-A-E-Q-S-L-A-Q-Q-P
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 K 1
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 L 9 E-L-E-K-M-K-Q-E-R-R-L-V-K-V-S-K-E-H-N-E-E-A-Q-K-L-L
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 L21 P-E-D-W-P-Y-K-Q-A-R-M-L-F-L-D-P-E-V-I-D-G-N-E-I-N-L
 L22 L-D-P-E-V-I-D-G-N-E-I-N-L-K-W-S-P-P-K-E-K-E-L-I-E-Y
 L23 K-W-S-P-P-K-E-K-E-L-I-E-Y-L-C-D-D-K-K-F-S-E-E-R-V-K
 L24 L-C-D-D-K-K-F-S-E-E-R-V-K-S-G-I-S-R-L-K-K-G-L-K-S-G

L25 S-G-I-S-R-L-K-K-G-L-K-S-G-I-Q-G-R-L-D-G-F-F-Q-V-V-P
L26 I-Q-G-R-L-D-G-F-F-Q-V-V-P-K-T-K-E-Q-L-A-A-A-A-K-R-A
L27 K-T-K-E-Q-L-A-A-A-A-K-R-A-Q-E-N-K-K-L-N-K-N-K-N-K-V
L28 L-A-A-A-A-K-R-A-Q-E-N-K-K-L-N-K-N-K-N-K-V-T-K-G-R-R
L29
L30 Csm3 M-D-Q-D-F-D-S-L-L-L-G-F-N-D-S-D-S-V-Q-K-D-P-T-V-P-N
M 1 D-S-D-S-V-Q-K-D-P-T-V-P-N-G-L-D-G-S-V-V-D-P-T-I-A-D
M 2 G-L-D-G-S-V-V-D-P-T-I-A-D-P-T-A-I-T-A-R-K-R-R-P-Q-V
M 3 P-T-A-I-T-A-R-K-R-R-P-Q-V-K-L-T-A-E-K-L-L-S-D-K-G-L
M 4 K-L-T-A-E-K-L-L-S-D-K-G-L-P-Y-V-L-K-N-A-H-K-R-I-R-I
M 5 P-Y-V-L-K-N-A-H-K-R-I-R-I-S-S-K-K-N-S-Y-D-N-L-S-N-I
M 6 S-S-K-K-N-S-Y-D-N-L-S-N-I-I-Q-F-Y-Q-L-W-A-H-E-L-F-P
M 7 I-Q-F-Y-Q-L-W-A-H-E-L-F-P-K-A-K-F-K-D-F-M-K-I-C-Q-T
M 8 K-A-K-F-K-D-F-M-K-I-C-Q-T-V-G-K-T-D-P-V-L-R-E-Y-R-V
M 9 V-G-K-T-D-P-V-L-R-E-Y-R-V-S-L-F-R-D-E-M-G-M-S-F-D-V
M10 S-L-F-R-D-E-M-G-M-S-F-D-V-G-T-R-E-T-G-Q-D-L-E-R-Q-S
M11 G-T-R-E-T-G-Q-D-L-E-R-Q-S-P-M-V-E-E-H-V-T-S-A-E-E-R
M12 P-M-V-E-E-H-V-T-S-A-E-E-R-P-I-V-A-D-S-F-A-Q-D-K-R-N
M13 P-I-V-A-D-S-F-A-Q-D-K-R-N-V-N-N-V-D-Y-D-N-D-E-D-D-D
M14 V-N-N-V-D-Y-D-N-D-E-D-D-D-I-Y-H-L-S-Y-R-N-R-R-G-R-V
M15 I-Y-H-L-S-Y-R-N-R-R-G-R-V-L-D-E-R-G-N-N-E-T-V-L-N-N
M16 L-D-E-R-G-N-N-E-T-V-L-N-N-V-V-P-P-K-E-D-L-D-A-L-L-K
M17 V-V-P-P-K-E-D-L-D-A-L-L-K-T-F-R-V-Q-G-P-V-G-L-E-E-N
M18 T-F-R-V-Q-G-P-V-G-L-E-E-N-E-K-K-L-L-L-G-W-L-D-A-H-R
M19 E-K-K-L-L-L-G-W-L-D-A-H-R-K-M-E-K-G-S-M-T-E-E-D-V-Q
M20 K-M-E-K-G-S-M-T-E-E-D-V-Q-L-I-Q-S-L-E-E-W-E-M-N-D-I
M21 L-I-Q-S-L-E-E-W-E-M-N-D-I-E-G-Q-H-T-H-Y-D-L-L-P-G-G
M22 E-G-Q-H-T-H-Y-D-L-L-P-G-G-D-E-F-G-V-D-Q-D-E-L-D-A-M
M23 H-Y-D-L-L-P-G-G-D-E-F-G-V-D-Q-D-E-L-D-A-M-K-E-M-G-F
M24
M25 Cdc9 M-R-R-L-L-T-G-C-L-L-S-S-A-R-P-L-K-S-R-L-P-L-L-M-S-S
M26 R-P-L-K-S-R-L-P-L-L-M-S-S-S-L-P-S-S-A-G-K-K-P-K-Q-A
M27 S-L-P-S-S-A-G-K-K-P-K-Q-A-T-L-A-R-F-F-T-S-M-K-N-K-P
M28 T-L-A-R-F-F-T-S-M-K-N-K-P-T-E-G-T-P-S-P-K-K-S-S-K-H
M29 T-E-G-T-P-S-P-K-K-S-S-K-H-M-L-E-E-R-M-D-N-V-S-G-E-E
M30 M-L-E-E-R-M-D-N-V-S-G-E-E-E-Y-A-T-K-K-L-K-Q-T-A-V-T
N 1 E-Y-A-T-K-K-L-K-Q-T-A-V-T-H-T-V-A-A-P-S-S-M-G-S-N-F
N 2 H-T-V-A-A-P-S-S-M-G-S-N-F-S-S-I-P-S-S-A-P-S-S-G-V-A
N 3 S-S-I-P-S-S-A-P-S-S-G-V-A-D-S-P-Q-Q-S-Q-R-L-V-G-E-V
N 4 D-S-P-Q-Q-S-Q-R-L-V-G-E-V-E-D-A-L-S-S-N-N-N-D-H-Y-S
N 5 E-D-A-L-S-S-N-N-N-D-H-Y-S-S-N-I-P-Y-S-E-V-C-E-V-F-N
N 6 S-N-I-P-Y-S-E-V-C-E-V-F-N-K-I-E-A-I-S-S-R-L-E-I-I-R
N 7 K-I-E-A-I-S-S-R-L-E-I-I-R-I-C-S-D-F-F-I-K-I-M-K-Q-S
N 8 I-C-S-D-F-F-I-K-I-M-K-Q-S-S-K-N-V-I-P-T-T-Y-L-F-I-N
N 9 S-K-N-V-I-P-T-T-Y-L-F-I-N-R-L-G-P-D-Y-E-A-G-L-E-L-G
N10 R-L-G-P-D-Y-E-A-G-L-E-L-G-L-G-E-N-L-L-M-K-T-I-S-E-T
N11 L-G-E-N-L-L-M-K-T-I-S-E-T-C-G-K-S-M-S-Q-I-K-L-K-Y-K
N12 C-G-K-S-M-S-Q-I-K-L-K-Y-K-D-I-G-D-L-G-E-I-A-M-G-A-R
N13 D-I-G-D-L-G-E-I-A-M-G-A-R-N-V-Q-P-T-M-F-K-P-K-P-L-T
N14 N-V-Q-P-T-M-F-K-P-K-P-L-T-V-G-E-V-F-K-N-L-R-A-I-A-K
N15 V-G-E-V-F-K-N-L-R-A-I-A-K-T-Q-G-K-D-S-Q-L-K-K-M-K-L

N16 T-Q-G-K-D-S-Q-L-K-K-M-K-L-I-K-R-M-L-T-A-C-K-G-I-E-A
 N17 I-K-R-M-L-T-A-C-K-G-I-E-A-K-F-L-I-R-S-L-E-S-K-L-R-I
 N18 K-F-L-I-R-S-L-E-S-K-L-R-I-G-L-A-E-K-T-V-L-I-S-L-S-K
 N19 G-L-A-E-K-T-V-L-I-S-L-S-K-A-L-L-L-H-D-E-N-R-E-D-S-P
 N20 A-L-L-L-H-D-E-N-R-E-D-S-P-D-K-D-V-P-M-D-V-L-E-S-A-Q
 N21 D-K-D-V-P-M-D-V-L-E-S-A-Q-Q-K-I-R-D-A-F-C-Q-V-P-N-Y
 N22 Q-K-I-R-D-A-F-C-Q-V-P-N-Y-E-I-V-I-N-S-C-L-E-H-G-I-M
 N23 E-I-V-I-N-S-C-L-E-H-G-I-M-N-L-D-K-Y-C-T-L-R-P-G-I-P
 N24 N-L-D-K-Y-C-T-L-R-P-G-I-P-L-K-P-M-L-A-K-P-T-K-A-I-N
 N25 L-K-P-M-L-A-K-P-T-K-A-I-N-E-V-L-D-R-F-Q-G-E-T-F-T-S
 N26 E-V-L-D-R-F-Q-G-E-T-F-T-S-E-Y-K-Y-D-G-E-R-A-Q-V-H-L
 N27 E-Y-K-Y-D-G-E-R-A-Q-V-H-L-L-N-D-G-T-M-R-I-Y-S-R-N-G
 N28 L-N-D-G-T-M-R-I-Y-S-R-N-G-E-N-M-T-E-R-Y-P-E-I-N-I-T
 N29 E-N-M-T-E-R-Y-P-E-I-N-I-T-D-F-I-Q-D-L-D-T-T-K-N-L-I
 N30 D-F-I-Q-D-L-D-T-T-K-N-L-I-L-D-C-E-A-V-A-W-D-K-D-Q-G
 O 1 L-D-C-E-A-V-A-W-D-K-D-Q-G-K-I-L-P-F-Q-V-L-S-T-R-K-R
 O 2 K-I-L-P-F-Q-V-L-S-T-R-K-R-K-D-V-E-L-N-D-V-K-V-K-V-C
 O 3 K-D-V-E-L-N-D-V-K-V-K-V-C-L-F-A-F-D-I-L-C-Y-N-D-E-R
 O 4 L-F-A-F-D-I-L-C-Y-N-D-E-R-L-I-N-K-S-L-K-E-R-R-E-Y-L
 O 5 L-I-N-K-S-L-K-E-R-R-E-Y-L-T-K-V-T-K-V-V-P-G-E-F-Q-Y
 O 6 T-K-V-T-K-V-V-P-G-E-F-Q-Y-A-T-Q-I-T-T-N-N-L-D-E-L-Q
 O 7 A-T-Q-I-T-T-N-N-L-D-E-L-Q-K-F-L-D-E-S-V-N-H-S-C-E-G
 O 8 K-F-L-D-E-S-V-N-H-S-C-E-G-L-M-V-K-M-L-E-G-P-E-S-H-Y
 O 9 L-M-V-K-M-L-E-G-P-E-S-H-Y-E-P-S-K-R-S-R-N-W-L-K-L-K
 O10 E-P-S-K-R-S-R-N-W-L-K-L-K-K-D-Y-L-E-G-V-G-D-S-L-D-L
 O11 K-D-Y-L-E-G-V-G-D-S-L-D-L-C-V-L-G-A-Y-Y-G-R-G-K-R-T
 O12 C-V-L-G-A-Y-Y-G-R-G-K-R-T-G-T-Y-G-G-F-L-L-G-C-Y-N-Q
 O13 G-T-Y-G-G-F-L-L-G-C-Y-N-Q-D-T-G-E-F-E-T-C-C-K-I-G-T
 O14 D-T-G-E-F-E-T-C-C-K-I-G-T-G-F-S-D-E-M-L-Q-L-L-H-D-R
 O15 G-F-S-D-E-M-L-Q-L-L-H-D-R-L-T-P-T-I-I-D-G-P-K-A-T-F
 O16 L-T-P-T-I-I-D-G-P-K-A-T-F-V-F-D-S-S-A-E-P-D-V-W-F-E
 O17 V-F-D-S-S-A-E-P-D-V-W-F-E-P-T-T-L-F-E-V-L-T-A-D-L-S
 O18 P-T-T-L-F-E-V-L-T-A-D-L-S-L-S-P-I-Y-K-A-G-S-A-T-F-D
 O19 L-S-P-I-Y-K-A-G-S-A-T-F-D-K-G-V-S-L-R-F-P-R-F-L-R-I
 O20 K-G-V-S-L-R-F-P-R-F-L-R-I-R-E-D-K-G-V-E-D-A-T-S-S-D
 O21 R-E-D-K-G-V-E-D-A-T-S-S-D-Q-I-V-E-L-Y-E-N-Q-S-H-M-Q
 O22 E-D-K-G-V-E-D-A-T-S-S-D-Q-I-V-E-L-Y-E-N-Q-S-H-M-Q-N
 O23
 O24 Nse1 M-E-V-H-E-E-Q-V-S-A-P-V-T-G-D-A-T-A-K-Y-L-L-Q-Y-I-L
 O25 G-D-A-T-A-K-Y-L-L-Q-Y-I-L-S-A-R-G-I-C-H-E-N-A-L-I-L
 O26 S-A-R-G-I-C-H-E-N-A-L-I-L-A-L-M-R-L-E-T-D-A-S-T-L-N
 O27 A-L-M-R-L-E-T-D-A-S-T-L-N-T-E-W-S-I-Q-Q-W-V-D-K-L-N
 O28 T-E-W-S-I-Q-Q-W-V-D-K-L-N-D-Y-I-N-A-I-N-V-K-L-N-L-L
 O29 D-Y-I-N-A-I-N-V-K-L-N-L-L-G-Y-K-I-I-R-I-N-H-G-I-G-R
 O30 G-Y-K-I-I-R-I-N-H-G-I-G-R-N-A-V-T-L-K-A-K-Q-N-F-E-S
 P 1 N-A-V-T-L-K-A-K-Q-N-F-E-S-F-E-D-N-T-A-I-R-A-H-N-N-D
 P 2 F-E-D-N-T-A-I-R-A-H-N-N-D-Y-A-V-L-Q-S-I-V-L-P-E-S-N
 P 3 Y-A-V-L-Q-S-I-V-L-P-E-S-N-R-F-F-V-Y-V-N-L-A-S-T-E-E
 P 4 R-F-F-V-Y-V-N-L-A-S-T-E-E-T-K-L-A-T-R-F-N-Q-N-E-I-E
 P 5 T-K-L-A-T-R-F-N-Q-N-E-I-E-F-M-K-W-A-I-E-Q-F-M-I-S-G
 P 6 F-M-K-W-A-I-E-Q-F-M-I-S-G-E-T-I-V-E-G-P-A-L-E-T-S-I

P 7 E-T-I-V-E-G-P-A-L-E-T-S-I-I-V-K-E-V-N-R-I-L-V-A-A-T
P 8 I-V-K-E-V-N-R-I-L-V-A-A-T-G-D-S-N-L-A-K-W-R-K-F-S-T
P 9 G-D-S-N-L-A-K-W-R-K-F-S-T-F-T-V-G-S-T-N-L-F-Q-F-Q-E
P10 F-T-V-G-S-T-N-L-F-Q-F-Q-E-L-T-A-T-D-I-E-D-L-L-L-R-L
P11 L-T-A-T-D-I-E-D-L-L-L-R-L-C-E-L-K-W-F-Y-R-T-Q-E-G-K
P12 C-E-L-K-W-F-Y-R-T-Q-E-G-K-F-G-I-D-L-R-C-I-A-E-L-E-E
P13 F-G-I-D-L-R-C-I-A-E-L-E-E-Y-L-T-S-M-Y-N-L-N-T-C-Q-N
P14 Y-L-T-S-M-Y-N-L-N-T-C-Q-N-C-H-K-L-A-I-Q-G-V-R-C-G-N
P15 C-H-K-L-A-I-Q-G-V-R-C-G-N-E-S-C-R-E-E-N-E-E-T-G-E-N
P16 E-S-C-R-E-E-N-E-E-T-G-E-N-S-L-S-Q-I-W-H-V-D-C-F-K-H
P17 S-L-S-Q-I-W-H-V-D-C-F-K-H-Y-I-T-H-V-S-K-N-C-D-R-C-G
P18 K-H-Y-I-T-H-V-S-K-N-C-D-R-C-G-S-S-L-I-T-E-G-V-Y-V-I
P19
P20 Nse3 M-S-S-I-D-N-D-S-D-V-D-L-T-E-D-L-A-V-A-K-I-V-K-E-N-P
P21 E-D-L-A-V-A-K-I-V-K-E-N-P-V-A-R-K-M-V-R-Y-I-L-S-R-G
P22 V-A-R-K-M-V-R-Y-I-L-S-R-G-E-S-Q-N-S-I-I-T-R-N-K-L-Q
P23 E-S-Q-N-S-I-I-T-R-N-K-L-Q-S-V-I-H-E-A-A-R-E-E-N-I-A
P24 S-V-I-H-E-A-A-R-E-E-N-I-A-K-P-S-F-S-K-M-F-M-D-I-N-A
P25 K-P-S-F-S-K-M-F-M-D-I-N-A-I-L-Y-N-V-Y-G-F-E-L-Q-G-L
P26 I-L-Y-N-V-Y-G-F-E-L-Q-G-L-P-S-K-N-N-M-N-A-G-G-N-G-S
P27 P-S-K-N-N-M-N-A-G-G-N-G-S-N-S-N-T-N-K-S-M-P-E-P-L-G
P28 N-S-N-T-N-K-S-M-P-E-P-L-G-H-R-A-Q-K-F-I-L-L-N-N-V-P
P29 H-R-A-Q-K-F-I-L-L-N-N-V-P-H-S-K-N-F-D-D-F-K-I-L-Q-S
P30 H-S-K-N-F-D-D-F-K-I-L-Q-S-A-H-T-Y-E-E-L-I-V-T-G-E-Y
Q 1 A-H-T-Y-E-E-L-I-V-T-G-E-Y-I-G-D-D-I-A-S-G-T-S-N-T-L
Q 2 I-G-D-D-I-A-S-G-T-S-N-T-L-E-S-K-L-S-T-D-R-D-L-V-Y-K
Q 3 E-S-K-L-S-T-D-R-D-L-V-Y-K-G-V-L-S-V-I-L-C-I-V-F-F-S
Q 4 G-V-L-S-V-I-L-C-I-V-F-F-S-K-N-N-I-L-H-Q-E-L-I-K-F-L
Q 5 K-N-N-I-L-H-Q-E-L-I-K-F-L-E-T-F-G-I-P-S-D-G-S-K-I-A
Q 6 E-T-F-G-I-P-S-D-G-S-K-I-A-I-L-N-I-T-I-E-D-L-I-K-S-L
Q 7 I-L-N-I-T-I-E-D-L-I-K-S-L-E-K-R-E-Y-I-V-R-L-E-E-K-S
Q 8 E-K-R-E-Y-I-V-R-L-E-E-K-S-D-T-D-G-E-V-I-S-Y-R-I-G-R
Q 9 D-T-D-G-E-V-I-S-Y-R-I-G-R-R-T-Q-A-E-L-G-L-E-S-L-E-K
Q10 R-T-Q-A-E-L-G-L-E-S-L-E-K-L-V-Q-E-I-M-G-L-E-K-E-Q-T
Q11 L-V-Q-E-I-M-G-L-E-K-E-Q-T-K-S-L-H-D-D-I-I-K-S-I-G-D
Q12 I-M-G-L-E-K-E-Q-T-K-S-L-H-D-D-I-I-K-S-I-G-D-S-Y-S-I

Appendix 3. Proteins binding to ORC. Proteins identified by mass spectrometry.

Protein names, the number of unique peptides and the confidence of protein identification are indicated. Taxonomy of all proteins is *Saccharomyces cerevisiae* unless otherwise indicated.

Protein	No. of unique peptides	Confidence
Origin recognition complex subunit 1	23	100%
Origin recognition complex subunit 4	21	100%
Origin recognition complex subunit 3	15	100%
Calcium/calmodulin-dependent protein kinase I	13	100%
Heat shock protein SSB1	13	100%
Seventh homolog of septin 1	13	100%
Heat shock protein SSA1	12	100%
Origin recognition complex subunit 6	12	100%
Bud site selection protein 3	11	100%
Origin recognition complex subunit 5	11	100%
Cell division control protein 12	10	100%
Uncharacterized transcriptional regulatory protein YKL038W	10	100%
Calcium/calmodulin-dependent protein kinase II	9	100%
Origin recognition complex subunit 2	9	100%
Protein ZDS1	7	100%
Putative serine carboxypeptidase YBR139W	7	100%
Cell division control protein 3	7	99%
Glucosidase 2 subunit alpha precursor	6	100%
Histone acetyltransferase type B catalytic subunit	6	98%
Coatomer subunit beta	6	93%
60S ribosomal protein L8-B	5	100%
ATP-dependent RNA helicase DED1	5	100%
Putative uncharacterized protein - Vanderwaltozyma polyspora DSM 70294	5	100%
40S ribosomal protein S3	4	100%
Mitochondrial clpX-like chaperone MCX1	4	100%
37S ribosomal protein S7, mitochondrial precursor	4	99%
40S ribosomal protein S4	4	99%
Uncharacterized protein YMR107W	4	96%
Glutamyl-tRNA synthetase, cytoplasmic	4	90%
Guanine nucleotide-binding protein subunit beta-like protein	4	90%
Glucosidase 2 subunit beta precursor	4	88%
Heat shock protein homolog SSE2	4	86%
Uncharacterized protein YJL145W	4	82%
Import inner membrane translocase subunit TIM44, mitochondrial precursor	4	81%
Serine/threonine-protein kinase GIN4	4	69%
Cell division control protein 10	3	100%
Ubiquitin-specific protease	3	99%

Casein kinase II subunit alpha	3	98%
60S ribosomal protein L19	3	97%
40S ribosomal protein S18	3	96%
Protein PBP4	3	94%
ATP synthase D chain, mitochondrial	3	93%
Putative thiosulfate sulfurtransferase YOR285W	3	88%
40S ribosomal protein S17-A	3	74%
40S ribosomal protein S24	3	65%
Casein kinase II subunit alpha'	3	60%
60S ribosomal protein L16-B	3	59%
Myb-like DNA-binding protein BAS1	3	45%
60S ribosomal protein L13-A	2	96%
PAB1-binding protein 1	2	96%
40S ribosomal protein S6	2	93%
40S ribosomal protein S8	2	93%
Histone acetyltransferase type B subunit 2	2	93%
40S ribosomal protein S7-A	2	90%
Ribosomal RNA-processing protein 15	2	90%
Transcription initiation factor TFIID subunit 10	2	90%
ATP synthase epsilon chain, mitochondrial	2	89%
ATP synthase gamma chain, mitochondrial precursor	2	87%
40S ribosomal protein S16	2	86%
Enolase - <i>Saccharomyces cerevisiae</i> YJM789	2	84%
Protein MKT1	2	82%
Streptavidin precursor - <i>Streptomyces avidinii</i>	2	82%
Glyceraldehyde-3-phosphate dehydrogenase 2	2	79%
40S ribosomal protein S5	2	78%
ALB protein - <i>Bos taurus</i> (Bovine)	2	78%
60S ribosomal protein L2	2	76%
Vacuole morphology and inheritance protein 14	2	75%
N(2),N(2)-dimethylguanosine tRNA methyltransferase, mitochondrial precursor	2	73%
Glutathione S-transferase I	2	71%
Protein BMH2	2	67%
Casein kinase II subunit beta'	2	66%
GU4 nucleic-binding protein 1	2	64%
Transcription factor SPT8	2	64%
60S ribosomal protein L10	2	53%
Uncharacterized protein YKL054C	2	51%
Elongation factor 2	2	50%
DNA-binding protein REB1	2	44%
60S ribosomal protein L26-A	2	39%
60S acidic ribosomal protein P0	2	33%
Heat shock protein SSA2	2	15%
60S ribosomal protein L7-A	1	90%

Casein kinase II subunit beta	1	90%
RNA exonuclease 4	1	90%
Thioredoxin - Escherichia coli B	1	90%
60S ribosomal protein L6-A	1	84%
Regulator of ribosome biosynthesis	1	83%
Eukaryotic initiation factor 4F subunit p150	1	82%
Transcription elongation factor SPT5	1	82%
60S ribosomal protein L9-A	1	80%
60S ribosomal protein L7-B	1	79%
Phosphoglycerate mutase 1	1	79%
60S ribosomal protein L14-A	1	78%
40S ribosomal protein S27-A	1	77%
40S ribosomal protein S9-A	1	76%
Nucleoporin NUP57	1	73%
40S ribosomal protein S28-A	1	72%
60S ribosomal protein L18	1	71%
Transcription initiation factor TFIID subunit 6	1	70%
60S ribosomal protein L17-A	1	67%
40S ribosomal protein S13	1	60%
60S ribosomal protein L20	1	58%
40S ribosomal protein S0-A	1	55%
Ubiquitin fusion degradation protein 4	1	53%
60S ribosomal protein L36-A	1	52%
60S ribosomal protein L3	1	50%
Protein AVL9	1	50%
Serine/threonine-protein kinase YPK1	1	50%
Heat shock protein 42	1	47%
40S ribosomal protein S26-A	1	36%
60S ribosomal protein L30	1	36%
Mitochondrial ATPase complex subunit ATP10	1	33%
60S ribosomal protein L31-A	1	30%
Phosphatidylinositol transfer protein CSR1	1	29%
RNA annealing protein YRA1	1	26%
Uncharacterized protein YPL146C	1	26%
60S ribosomal protein L35	1	24%
40S ribosomal protein S12	1	22%
Importin subunit alpha	1	20%

Appendix 3. Loading assay, specifically bound proteins. Proteins identified by mass spectrometry. Protein names, the number of unique peptides and the confidence of protein identification are indicated. Taxonomy of all proteins is *Saccharomyces cerevisiae*.

Protein	No. of unique peptides	Confidence
Origin recognition complex subunit 1	25	100%
Origin recognition complex subunit 4	19	100%
Origin recognition complex subunit 2	10	100%
Origin recognition complex subunit 5	10	100%
Cell division control protein 6	7	100%
Histone acetyltransferase type B catalytic subunit	5	99%
DNA repair and recombination protein RAD52	5	94%
DNA-directed RNA polymerase I subunit RPA34	4	100%
Histone acetyltransferase type B subunit 2	4	99%
Protein BMH2	3	98%
DNA replication licensing factor MCM3	3	33%
Nuclear polyadenylated RNA-binding protein 4	2	94%
Protein LSM12	2	94%
Protein MKT1	2	89%
ARS-binding factor 2, mitochondrial precursor	2	87%
60S ribosomal protein L19	2	68%
Coatomer subunit zeta	2	59%
RNA polymerase II transcriptional coactivator SUB1	2	50%
DNA-(apurinic or apyrimidinic site) lyase 1	2	44%
DNA replication licensing factor MCM6	2	29%
M1-1 protoxin precursor	1	90%
Coatomer subunit epsilon	1	89%
Repression factor of MSEs protein 1	1	77%
60S ribosomal protein L13-A	1	75%
40S ribosomal protein S7-A	1	70%
Negative growth regulatory protein NGR1	1	50%
Ubiquitin domain-containing protein DSK2	1	50%
Nucleolar protein 3	1	49%
Histone H1	1	47%
Proliferating cell nuclear antigen	1	36%
60S ribosomal protein L17-A	1	34%
ISWI chromatin-remodeling complex ATPase ISW1	1	29%
RNA-binding suppressor of PAS kinase protein 1	1	27%