Functional proteomic identification of DNA replication proteins by induced proteolysis *in vivo*

Masato Kanemaki*, Alberto Sanchez-Diaz*, Agnieszka Gambus & Karim Labib

** Cancer Research UK, Paterson Institute for Cancer Research, Christie Hospital NHS Trust, Wilmslow Road, Manchester, M20 4BX, UK*
**These authors contributed equally to this work*

Evolutionarily diverse eukaryotic cells share many conserved proteins of unknown function. Some are essential for cell viability[1,2], emphasising their importance for fundamental processes of cell biology but complicating their analysis. We have developed an approach to the large-scale characterization of such proteins, based on conditional and rapid degradation of the target protein *in vivo*, so that the immediate consequences of bulk protein depletion can be examined[3]. Budding yeast strains have been constructed in which essential proteins of unknown function have been fused to a 'heat-inducible-degron' cassette that targets the protein for proteolysis at 37 °C (ref. 4). By screening the collection for defects in cell-cycle progression, here we identify three DNA replication factors that interact with each other and that have uncharacterized homologues in human cells. We have used the degron strains to show that these proteins are required for the establishment and normal progression of DNA replication forks. The degron collection could also be used to identify other, essential, proteins with roles in many other processes of eukaryotic cell biology.

Analysing the role of eukaryotic proteins that are essential for cell viability requires a method by which protein function can be inactivated conditionally. Until now, the only methods suitable for characterizing large numbers of such proteins involve indirect approaches to protein inactivation, either by replacing the promoter of the encoding gene with another that is repressible[5–7], or else by RNA interference[1]. In these cases the messenger RNA can rapidly be depleted, but decay of the protein is often much slower, so that the phenotype may be complicated by secondary responses to the initial consequences of partially inactivating the protein.

Recently we described a simple method by which proteins of the budding yeast *Saccharomyces cerevisiae* can be depleted rapidly in a manner that is direct and conditional[3]. The chromosomal locus of a gene is modified so that the amino-terminus of the encoded protein is fused to a 'heat-inducible-degron' cassette that targets the fusion protein for degradation at 37 °C (ref. 4). The degron is recognized by the Ubr1 protein that is associated with a ubiquitin-conjugating enzyme, and proteolysis is stimulated at higher temperatures, probably because unfolding of the degron cassette exposes lysine residues that are the sites of ubiquitylation[4]. We showed previously that essential proteins fused to the degron are functional at 37 °C in the absence of Ubr1, but are degraded efficiently at 37 °C when the Ubr1 protein is subsequently expressed to high levels[3].

To compare direct inactivation of a protein using the degron method with depletion of the mRNA by repressing transcription, we inactivated the *S. cerevisiae* DNA replication protein Mcm4 using the two approaches. When inactivation was assayed by the ability of cells to form colonies under restrictive conditions, both methods appear equally efficient (Fig. 1a). However, more careful analysis of the phenotype in liquid culture experiments revealed important differences. Repressing transcription of the *MCM4* gene produced a rapid decrease in the level of *MCM4* mRNA, but depletion of Mcm4 protein was more gradual (Fig. 1b). This caused cells to become arrested with a DNA content of 2C, typical of defects in G2 phase or mitosis, probably because subtle problems in chromosome replication occur as Mcm4 is gradually depleted, activating a checkpoint response that blocks completion of the cell cycle. In contrast, Mcm4 protein was rapidly depleted in the degron strain, despite persistence of the mRNA, producing a marked and obvious defect in chromosome replication (Fig. 1b). This qualitative difference in phenotype demonstrates the importance of rapidly depleting proteins essential for cell viability, rather than their corresponding mRNAs, in order to assign a biological function.

We have made yeast strains in which the degron tag has been added to more than half of the essential *S. cerevisiae* proteins of unknown function, using a one-step polymerase chain reaction (PCR) method (see Methods and Supplementary Table 1 for a complete list of the proteins). Most of these proteins have homologues of unknown function in other eukaryotes, and their functions cannot be predicted from the amino acid sequence. We were able to make viable yeast strains carrying degron fusions for 94% of the essential proteins tested, and about 60% of these are specifically unable to form colonies at 37 °C in the presence of Ubr1 (the full data set is described in Supplementary Table 1 and Supplementary Fig. 1).

The resulting collection can be screened for defects in many fundamental processes of cell biology. Initially, we have screened the collection for strains defective in progression through the cell cycle at 37 °C. For each of the temperature-sensitive strains, we grew an asynchronous culture at 24 °C and then expressed Ubr1 before raising the temperature to 37 °C and examining cellular and nuclear morphology together with DNA content. In addition, we screened the degron strains that are viable at 37 °C (suggesting insufficient inactivation of the protein; see Supplementary Fig. 2) for ones where cell size was markedly increased at the high temperature, indicating a 'leaky' defect in cell-cycle progression.

In a control yeast strain, growth was unaffected after shifting to 37 °C after a brief period of adaptation (Fig. 2). Inactivating most of the essential proteins of unknown function blocked cell growth in a manner that does not correlate with position in the cell cycle. In contrast, however, we identified three new DNA replication factors that we call Cdc101, Cdc102 and Cdc105 (proteins with other roles in the cell cycle will be described elsewhere). For two of these—Cdc102/Yjl072c and Cdc105/Ydr489w—the corresponding degron strains are specifically unable to grow at 37 °C in the presence of high levels of Ubr1 (Fig. 2a) and accumulate as large-budded cells with largely unreplicated DNA (Fig. 2b; see also Supplementary Fig. 3). The Cdc101/Ydr013w degron strain (*cdc101-td*, where td denotes a

temperature-sensitive degron) is leaky, as it is able to grow at 37 °C even in the presence of Ubr1 (Fig. 2a); however, we observed a clear, although transient, defect in chromosome replication, and once again there is a marked accumulation of large-budded cells with a single nucleus (Fig. 2b; see also Supplementary Fig. 3).

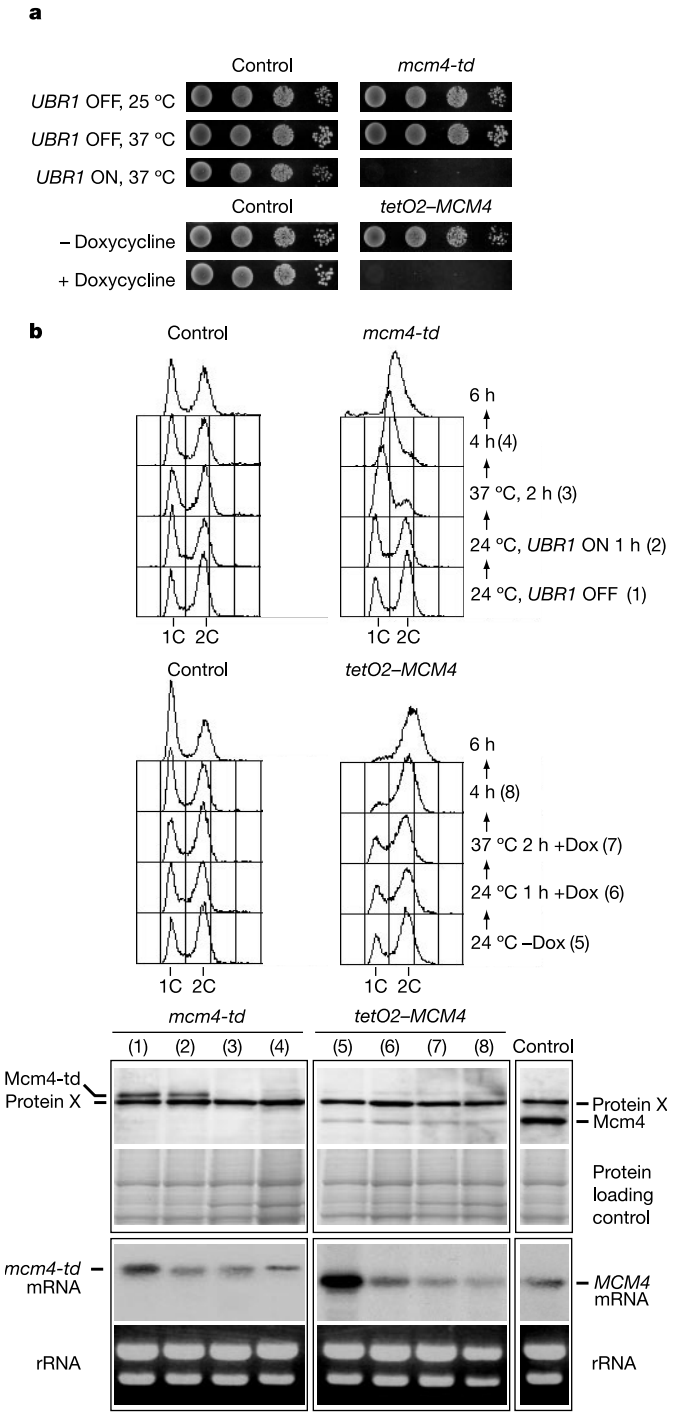

Figure 1 Direct inactivation of essential proteins by fusion to a heat-inducible degron. **a**, Serial dilutions were made of an *MCM4* degron strain (*mcm4-td*) or a strain in which transcription of *MCM4* can be repressed (*tetO2–MCM4*), together with appropriate control strains. **b**, The DNA content of *mcm4-td*, *tetO2–MCM4* and control strains was compared after transferring cells from permissive to restrictive conditions (identical except 20 μg ml^{-1} doxycycline was added to the *tetO2–MCM4* strain and its control). The level of *MCM4* mRNA and Mcm4 protein was determined during the same experiment (the protein loading control shows the equivalent portion of a gel stained with Coomassie blue).

The Cdc101, Cdc102 and Cdc105 proteins are unrelated to each other, but all three have homologues of unknown function in other eukaryotes, including humans. Their function cannot be predicted from their amino acid sequence, but all three proteins interact with each other in genome-wide two-hybrid screens[8,9]. We purified Cdc102 from yeast extracts and used antibodies to Cdc102 and Cdc105 to show that these proteins do indeed form part of a new complex required for chromosome replication (Fig. 2c). We used mass spectrometry to identify two other components of the complex: Cdc101 and Yol146w (data not shown). Yol146w is essential and of unknown function, but the corresponding degron strain is viable at 37 °C (Supplementary Fig. 1). The same four proteins have recently been shown to form a complex required for an early step of chromosome replication, both in *S. cerevisiae* and in extracts of *Xenopus laevis* eggs[10,11].

To examine the localization of Cdc102, we made yeast strains in which green fluorescent protein (GFP) or nine copies of the c-Myc epitope was added to the carboxy terminus of Cdc102 so that the mRNA expressing each fusion protein was still expressed from the *CDC102* promoter as the only version in the cell. Cdc102–GFP showed nuclear localization throughout the cell cycle (not shown), consistent with its role in chromosome replication. We then used chromatin immunoprecipitation (ChIP) experiments to examine the association of Cdc102–9Myc with replicating DNA. We released cells from G1 arrest in the presence of hydroxyurea, which inhibits ribonucleotide reductase and so reduces cellular dNTP pools. Under such conditions, initiation occurs at early origins but DNA replication forks then stall within a few kilobases[12,13]. Cdc102–9Myc associated specifically with early origins in hydroxyurea (Fig. 2d), with similar kinetics to the establishment of DNA replication forks[12]. Loading of Cdc102–9Myc required activation of both the Cdc7 kinase and the cyclin-dependent kinase Cdc28 (Fig. 2d), which are essential for the initiation of chromosome replication[14–16]. These experiments indicate that the complex containing Cdc102 and Cdc105 has a direct role in chromosome replication downstream of the two kinases that together trigger initiation.

We then examined the role of Cdc102 and Cdc105 in the establishment and progression of DNA replication forks *in vivo* at individual replicons. The Cdc102 and Cdc105 degron strains (*cdc102-td* and *cdc105-td*, respectively), together with a control strain, were grown for seven generations in medium containing 'heavy' isotopes of carbon and nitrogen, so that the chromosomal DNA was fully substituted (see Methods). Cells were then switched into 'light' medium and synchronized in the G1 phase of the cell cycle using mating pheromone. This procedure allowed us subsequently to distinguish unreplicated heavy–heavy DNA from replicated heavy–light DNA in CsCl gradients, by virtue of their differing densities[17]. In combination with 'slot blots' using probes for specific genomic sequences it is thus possible to follow the establishment and progression of individual DNA replication forks. Expression of Ubr1 was induced in the G1-arrested cells and the temperature was raised to 37 °C to inactivate Cdc102 or Cdc105. We then released cells from G1 arrest and examined their ability to proceed with chromosome replication.

In contrast to the control strain, which rapidly completed DNA replication within 60 min, very little replication was observed by flow cytometry after inactivation of Cdc102 or Cdc105, even after 90 min (Fig. 3a), although cells formed buds with normal kinetics (not shown). We assayed the replication of two of the earliest replicons in the *S. cerevisiae* genome: between the origin ARS306 and the left end of chromosome III, and between ARS607 and the right end of chromosome VI. Replication of each origin was found to be profoundly defective (Fig. 3b, probes 1 and 3), indicating that Cdc102 and Cdc105 are required for the establishment of DNA replication forks at these sites. Consistent with this defect, replication of the end of each replicon was similarly defective (Fig. 3b, probes 2 and 4).

To determine whether Cdc102 and Cdc105 are important for the

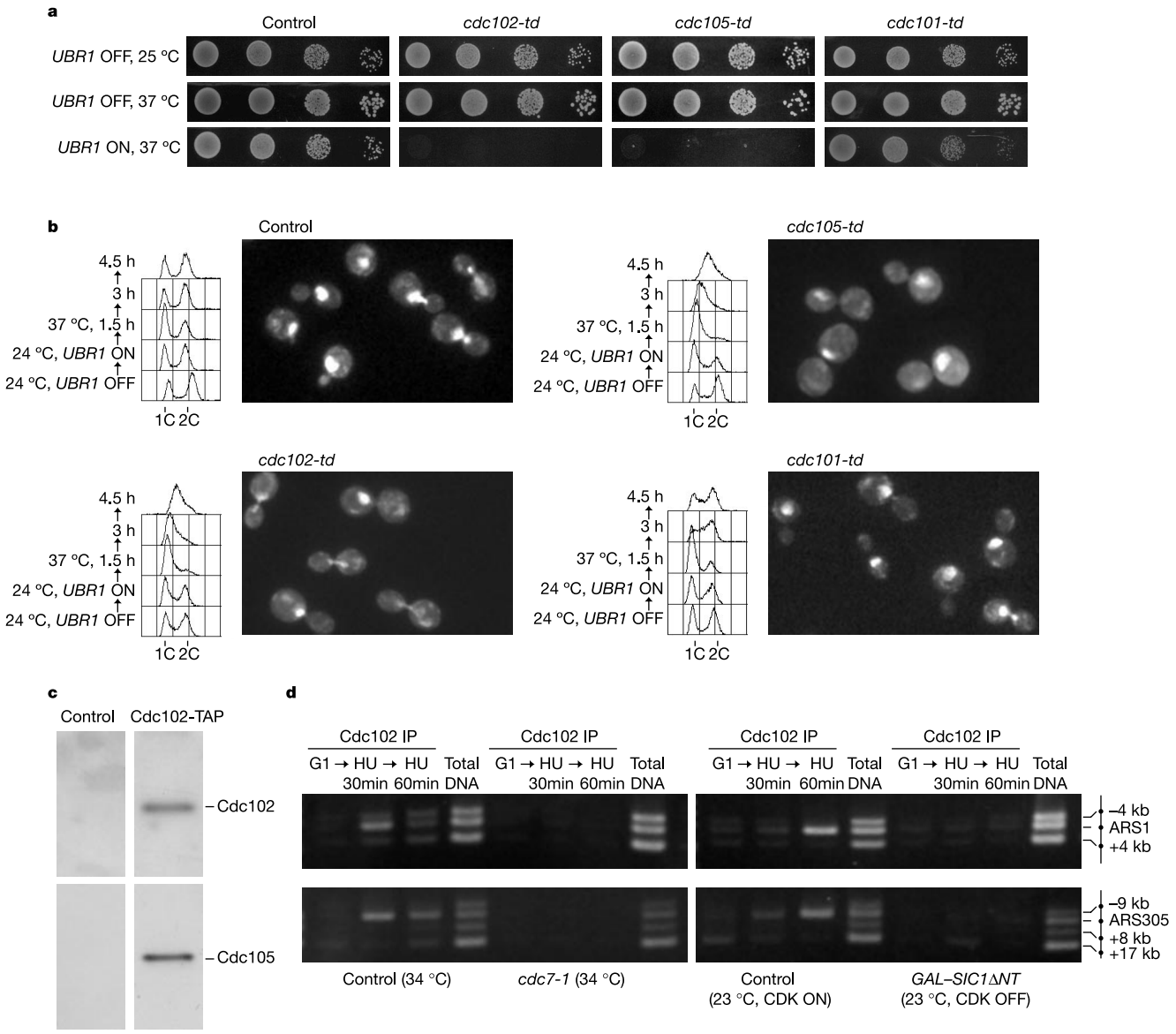

Figure 2 Three new DNA replication proteins identified by functional proteomics. **a**, Serial dilutions of control or degron strains were grown under the indicated conditions and examined after 48 h. **b**, Liquid culture experiments for the same strains. DNA content was measured by flow cytometry throughout the experiment (left panels). Cells from the 3-h time point were stained with the DNA-binding dye 4,6-diamidino-2-phenylindole (DAPI) and nuclear morphology was examined by fluorescence microscopy (right panels). **c**, Yeast extracts from control or *CDC102-TAP* strains were purified sequentially over IgG–Sepharose and calmodulin affinity resin, before detection of Cdc102 and Cdc105 proteins by immunoblotting. **d**, Association of Cdc102–9Myc with the early origins ARS1 and ARS305 was examined in the indicated strains by chromatin immunoprecipitation (IP). HU, hydroxyurea.

progression of DNA replication forks away from origins, we labelled the same strains as before with heavy isotopes, synchronized cells in G1 phase, and then allowed them to progress into S phase at 24 °C in the presence of hydroxyurea, so that DNA replication forks from early origins stalled within a few kilobases. We then expressed Ubr1 and shifted cells to 37 °C to inactivate Cdc102 or Cdc105, before transferring them into fresh medium lacking hydroxyurea, to determine whether chromosome replication could continue. In the control strain, replication of the genome was essentially complete within 30 min, as detected by flow cytometry (Fig. 4a). In contrast, the continuation of replication was severely impaired after inactivation of Cdc102 or Cdc105 (Fig. 4a; a similar experiment showing even later time points is given in Supplementary Fig. 4), suggesting that the progression of DNA replication forks is defective in their absence. By examining individual replicons we saw, even at 24 °C in hydroxyurea, that the replication of regions containing the early origins ARS306 and ARS607 is relatively defective in the *cdc102-td* and *cdc105-td* degron strains in comparison to the control (Fig. 4b). Replication from early origins under such conditions is, however, sufficient to activate the 'origin-firing checkpoint'[12], as mitosis is blocked in all cells and replication of the late origin ARS501 is completely inhibited during hydroxyurea arrest (see Supplementary Fig. 5).

In the control strain, it takes less than 30 min for forks established at ARS306 or ARS607 to reach the end of the replicon (Fig. 4b; compare the replication of probes 1 and 3 or probes 4 and 6). In contrast, although some forks do reach the end of the replicon with apparently similar kinetics in the *cdc102-td* and *cdc105-td* degron strains, a significant proportion is defective (21% of forks from ARS306 and 32% from ARS607 in the *cdc102-td* degron strain; 33% of forks from ARS306 and 37% from ARS607 in the *cdc105-td* degron strain; see Methods). These forks travel slowly through the

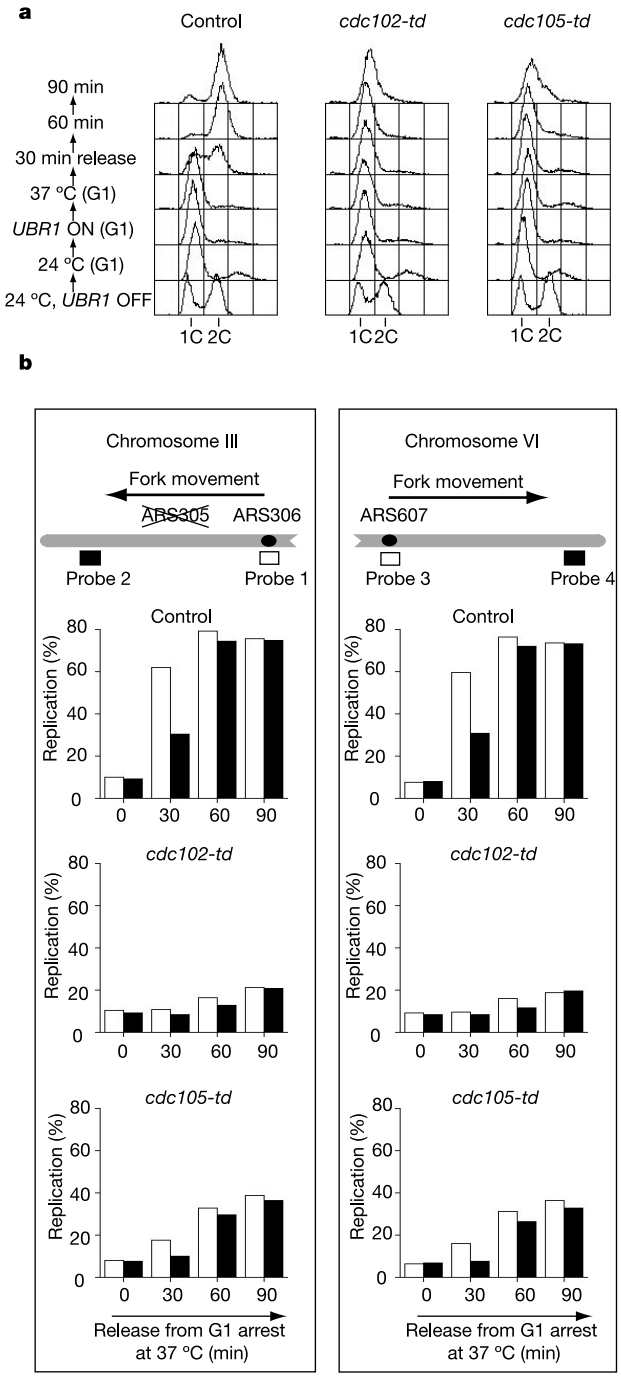

Figure 3 Cdc102 and Cdc105 are essential for an early step of origin replication. **a**, Flow cytometry was used to measure the DNA content of control, *cdc102-td* and *cdc105-td* strains after release from G1 arrest at 37 °C. **b**, Replication of two individual replicons was examined: at the left end of chromosome III (left panel) and the right end of chromosome VI (right panel).

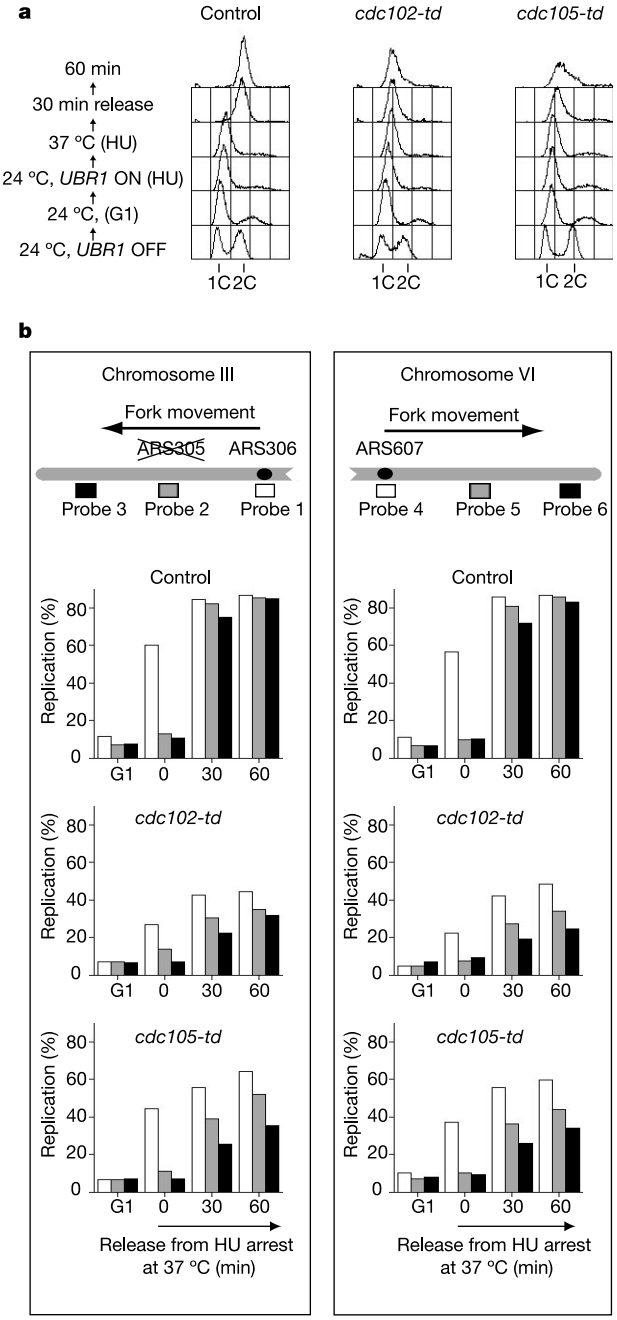

Figure 4 Cdc102 and Cdc105 are important for the normal progression of DNA replication forks away from origins. **a**, DNA content was measured by flow cytometry as before. **b**, Replication of the left end of chromosome III (left panel) and the right end of chromosome VI (right end) was measured as above.

two replicons, as shown by the progressive decrease in replication with increasing distance from the origin (Fig. 4b; compare probes 2 and 3 and probes 5 and 6). This may reflect a reduced rate of synthesis at the forks or repeated stalling throughout the replicon.

Taken together, these experiments indicate that the complex containing Cdc102 and Cdc105 has an important role in the establishment of DNA replication forks at origins of DNA replication, and is also important for the normal progression of forks away from origins. Future screens of the degron collection will probably allow the identification of new proteins required for many other processes of eukaryotic cell biology. The collection will also facilitate the testing of predictions made by other genomic or proteomic approaches, such as 'global' studies of gene expression, protein localization, protein interactions, and so on[8,9,18–20].

We note that the degron approach described here for *S. cerevisiae* could, in principle, be applied to other eukaryotes. The degron is unstable in both fission yeast[21] and mouse cells[22] at 37 °C, although efficient degradation through increased expression of Ubr1 has yet to be achieved in these systems. Although genetic modification is clearly much more challenging in higher eukaryotes, it may be possible to use the techniques of RNA interference to replace an endogenous protein with a degron version, so that the immediate consequences of rapidly depleting a protein can be examined. □

Methods

Construction of degron strains

Degron strains were made by a one-step PCR approach using a yeast strain in which the only copy of the *UBR1* gene was regulated by the galactose-inducible *GAL1,10* promoter[3] and the degron cassette described in Supplementary Fig. 6. For each essential gene of unknown function, a PCR product was generated using 70-base oligonucleotides beginning with 50 nucleotide homology to either the promoter region or the start of the open reading frame, and ending with 20 nucleotides equivalent to sequences within the degron cassette (see Supplementary Fig. 6; details will be provided on request).

We transformed the PCR products into a haploid *GAL–UBR1* strain or a diploid strain heterozygous for *GAL–UBR1*, and cells were grown at 24 °C on YPD medium containing 0.1 mM $CuSO_4$. Integrations were checked by a series of PCR reactions using combinations of oligonucleotides corresponding to sequences either side of the integration site or within the degron cassette. For each essential protein of unknown function we generated two independent haploid clones, and confirmed that they behaved in the same way.

Growth of degron strains

Asynchronous cultures were grown at 24 °C in YP medium containing 2% raffinose as the carbon source (YPRaff) and 0.1 mM $CuSO_4$. To inactivate a degron fusion protein, expression of Ubr1 was induced by transferring cells to YP medium containing 2% galactose (YPGal) and 0.1 mM $CuSO_4$ for 35–60 min at 24 °C, before changing to YPGal medium pre-warmed to 37 °C, and continuing incubation for at least 45–60 min.

Dense-isotope substitution experiments

These experiments were performed essentially as described previously[3,23], using strains in which the early origin of DNA replication ARS305 had been deleted. See Supplementary Information for full details of the experimental protocol together with details of the DNA sequences examined.

The defect in fork progression after inactivation of Cdc102 or Cdc105 was calculated using the data in Fig. 4. In control cells, forks from ARS306 or ARS607 take 20–30 min to reach the end of the corresponding replicons on release from hydroxyurea at 37 °C (Fig. 4b and M.K., unpublished data). Therefore, we can estimate the defect in fork progression over any 30-min period by dividing the percentage replication of a fragment close to the end of the replicon (probe 3 for chromosome III; probe 6 for chromosome VI) by the percentage replication of the corresponding origin 30-min earlier (probe 1 for chromosome III; probe 4 for chromosome VI). This was done for the two periods 0–30 min and 30–60 min, and the mean value was determined.

Construction and use of the *tetO2–MCM4* strain

The chromosomal locus of the *MCM4* gene was modified to replace the endogenous promoter with the doxycycline-regulatable *tetO2* promoter using a one-step PCR method as described previously[5]. For the experiment shown in Fig.1b, the *mcm4-td* and control strains were grown in YPRaff plus 0.1 mM $CuSO_4$ at 24 °C, before transferring to YPGal at 24 °C for 60 min, and then shifting cells to 37 °C in YPGal medium. The *tetO2–MCM4* strain and its control (both containing Tet-SSN6[5]) were treated identically to the degron strain except that 20 $\mu g\,ml^{-1}$ doxycycline was added after transferring cells to the medium containing galactose.

RNA and protein analysis

RNA was prepared from cells using the Qiagen RNeasy mini-prep kit. *MCM4* mRNA was detected using a probe corresponding to nucleotides 1–450 of the *MCM4* open reading frame. Mcm4 protein was detected in immunoblots using an antibody raised against a peptide from the C terminus of the protein (Santa Cruz sc-6685). Cdc102 and Cdc105 were detected using polyclonal antibodies raised against six-histidine-tagged recombinant proteins purified from *Escherichia coli* extracts. A complex containing Cdc102 and Cdc105 was purified from yeast extracts using the tandem affinity purification (TAP) method[24,25].

Flow cytometry

We measured DNA content as described previously[26].

Chromatin immunoprecipitation

ChIP experiments were performed as described previously using PCR primers specific for ARS305 and flanking sequences[27]. To examine the association of Cdc102–9Myc with ARS1 and adjacent sequences, we used the following PCR primers: chromosome IV (485.5 kb) 5′-AAGCGCCCCTGATTGACAAG-3′ and 5′-GCTGGAGGAATTCGAGAATG-3′; chromosome IV (462.5 kb) 5′-TGGTGTTGATGTAAGCGGAG-3′ and 5′-AAAGTCAACCCCCTGCGATG-3′; chromosome IV (466.5 kb) 5′-AGTGCCCTAGAAGGTGCTAC-3′ and 5′-GGAAACACCACCGGCAAACT-3′. To show that the Cdc7 kinase is required for association of Cdc102–9Myc with chromatin, *CDC102–9Myc* and *cdc7-1 CDC102–9Myc* strains were grown in YPRaff medium at 24 °C, arrested in G1 with α-factor, then incubated at 34 °C with α-factor for 50 min before releasing from G1 arrest at 34 °C in the presence of 0.2 M hydroxyurea. To show that association of Cdc102–9Myc with chromatin is CDK-dependent, *CDC102–9Myc* and *GAL–SIC1ΔNT CDC102–9Myc* strains were grown in YPRaff medium at 23 °C, arrested in G1 with α-factor, then transferred for 45 min to YPGal medium containing α-factor to induce expression of Sic1ΔNT[28,29], before release from G1 arrest at 23 °C in the presence of 0.2 M hydroxyurea.

Received 25 February; accepted 6 May 2003; doi:10.1038/nature01692.
Published online 25 May 2003.

1. Kamath, R. S. *et al.* Systematic functional analysis of the *Caenorhabditis elegans* genome using RNAi. *Nature* **421,** 231–237 (2003).
2. Giaever, G. *et al.* Functional profiling of the *Saccharomyces cerevisiae* genome. *Nature* **418,** 387–391 (2002).
3. Labib, K., Tercero, J. A. & Diffley, J. F. X. Uninterrupted MCM2-7 function required for DNA replication fork progression. *Science* **288,** 1643–1647 (2000).
4. Dohmen, R. J., Wu, P. & Varshavsky, A. Heat-inducible degron: a method for constructing temperature-sensitive mutants. *Science* **263,** 1273–1276 (1994).
5. Belli, G., Gari, E., Aldea, M. & Herrero, E. Functional analysis of yeast essential genes using a promoter-substitution cassette and the tetracycline-regulatable dual expression system. *Yeast* **14,** 1127–1138 (1998).
6. Dragon, F. *et al.* A large nucleolar U3 ribonucleoprotein required for 18S ribosomal RNA biogenesis. *Nature* **417,** 967–970 (2002).
7. Wu, L. F. *et al.* Large-scale prediction of *Saccharomyces cerevisiae* gene function using overlapping transcriptional clusters. *Nature Genet.* **31,** 255–265 (2002).
8. Uetz, P. *et al.* A comprehensive analysis of protein–protein interactions in *Saccharomyces cerevisiae*. *Nature* **403,** 623–627 (2000).
9. Ito, T. *et al.* A comprehensive two-hybrid analysis to explore the yeast protein interactome. *Proc. Natl Acad. Sci. USA* **98,** 4569–4574 (2001).
10. Takayama, Y. *et al.* GINS, a novel multiprotein complex required for chromosomal DNA replication in budding yeast. *Genes Dev.* **17,** 1153–1165 (2003).
11. Kubota, Y. *et al.* A novel ring-like complex of *Xenopus* proteins essential for the initiation of DNA replication. *Genes Dev.* **17,** 1141–1152 (2003).
12. Santocanale, C. & Diffley, J. F. X. A Mec1- and Rad53-dependent checkpoint controls late-firing origins of DNA replication. *Nature* **395,** 615–618 (1998).
13. Yabuki, N., Terashima, H. & Kitada, K. Mapping of early firing origins on a replication profile of budding yeast. *Genes Cells* **7,** 781–789 (2002).
14. Bousset, K. & Diffley, J. F. X. The Cdc7 protein kinase is required for origin firing during S phase. *Genes Dev.* **12,** 480–490 (1998).
15. Donaldson, A. D., Fangman, W. L. & Brewer, B. J. Cdc7 is required throughout the yeast S phase to activate replication origins. *Genes Dev.* **12,** 491–501 (1998).
16. Donaldson, A. D. *et al.* CLB5-dependent activation of late replication origins in *S. cerevisiae*. *Mol. Cell* **2,** 173–183 (1998).
17. McCarroll, R. M. & Fangman, W. L. Time of replication of yeast centromeres and telomeres. *Cell* **54,** 505–513 (1988).
18. Gavin, A. C. *et al.* Functional organization of the yeast proteome by systematic analysis of protein complexes. *Nature* **415,** 141–147 (2002).
19. Ho, Y. *et al.* Systematic identification of protein complexes in *Saccharomyces cerevisiae* by mass spectrometry. *Nature* **415,** 180–183 (2002).
20. Kumar, A. *et al.* Subcellular localization of the yeast proteome. *Genes Dev.* **16,** 707–719 (2002).
21. Lindner, K., Gregan, J., Montgomery, S. & Kearsey, S. E. Essential role of MCM proteins in premeiotic DNA replication. *Mol. Biol. Cell* **13,** 435–444 (2002).
22. Levy, F., Johnston, J. A. & Varshavsky, A. Analysis of a conditional degradation signal in yeast and mammalian cells. *Eur. J. Biochem.* **259,** 244–252 (1999).
23. Tercero, J. A., Labib, K. & Diffley, J. F. X. DNA synthesis at individual replication forks requires the essential initiation factor, Cdc45p. *EMBO J.* **19,** 2082–2093 (2000).
24. Rigaut, G. *et al.* A generic protein purification method for protein complex characterization and proteome exploration. *Nature Biotechnol.* **17,** 1030–1032 (1999).
25. Tasto, J. J., Carnahan, R. H., McDonald, W. H. & Gould, K. L. Vectors and gene targeting modules for tandem affinity purification in *Schizosaccharomyces pombe*. *Yeast* **18,** 657–662 (2001).
26. Labib, K., Diffley, J. F. X. & Kearsey, S. E. G1-phase and B-type cyclins exclude the DNA-replication factor Mcm4 from the nucleus. *Nature Cell Biol.* **1,** 415–422 (1999).
27. Kamimura, Y., Tak, Y.-S., Sugino, A. & Araki, H. Sld3, which interacts with Cdc45 (Sld4), functions for chromosomal DNA replication in *Saccharomyces cerevisiae*. *EMBO J.* **20,** 2097–2107 (2001).
28. Drury, L. S., Perkins, G. & Diffley, J. F. X. The Cdc4/34/53 pathway targets Cdc6p for proteolysis in budding yeast. *EMBO J.* **16,** 5966–5976 (1997).
29. Desdouets, C. *et al.* Evidence for a Cdc6p-independent mitotic resetting event involving DNA polymerase α. *EMBO J.* **17,** 4139–4146 (1998).

Supplementary Information accompanies the paper on **www.nature.com/nature**.

Acknowledgements We thank J. Diffley for support and encouragement in the early stages of this project and for comments on the manuscript, together with N. Jones. We are grateful to members of our laboratory for helpful discussions, S. Pepper for helping to analyse mass spectrometry data, and to M. Segurado and E. Rawson for their assistance. We thank E. Schiebel and K. Gould for plasmids; E. Schiebel for help in growing fermentor cultures; and H. Araki and Y. Kamimura for assistance with ChIP and for communicating unpublished data, together with H. Takisawa. A.S.-D. thanks F. Perez-Campo for her support. This work was funded by Cancer Research UK, from whom K.L. receives a Senior Cancer Research Fellowship, and by the European Community, from whom A.S.-D. receives a Marie Curie Fellowship.

Competing interests statement The authors declare that they have no competing financial interests.

Correspondence and requests for materials should be addressed to K.L. (klabib@picr.man.ac.uk).