SCF<sup>Dia2</sup> regulates DNA replication forks during S-phase in budding yeast

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Dia2 is an F-box protein, which is involved in the regulation of DNA replication in the budding yeast *Saccharomyces cerevisiae*. The function of Dia2, however, remains largely unknown. In this study, we report that Dia2 is associated with the replication fork and regulates replication fork progression. Using modified yeast two-hybrid screening, we have identified components of the replisome (Mrc1, Ctf4 and Mcm2), as Dia2-binding proteins. Mrc1 and Ctf4 were ubiquitinated by SCF<sup>Dia2</sup> both in vivo and in vitro. Domain analysis of Dia2 revealed that the leucine-rich repeat motif was indispensable for the regulation of replisome progression, whereas the tetra-tripeptide repeat (TPR) motif was involved in the interaction with replisome components. In addition, the TPR motif was shown to be involved in Dia2 stability; deleting the TPR stabilized Dia2, mimicking the effect of DNA damage. ChIP-on-chip analysis illustrated that Dia2 localizes to the replication fork and regulates fork progression on hydroxurea treatment. These results demonstrate that Dia2 is involved in the regulation of replisome activity through a direct interaction with replisome components. The EMBO Journal (2009) 28, 3693–3705. doi:10.1038/emboj.2009.320; Published online 12 November 2009

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Introduction

To ensure thorough and accurate genome duplication during proliferation, it is likely that DNA replication is highly regulated by numerous factors during the cell cycle. A recent biochemical study revealed that a large protein complex, the replisome progression complex (RPC), assembles specifically during S-phase (Gambus et al., 2006). Identification of RPC components by mass spectrometry revealed that the replication fork contains numerous replication fork-associated factors. For example, the Cdc45–MCM–GINS complex is thought to be a replicative helicase (Aparicio et al., 2006; Moyer et al., 2006). Although the Cdc45–MCM–GINS complex is essential for DNA replication, the RPC also contains non-essential proteins. Mrc1 is required for checkpoint activation and fork stabilization on nucleotide depletion by hydroxyurea (HU) (Alcasabas et al., 2001; Katou et al., 2003). It has also been shown that Mrc1 binds directly to the C-terminal region of Mcm6, and that this interaction is essential for checkpoint activation against methyl methane sulphonate (MMS)-induced DNA damage (Komata et al., 2009). Tof1 and Csm3 form a fork-pausing complex involved in stable fork arrest at various replication-pausing sites (Calzada et al., 2005; Hodgson et al., 2007). CTF4 is involved in the establishment of sister chromatid cohesion, which is coupled to DNA replication (Hanna et al., 2001). More recently, it has been proposed that Ctf4 is a key connector between the Cdc45–MCM–GINS complex and DNA polymerase-α, and is required for the coordinated progression of the replisome (Gambus et al., 2009; Tanaka et al., 2009). The FACT complex is a histone chaperone, which is thought to be involved in the disassembly of nucleosomes at the replication fork. It is not fully understood, however, how these RPC components are regulated during DNA replication.

DNA replication must be completed thoroughly, even when the DNA replication fork encounters problems, such as damaged DNA or DNA–protein complexes. When replication is impeded, activation of the intra-S phase checkpoint is critical for maintaining cell viability. Recent studies have shown that the stabilization of stalled replication forks is central to the function of the checkpoint (Tercero and Diffley, 2001; Tercero et al., 2003). A failure in this process leads to DNA gaps and double-strand gaps, which are repaired by a recombination-mediated process and could result in genomic instability (Branzei and Foiani, 2007). The molecular targets through which checkpoints promote fork stabilization remain largely unknown.

Ubiquitin proteasome-dependent protein degradation is involved in almost all biological processes in eukaryotic organisms. Ubiquitin is activated by E1, conjugated to E2 and finally transferred to substrates with the aid of E3, which bridges E2 and the substrate. Polyubiquitinated substrates are recognized by the proteasome and rapidly degraded. E3s, also known as ubiquitin ligases, recognize and bind to their substrates; thus, E3s determine substrate specificity. The Skp1–Cdc53–F-box (SCF) complex is one of the most well-studied E3s. This complex consists of a RING finger protein, Rbx1; Cullin, Cdc53; the adapter protein, Skp1; and the substrate recognition subunit, F-box protein. *Saccharomyces cerevisiae* has 21 putative F-box proteins, many of whose functions are largely unknown (Willems et al., 2004). One of

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these F-box proteins, Dia2, differs from the others in having two protein–protein interaction motifs in addition to the F-box: a tetratricopeptide repeat (TPR) at its N-terminal region and a leucine-rich repeat (LRR) at its C-terminal region. Dia2 was originally isolated as a mutant that showed invasive growth (Palecek et al., 2000). However, recent studies have clarified that Dia2 is involved in the regulation of DNA replication (Blake et al., 2006; Koepp et al., 2006; Pan et al., 2006). DIA2 deletion (dia2Δ) cells accumulate at the S/G2/M phase (Blake et al., 2006; Koepp et al., 2006) and show constitutive activation of the checkpoint kinase Rad53 (Blake et al., 2006; Pan et al., 2006). Moreover, dia2Δ cells show sensitivity to HU, MMS and camptothecin, which cause DNA replication stress. This results in a very slow progression of S phase in the mutant in the presence of low amounts of MMS (Blake et al., 2006; Koepp et al., 2006). Consistent with this observation, dia2Δ cells show a prevalence of gross chromosomal rearrangements, especially in the rRNA region in which the DNA replication fork should pause at the fork barrier site (Blake et al., 2006). Furthermore, DIA2 has genetic interactions with a wide variety of genes that are involved in genome maintenance (Blake et al., 2006; Pan et al., 2006). As homologous recombination repair and non-homologous end joining were not affected in dia2Δ cells, these results suggest that Dia2 is required to overcome replication impedances and that failure in this process induces genomic instability (Blake et al., 2006).

Dia2 has been reported to bind to DNA replication origins in G2/M-arrested cells (Koepp et al., 2006; Swaminathan et al., 2007), but the phenotype of dia2Δ cells suggested that Dia2 might have a more direct role in replication fork activity. Actually, interactions between Dia2 and some replication fork-associated proteins have been found by genome-wide mass spectrometric analysis (Ho et al., 2002). In addition, the fission yeast Schizosaccharomyces pombe Dia2 homologue, Pof3, interacts with Chl1, a counterpart of Ctf4 (Mamnun et al., 2006). Nevertheless, the functions of Dia2 for replication fork have yet to be clarified. As Dia2 forms an SCFDia2 complex and the F-box of Dia2 is necessary for its function (Blake et al., 2006; Koepp et al., 2006), ubiquitination of target proteins by SCFDia2 would presumably be crucial for Dia2 function. We do not yet know what the SCFDia2 substrates are and how SCFDia2 activity is regulated. Here, we report that Dia2 localizes at the replication fork and is required for replication fork stability. We identified certain replisome components as SCFDia2-binding proteins. By domain analysis, we also found that Dia2 is regulated by protein degradation through its N-terminal TPR.

**Results**

*Modified two-hybrid screening identified the replisome components MRC1, CTF4 and MCM2*

To clarify the function of Dia2, we set out to identify Dia2-binding proteins using yeast two-hybrid screening. In general, it is difficult to identify E3 substrates by two-hybrid screening; E3–substrate interactions are unstable because substrate proteins are ubiquitinylated by E3 and degraded by proteasomes. A previous study overcame this problem by modifying conventional yeast two-hybrid screening to identify SCF proteins (Kishi et al., 2007, 2008). To identify SCFDia2 substrates efficiently, we made two modifications. First, the endogenous DIA2 promoter of the host cell was replaced with the GAL1 promoter. As two-hybrid screening was performed in the presence of glucose, a condition in which DIA2 expression is repressed, SCFDia2 substrates were not degraded by endogenous Dia2. Second, we used Dia2Δ, which lacks the F-box, as the bait (Figure 1A). Dia2Δ does not bind to Skp1 but presumably binds to its substrates as it has intact substrate recognition motifs. Thus, substrates were not degraded by the bait protein. With this modified yeast two-hybrid screen, we identified seven MRCl clones, three CTF4 clones and one MCM2 clone. The MRCl clone was truncated at its C-terminal region and consisted of the first 557 amino acids of Mrcl. We found that full-length Mrcl also interacts with Dia2AF (Figure 1B). If Mrcl were the substrate for SCFDia2, one prediction would be that the introduction of wild-type (WT) Dia2 would decrease the two-hybrid interaction between Dia2 and Mrcl, as active SCFDia2 could ubiquitinate and degrade Mrcl. Figure 1B shows that this is the case, as MRCl showed a faint two-hybrid interaction with WT LexA-DIA2 when WT L40 (DIA2) was used as a host strain. This interaction was clearly enhanced either by deleting the endogenous DIA2 gene (dia2Δ) or by using DIA2AF as bait. A combination of these modifications resulted in the strongest two-hybrid interaction. These results are consistent with the hypothesis that Mrcl is a substrate for SCFDia2.

*Physical interaction between Dia2 and Mrcl*

To examine the physical interaction between Dia2 and Mrcl in more detail, these proteins were co-expressed in Sf21 cells using a baculovirus expression system. Recombinant proteins were immunoprecipitated by antibodies against their epitope tags. We found reciprocal co-immunoprecipitation of Dia2 and Mrcl, but not of Dia2 and Tof1, which were used as negative controls (Figure 1C). To test whether endogenous Dia2 and Mrcl bind to each other in yeast, endogenous Mrcl and Dia2 were FLAG- and Myc-tagged, respectively, at their C-termini. We considered Dia2His10FLAG to be functional because Dia2-His10FLAG strains were resistant to MMS, unlike the dia2Δ strain (Supplementary Figure 1). We found co-immunoprecipitation of Mrcl with Dia2 by using an anti-FLAG antibody, but not by using a control antibody (Figure 1D). These results suggested that endogenous Mrcl binds to endogenous Dia2 in vivo. To test whether not only Mrcl but also Ctf4 physically interacts with Dia2 in yeast, Dia2AF was expressed from the GAL1 promoter, and co-immunoprecipitation of endogenous Ctf4 was examined. Figure 1E shows that both Mrcl and Ctf4 bind to FLAG-tagged Dia2AF, consistent with a previous genome-wide protein interaction study (Ho et al., 2002). Thus, Dia2 physically interacts with Mrcl and Ctf4 in vivo.

*Regions required for Dia2–Mrcl interaction*

Besides the F-box, Dia2 has two putative protein–protein interaction motifs, the TPR and LRR, at the N- and C-terminal regions, respectively (Figure 1A). We assessed which motif binds to Mrcl, Ctf4 or Mcm2 by two-hybrid assay. Deletion of the TPR motif diminished the two-hybrid interactions between Dia2 and Mrcl, Ctf4 and Mcm2, whereas deletion of the LRR motif did not affect any of these two-hybrid interactions (Figure 2A). Thus, the TPR motif of Dia2 is both necessary and sufficient for binding to Mrcl, Ctf4 and Mcm2. We also found that Mrcl and Mcm2 co-immunoprecipitated with WT HA-Dia2 from yeast extracts,
DIA2 ΔF lacks an F-box (amino acid 224–263). (B) Two-hybrid interaction between Dia2 and Mrc1. Host yeast strains, either L40 or L40 dia2Δ, were transformed with either LexA-DIA2 WT or LexA-DIA2 ΔF plasmid and B42-MRC1 plasmid and then plated onto an SD – Leu, – Trp plate containing X-gal. (C) Co-immunoprecipitation of Dia2 and Mrc1 in Sf21 cells. Baculoviruses expressing the DIA2-6His3HA and 6His3FLAG proteins indicated above were co-transfected into Sf21 cells. Cell extracts were prepared and proteins were immunoprecipitated with either αHA or αFLAG. Bound proteins were separated by SDS–PAGE and analysed by immunoblotting. (D) Co-immunoprecipitation of Dia2 and Mrc1 in budding yeast. Endogenous Dia2 and Mrc1 were tagged with 6His10FLAG and 18Myc, respectively. Cells were cultured in YPD medium and treated with 0.1 mM of the proteasome inhibitor MG132 for 45 min, and then cell extracts were prepared by bead beating. DIA2-6His10FLAG was expressed from the GAL1 promoter and immunoprecipitated with αFLAG. Co-immunoprecipitation of Mrc1-18Myc and Ctf4-3HA was analysed by immunoblotting. (E) Dia2 ΔF-3FLAG was expressed from the GAL1 promoter and immunoprecipitated with αFLAG. Co-immunoprecipitation of Mrc1-18Myc and Ctf4-3HA was analysed by immunoblotting.

Figure 1 A physical interaction between Dia2 and Mrc1. (A) Schematic diagram of the DIA2 construct. DIA2 ΔF lacks an F-box (amino acid 224–263). (B) Two-hybrid interaction between Dia2 and Mrc1. Host yeast strains, either L40 or L40 dia2Δ, were transformed with either LexA-DIA2 WT or LexA-DIA2 ΔF plasmid and B42-MRC1 plasmid and then plated onto an SD – Leu, – Trp plate containing X-gal. (C) Co-immunoprecipitation of Dia2 and Mrc1 in Sf21 cells. Baculoviruses expressing the DIA2-6His3HA and 6His3FLAG proteins indicated above were co-transfected into Sf21 cells. Cell extracts were prepared and proteins were immunoprecipitated with either αHA or αFLAG. Bound proteins were separated by SDS–PAGE and analysed by immunoblotting. (D) Co-immunoprecipitation of Dia2 and Mrc1 in budding yeast. Endogenous Dia2 and Mrc1 were tagged with 6His10FLAG and 18Myc, respectively. Cells were cultured in YPD medium and treated with 0.1 mM of the proteasome inhibitor MG132 for 45 min, and then cell extracts were prepared by bead beating. DIA2-6His10FLAG was immunoprecipitated with αFLAG, and bound proteins were separated by SDS–PAGE and analysed by immunoblotting. (E) Dia2 ΔF-3FLAG was expressed from the GAL1 promoter and immunoprecipitated with αFLAG. Co-immunoprecipitation of Mrc1-18Myc and Ctf4-3HA was analysed by immunoblotting.

but co-immunoprecipitation was minimal with HA-Dia2 ΔFPR (Figure 2B). Using immunoprecipitation data from Sf21 cells, we found that Mrc1 binds strongly to WT Dia2 and Dia2-N but binds very weakly to Dia2-C (Figure 2C). These results also support the hypothesis that Mrc1, Ctf4 and Mcm2 bind to the N-terminal TPR motif of Dia2. To determine the Dia2-binding site of Mrc1, various Mrc1 truncation mutants were constructed, and interactions with DIA2 AF were analysed by yeast two-hybrid assay. We found that Dia2 interacts with Mrc1 through two regions: one between a.a. 380–557, and the other between a.a. 701–800 (Figure 2D). These results suggest that Dia2 binds two separate domains of Mrc1.

**The LRR is indispensable for Dia2 function**

Known F-box proteins have substrate recognition motifs at C-terminal region (Willems et al., 2004). We hypothesized that TPR is involved in the regulation of Dia2 by interacting with replisome components, whereas LRR is important for substrate recognition. To test this hypothesis, we examined whether TPR and LRR are essential for Dia2 function. dia2Δ cells are sensitive to HU, MMS and camptothecin, accumulate in S and G2/M phases and have increased cell size (Blake et al., 2006). Previous studies have shown that the F-box is required for Dia2 function, suggesting that E3 ubiquitin ligase activity is necessary for Dia2 function. Various Dia2 mutants were expressed in dia2Δ cells, and we tested whether they could suppress the phenotypes of dia2Δ cells. With a vector control, dia2Δ cells were sensitive to HU and MMS (Figure 3A). As reported previously, DIA2 WT but not DIA2 ΔF suppressed this drug sensitivity. DIA2 ΔLRR also suppressed this drug sensitivity, but DIA2 ΔLRR did not. We found that Dia2 ΔTPR, but not Dia2 ΔLRR, could suppress accumulation in S and G2/M phase by FACs analysis (Figure 3B). We also observed an increase in the cell size of dia2Δ cells (Figure 3C and D). In comparison with vector control cells, DIA2 WT and DIA2 ΔTPR cells are smaller (P<0.001, χ²-test). By contrast, DIA2 ΔF and DIA2 ΔLRR cells showed size distributions very similar to those of vector control cells (P>0.1, χ²-test). These results suggest that the LRR, but not the TPR, motif is indispensable for Dia2 function under the tested conditions. However, we could not rule out...
Figure 2 Binding regions of Dia2 and Mrc1. (A) The interactions between Dia2 fragments and Mrc1, Ctf4 and Mcm2 were examined by plating cells onto either SD–Leu–Trp plates or SD–Leu–Trp–His + 1 mM 3-AT plates. Dia2 ΔF lacks amino acids 224–263, ΔN lacks amino acids 1–263, ΔC lacks amino acids 224–746, ΔFΔTPR lacks amino acids 1–173 and 224–263, and ΔFΔLRR lacks amino acids 224–263 and 347–746. (B) Co-immunoprecipitation of Dia2 fragments and Mrc1 and Mcm2 in yeast. Either 3HA-Dia2 WT or 3HA-Dia2 ΔTPR was expressed from ADH1 promoter. Yeast cells were cultured in YPD medium, arrested by α-factor for 3 h, then released into 0.2 M HU-containing medium for 1 h. Yeast extracts were prepared, and proteins were immunoprecipitated with α-HA antibody. Bound proteins were separated by SDS–PAGE and analysed by immunoblotting. (C) Co-immunoprecipitation of Dia2 fragments and Mrc1 in Sf21 cells. Baculoviruses expressing His6-3HA DIA2 WT (1–746 a.a.), N (1–223 a.a.) or C (264–746 a.a.), and 6His3FLAG Mrc1 were co-transfected into Sf21 cells. At 3 days after infection, cell extracts were prepared and immunoprecipitated with α-HA antibody. Bound proteins were separated by SDS–PAGE and analysed by immunoblotting. (D) Two-hybrid interaction between Dia2 and Mrc1 fragments. L40 was transformed with LexA-DIA2 ΔF plasmid and B42-MRC1 fragment plasmids, as indicated. Cells were plated onto SD–Leu–Trp–His plates, some of which contained the appropriate ingredients, as stated below.
**Figure 3** The LRR motif of Dia2 is indispensable for its function. (A) Suppression of drug sensitivity of dia2Δ cells. The dia2Δ strain was transformed with single-copy plasmids that expressed the DIA2 mutant from the ADH1 promoter. Cells were plated onto SD -Leu plates containing either HU or MMS. DIA2 ΔF lacks amino acids 224–263, DIA2 ΔLRR lacks amino acids 347–746 and DIA2 ΔTPR lacks amino acids 1–173. (B) The cell-cycle profile of strains used in (A). Log-phase cells were analysed by flow cytometry. (C) The strain used in (A) was observed by microscopy. (D) From the obtained images in (C), the lengths of the cells were measured and plotted as histograms. The significance of the differences in size distributions between + vector and either + DIA2 WT, + DIA2 ΔF, + DIA2 ΔTPR and + DIA2 ΔLRR were tested by the \( \chi^2 \)-test. P-values were 0.000738, 0.109043, 1.19E–05 and 0.220063, respectively. (E) Synthetic growth defects between cul8Δ and DIA2 ΔTPR. dia2Δ, cul8Δ or dia2Δ hst3Δ double mutants, which have DIA2 WT (URA3) plasmid and an empty, DIA2 WT or DIA2 ΔTPR plasmid (LEU2), were cultured in YPD medium overnight and then plated onto either YPD or SD –Leu plates containing 5-FOA.
the possibility that the TPR motif is essential in specific conditions. dia2Δ shows a synthetic lethal/growth defect phenotype with various other genes (Blake et al., 2006; Pan et al., 2006; Collins et al., 2007). Among them, CUL8/RIT101 encodes a cullin that is involved in resumption of stalled replication forks (Luke et al., 2006; Collins et al., 2007; Duro et al., 2008). HST3 encodes a histone deacetylase responsible for deacetylation of histone H3 K56 (Ozdemir et al., 2006). Recent studies suggest that CUL8 functions downstream of HST3 (Collins et al., 2007). We tested whether DIA2 ATRP has a synthetic lethal/slow growth phenotype with those genes using a plasmid-shuffling experiment (Figure 3E). Double mutant cells, with DIA2 WT on a URA3 plasmid and a vector control, DIA2 WT or DIA2 ATRP on a LEU2 plasmid, were plated on YPD or 5-FOA-containing plates. Both cul8/rtt101Δ and hst3Δ showed synthetic lethal/slow growth phenotypes with dia2Δ. WT DIA2 could suppress the phenotype, and DIA2 ATRP could suppress the synthetic lethal/slow growth phenotype with hst3Δ, but not with cul8Δ. These results indicate that the TPR motif of Dia2 has an essential role in the absence of Cul8. Expression of DIA2 ATRP slightly affected the growth of cul8Δ, suggesting that DIA2 ATRP might have a dominant function in a cul8Δ mutant background. Cul8 has been proposed to promote sister chromatid exchanges (SCE) at stalled forks. (Duro et al., 2008). We tested whether Dia2 is also involved in this process (Supplementary Figure 2). We found that, unlike cul8Δ cells, dia2Δ cells were able to activate unequal SCE on MMS treatment. We noticed that the uSCE rate was about six times higher in dia2Δ cells in the absence of DNA damage compared with WT cells. These results imply that, in dia2Δ cells, replication forks stall spontaneously and Cul8 is necessary for their recovery.

SCF<sup>Dia2</sup> destabilizes Mrc1 in vivo and ubiquitinates Mrc1 in vitro

Although Mrc1 binds to the TPR motif of Dia2, it is still possible that Mrc1 is ubiquitinates by SCF<sup>Dia2</sup>. Thus, we asked whether Mrc1 is destabilized in vivo in an SCF<sup>Dia2</sup>, and proteasome-dependent manner. To address this question, the proteasome inhibitor, MG132, was added to the yeast culture to prevent proteasome-dependent protein degradation. Next, cycloheximide (CHX) was added to prevent new protein synthesis. The amount of endogenous Mrc1 was decreased gradually after CHX addition, whereas in the absence of DNA damage compared with WT cells. These results imply that, in dia2Δ cells, replication forks stall spontaneously and Cul8 is necessary for their recovery.

Regulation of Dia2 stability by TPR motif and DNA damage

Although TPR is dispensable for Dia2 function under the tested conditions, it is possible that TPR participates in the regulation of Dia2 function. In our experiments, Dia2 expressed from a plasmid under the control of the ADH1 promoter was unstable in vivo but was greatly stabilized on the removal of the TPR motif (Figure 5A). These observations enabled us to assume that although the TPR motif is dispensable for Dia2 function, it might be involved in its regulation through protein stabilization. We also found that WT Dia2 and Dia2 ALRR were unstable under normal conditions but were stabilized on MMS treatment (Figure 5A). Both exogenously expressed Dia2 and endogenous Dia2 are unstable in log phase, α-factor- or nocodazole-arrested cells, but they are slightly stabilized in HU-arrested cells and highly stabilized in MMS-treated cells (Figure 5B). Taken together, Dia2 is a modestly unstable protein that is dependent on its TPR motif in vivo and is stabilized on DNA damage during S phase. Dia2 destabilization is at least partially dependent on proteasome activity, as MG132 stabilized Dia2 (Figure 5C). It has been reported that some F-box proteins are autoubiquitinated (Zhou and Howley, 1998). We found that Dia2 is stabilized in both cdc34 and skp1 ts mutants, but not in a cdc4 ts mutant (Figure 5D). These results are consistent with the idea that Dia2 is degraded through autoubiquitination.
Dia2 co-localizes with replication fork-associated proteins and is required for fork stability

The interaction between the TPR motif and replisome components implied that Dia2 localizes at replication forks. To determine the chromosomal localization of Dia2 in early S-phase cells, we performed ChIP-on-chip analysis along all of chromosome VI (Figure 6A). Mrc1 and Cdc45 are components of the replisome and were used as positive controls for replisome localization. Cells were arrested at G1 by α-factor, HU and nocodazole, respectively, for 3 h. Then, 500 μg/ml of cycloheximide was added. The stability of Mrc1 was analysed as in (A). (D) Cells were cultured in YPD medium and cell extracts were prepared at 0, 30 or 60 min after CHX addition. Mcm4-5FLAG was immunoprecipitated with α-FLAG antibody and co-immunoprecipitated proteins were analysed by immunoblotting. (E) In in vitro ubiquitination of Mrc1 using Ni2+-NTA agarose-purified SCFΔDia2. Baculoviruses expressing the four components of SCFΔDia2 were co-transfected into S21 cells. Both Rbx1 and Dia2 were 6His tagged. At 3 days after transfection, cell extracts were prepared and SCFΔDia2 was purified by Ni2+-NTA agarose chromatography. Purified SCFΔDia2 was used as E3 for the in vitro ubiquitination assay. Either 6His3FLAG-Mrc1 or 6His3FLAG-Tof1 was purified from S21 cells by Ni2+-NTA agarose beads and used as a substrate. The amount of SCFΔDia2 was titrated two-fold. After the reaction, the proteins were analysed by immunoblotting following SDS-PAGE. Mrc1 was detected by α-Myc. Total proteins on the membrane were stained with Ponceau S and were used as loading controls. (F, G, H) Experiments were performed as shown in (D) and (E), respectively.

Figure 4 Destabilization of Mrc1 by SCFΔDia2. (A) Stabilization of Mrc1 by MG132. The yeast strain YSM396, in which endogenous MRC1 had been tagged with 18MYC and PDR5 had been deleted to sensitize the cells to MG132, was used and cultured in YPD medium. Cells were treated with a 0.1 mM concentration of the proteasome inhibitor MG132 for 60 min, and then 500 μg/ml of cycloheximide was added to prevent new protein synthesis. At the indicated time point, cells were collected, and cell extracts were prepared with TCA. Proteins were analysed by immunoblotting using α-Myc. Total proteins on the membrane were stained with Ponceau S and were used as loading controls. (B) Stabilization of Mrc1 by Dia2 deletion. The yeast strain YSM280 (MRC1-MYC18, DIA2) or YSM368 (MRC1-MYC18, dia2Δ) was cultured in YPD medium, and 500 μg/ml of cycloheximide was added to prevent new protein synthesis. (C) Mrc1 was particularly unstable in HU-arrested S-phase cells. YSM280 was cultured in YPD medium. The cells were arrested at G1, S and G2/M by α-factor, HU and nocodazole, respectively, for 3 h. Then, 500 μg/ml of cycloheximide was added. The stability of Mrc1 was analysed as in (A). (D) Cells were cultured in YPD medium and cell extracts were prepared at 0, 30 or 60 min after CHX addition. Mcm4-5FLAG was immunoprecipitated with α-FLAG antibody and co-immunoprecipitated proteins were analysed by immunoblotting. (E) In vitro ubiquitination of Mrc1 using Ni2+-NTA agarose-purified SCFΔDia2. Baculoviruses expressing the four components of SCFΔDia2 were co-transfected into S21 cells. Both Rbx1 and Dia2 were 6His tagged. At 3 days after transfection, cell extracts were prepared and SCFΔDia2 was purified by Ni2+-NTA agarose chromatography. Purified SCFΔDia2 was used as E3 for the in vitro ubiquitination assay. Either 6His3FLAG-Mrc1 or 6His3FLAG-Tof1 was purified from S21 cells by Ni2+-NTA agarose beads and used as a substrate. The amount of SCFΔDia2 was titrated two-fold. After the reaction, the proteins were analysed by immunoblotting following SDS-PAGE. Mrc1 was detected by α-Myc. Total proteins on the membrane were stained with Ponceau S and were used as loading controls. (F, G, H) Experiments were performed as shown in (D) and (E), respectively.

### Dia2 co-localizes with replication fork-associated proteins and is required for fork stability

The interaction between the TPR motif and replisome components implied that Dia2 localizes at replication forks. To determine the chromosomal localization of Dia2 in early S-phase cells, we performed ChIP-on-chip analysis along all of chromosome VI (Figure 6A). Mrc1 and Cdc45 are components of the replisome and were used as positive controls for replisome localization. Cells were arrested at G1 by α-factor and released into HU-containing media for 60 min to induce
arrest at early S-phase. Mrc1 and Cdc45 localized in the vicinity of early firing origins but did not localize to late firing origins, as previously reported (Katou et al, 2003). Compared with Cdc45, Mrc1 was observed much closer to origins. This might reflect the fact that Cdc45 gives a stronger ChIP-on-chip signal than Mrc1, or that Cdc45 and Mrc1 position differently in RPC. We found that Dia2 also localized to early firing origins, similar to Mrc1, but did not localize to late firing origins. These results suggest that Dia2 associates with replication forks in early S-phase. Next, we examined the localization of Cdc45 and incorporation of bromodeoxyuridine (BrdU) in HU-arrested cells in the absence of Dia2. Both Dia2 and dia2Δ cells were arrested by α-factor and released into HU-containing media for 60 min (Figure 7A). In Dia2 cells, both Cdc45 localization and BrdU incorporation were limited to the vicinity of early firing origins, as expected. In dia2Δ cells, however, Cdc45 localized more broadly, and BrdU was incorporated into more dispersed regions. These results suggested that the replication fork progresses slightly even in the presence of HU in dia2Δ cells. We also found that Cdc45 accumulated at inter-origin regions in dia2Δ cells. These results imply that collided forks were not resolved and were stably present in this specific condition. Using a time course experiment, we found that Cdc45 signals were present at the right hand fork from ARS607 and at the left hand fork from ARS603.5 at the early time point, but became fainter at the late time point (Figure 7B). We analysed Cdc45 localization and BrdU incorporation not only along chromosome VI, but also in all S. cerevisiae chromosomes (Supplementary Figures 4–7) and obtained consistent results. Cdc45 did not localize to late origins in either Dia2 or dia2Δ cells, indicating that suppression of late origin firing by the intra-S checkpoint normally occurs in the absence of Dia2. This is consistent with previous observations that checkpoint activation on HU treatment is normal in dia2Δ cells (Blake et al, 2006; Pan et al, 2006). ChIP-on-chip analysis showed that Dia2 is involved in the progression of replication forks in the presence of HU. We next investigated S-phase progression of either Dia2 or dia2Δ cells in the presence of a low concentration of HU by FACS (Figure 7C). Cells were arrested at early G1 phase by α-factor and then released into media containing the indicated amount of HU. In the absence of HU, S-phase progression is very similar in either Dia2 or dia2Δ cells, suggesting that S-phase progresses at a similar rate in

Figure 5 The TPR motif of Dia2 is involved in protein stability. (A) Stability of the Dia2 mutant on MMS treatment. Cells containing single-copy plasmids expressing the 3HA-Dia2 mutant from the ADH1 promoter were cultured in YPD medium to mid-log phase. Cells were arrested by α-factor then released into YPD medium containing MMS for 1 h. Next, 200 μg/ml of cycloheximide was added to prevent new protein synthesis. At the indicated time point, cells were collected and cell extracts were prepared by alkaline lysis. The proteins were analysed by immunoblotting using 3HA antibody. Total proteins on the membrane were stained with Ponceau S and used as loading control. (B) Stability of endogenous Dia2. Endogenous Dia2 was tagged with His6-10FLAG. The cells were cultured in YPD medium and arrested by either α-factor or nocodazole for 3 h. For HU and MMS arrest, α-factor-arrested cells were released into medium containing either HU or MMS for 1 h. The stability of endogenous Dia2 was examined as in Figure 4A. (C) The yeast strain YSM403, in which endogenous Dia2 was tagged with His6-10FLAG and PDR5 was deleted to sensitize cells to MG132, was cultured in YPD medium. Cells were treated with 0.1 mM of the proteasome inhibitor MG132 for 60 min, and 200 μg/ml of cycloheximide was added. The protein stability of Dia2 was examined as in (A). (D) Stability of endogenous Dia2 in SCF mutants. Endogenous Dia2 was tagged with His6-10FLAG in WT, cdc34-1, skp1-11, and cdc4-1 strains, respectively. Cells were cultured at 25°C and then the temperature was shifted to 37°C. After 1 h of incubation, cycloheximide was added. The stability of the Dia2 protein was examined as in (A).
the absence of Dia2. In the presence of 200 mM HU, the FACS peak did not move in dia2Δ cells. In the presence of 50 mM HU, DIA2 cells responded to HU, and the FACS peak at 1c DNA content did not move up to 60 min after release. In dia2Δ cells, however, the FACS peak gradually moved toward 2c content. These results demonstrated that dia2Δ cells cannot suppress DNA replication in response to low amounts of HU.

Discussion

Faithful DNA replication is one of the most fundamental prerequisites for cell proliferation. Recent studies have shown that many proteins are associated with the replication fork. It is important to regulate these proteins against obstacles impeding DNA replication, such as damaged DNA or protein-DNA complexes. Here, we show that the F-box protein, Dia2, co-localizes with replication fork-associated proteins and participates in the regulation of the replication fork. Dia2 localized at early replication origin in HU-arrested cells, like Mrc1 does. We have tried to observe that Dia2 actually moves along chromosome in the absence of HU, but we have not got clear results, due to the weak ChIP-on-chip signal of Dia2. Thus, it is still formally possible that Dia2 localizes at origins but not at ongoing forks. However, evidences suggest that Dia2 is likely to be a replication fork protein: (1) the interaction of Dia2 with Mrc1, Ctf4 and Mcm2, which are established fork factors (Figure 1 and 2), (2) Sensitivity of the cells to fork block agents (Blake et al., 2006), and (3) abnormal progression of replication fork on HU treatment (Figure 7). Localization of Dia2 at the replication fork suggests that SCF Dia2 has a direct role in the regulation of DNA replication. We speculate that the interaction between the TPR motif and replisome components contributes to Dia2 localization (discussed in detail later). In the absence of Dia2, replication forks tend to progress in the presence of HU. Moreover, at some forks, such as the right hand fork of ARS607 or the left hand fork of ARS603.5, Cdc45 signals become less recognizable in HU-arrested cells. This observation could be explained in two ways. First, in dia2Δ cells, replication forks cannot stall properly responding to low nucleotide levels, resulting in randomly stalled replication forks. Random distributions of replication forks along a chromosome would result in lower Cdc45 signals, which cannot be recognized by ChIP-on-chip analysis. Alternatively, Cdc45 might be dissociated from the chromosome under such conditions. Currently, we prefer the former explanation because, as BrdU is incorporated into more dispersed regions, replication forks should progress; in addition, dia2Δ cells did not show severe defects after the release from HU arrest (data not shown). Thus, Dia2 might be involved in the process that actively arrests the fork, responding to reduced nucleotide levels. Some replication forks, however, accumulated at inter-origin regions. This suggests that collided forks were not resolved under these conditions. These results imply that Dia2 might function in replication termination.
Dia2 seems to be critical for fork regulation, but its function remains to be elucidated. We have found that the replication fork-associated proteins Mrc1, Ctf4 and Mcm2 interact with Dia2. Perhaps Dia2 is localized to replication forks through interactions with these proteins. We also found that Mrc1 binds to Dia2, is destabilized \textit{in vivo} in a

![Figure 7 Behaviour of replication forks in the presence of HU.](image)

\textbf{Figure 7} Behaviour of replication forks in the presence of HU. (A) Distribution of Cdc45 and BrdU-incorporated regions on chromosome VI in wild-type cells and a \textit{dia2} mutant in S-phase under the presence of HU. Cells expressing HA-tagged version of Cdc45 and adenovirus thimidine kinase (TK) for efficient incorporation of BrdU were arrested in G1 phase by α-factor and then released into S-phase at 23°C in the presence of 200 mM HU for 60 min. Blue and red bars represent regions of Cdc45 binding and BrdU incorporation, respectively. (B) The time course of Cdc45 localization on chromosome VI in \textit{dia2} cells. After release from G1 arrest into S-phase in the presence of 200 mM HU at 23°C, cells were withdrawn at indicated time points from culture and ChIP-on-chip analysis was performed. (C) FACS analysis of HU arrested cells. Cells were arrested by α-factor for 3 h then released into YPD medium containing indicated amounts of HU. Cells were collected at time point indicated on left, and processed for FACS analysis.
Dia2-dependent manner and is ubiquitinated by SCF\textsuperscript{Dia2} in vitro. Mrc1 is less stable in HU-arrested cells. This is consistent with the fact that both Dia2 and Mrc1 localize to the replication fork. However, degradation of Mrc1 is very slow compared with known SCF substrates like Sic1 or Cdc6; in addition, only a very limited amount of Mrc1 seemed to be degraded. It is possible that degradation might occur only locally; thus, only limited amounts of Mrc1 are degraded. Actually, we showed that the amount of Mrc1, that was co-immunoprecipitated with Mcm4, was dramatically decreased during a CHX chase. We observed similar results with Ctf4, another RPC component. Thus, we presume that SCF\textsuperscript{Dia2} localizes to replication forks during S-phase and destabilizes Mrc1 and Ctf4. Having said that, however, at the moment, we do not know the significance of Mrc1 and Ctf4 degradation. The TPR motif is required for the interaction with Mrc1, Ctf4 and Mcm2, but it is dispensable for drug sensitivity. It is possible that Mrc1 and Ctf4 might be degraded not as real targets but as collateral damage. This possibility is supported by the fact that known F-box proteins recognize their substrates through the C-terminal region, but Mrc1 and Ctf4 bind to the N-terminal TPR motif of Dia2. As some F-box proteins have been reported to be autoubiquitinated (Zhou and Howley, 1998), Mrc1 and Ctf4, bound to TPR, might be ubiquitinated in a similar way. Alternatively, it is possible that degradation of Mrc1 and Ctf4 by SCF\textsuperscript{Dia2} might be involved in the dissociation of SCF\textsuperscript{Dia2} from the replication fork. Further experiments will be required to clarify the importance of Mrc1 destabilization.

The N-terminal TPR motif of Dia2 was necessary and sufficient for binding to Mrc1, Ctf4 and Mcm2 in the two-hybrid assay. However, the TPR motif is dispensable for Dia2 function for suppressing drug sensitivity. These results suggest that the interactions with Mrc1, Ctf4 and Mcm2, and the destabilization of Mrc1 are probably not essential for Dia2’s function in DNA damage response under these conditions. However, as truncation of the TPR motif results in significant stabilization of Dia2, it is possible that overexpression of Dia2\_ATPR by this stabilization might suppress its partial functional defect. In addition, we cannot exclude the possibility that the TPR motif has a significant role in Dia2 function under specific circumstances. Morohashi et al (personal communication) observed that DIA2 ATPR could not suppress synthetic lethality with rrm3Δ (Dr Karim Labib, personal communication), suggesting that the interaction with the replosome through the TPR motif is extremely important for dealing with DNA damage caused by RR3M deletion. Furthermore, we found that DIA2\_ATPR shows a synthetic growth defect with cul8A. Thus, we presume that loading of SCF\textsuperscript{Dia2} onto a replication fork through the TPR motif and/or destabilization of Mrc1 and Ctf4 are important under specific circumstances.

We have shown that Dia2 is a mildly unstable protein and that TPR deletion causes stabilization of Dia2. Destabilization of Dia2 is at least partially proteasome dependent. We have also found that Dia2 is greatly stabilized by MMS treatment. This stabilization might be a way of regulating SCF\textsuperscript{Dia2} by permitting its activity only when DNA damage is present. The mechanism of stabilization is currently unknown. One attractive idea, however, is that binding between the Dia2 TPR motif and Mrc1, Ctf4 or Mcm2 might change on DNA damage; thus, degradation would be blocked. Recently, it has been reported that Mrc1 binds to DNA polymerase-\(\epsilon\), which is responsible for leading strand DNA synthesis (Lou et al, 2008). There are two separate polymerase-\(\epsilon\)-binding sites in Mrc1. Interestingly, the N-terminal-binding site of Mrc1 dissociates from polymerase-\(\epsilon\) on DNA damage. Similar to polymerase-\(\epsilon\), Dia2 binds to Mrc1 through two separate binding sites: 380–557 a.a. and 701–800 a.a. These regions partially overlap with polymerase-\(\epsilon\)-binding regions. Thus, it may be possible that these two proteins compete for binding. On DNA damage, the N-terminal-binding site of Mrc1 dissociates from polymerase-\(\epsilon\) and might provide a further binding site for Dia2, possibly resulting in Dia2 stabilization. Therefore, although the TPR motif is dispensable for Dia2 function, we would like to propose that it might have a role in the regulation of Dia2 stability and localization through interactions with Mrc1, Ctf4 and Mcm2.

Unlike the TPR motif, the F-box and C-terminal LRR motif are indispensable for Dia2 function. These results clearly indicate that Dia2 recognizes its substrates through the LRR motif and then ubiquitinates them. The identification of such substrates will greatly contribute to our understanding of how replication forks are regulated upon replication stress through ubiquitination.

**Materials and methods**

**Yeast techniques**

The strains used in this study were constructed by PCR-based homologous recombination and are listed in Table I. The cells were grown in either YPD or SD medium at 30°C to maintain the plasmid. Cells were synchronized in G1, S and G2/M phase by 10μg/ml α-factor, 0.2 M HU and 10μg/ml of nocodazole, respectively, for 3 h. For MMS treatment, the cells were arrested using α-factor and then released into YPD medium containing 0.015% MMS for 1 h. For the spot assays, cells were grown in either YPD or SD medium at 30°C for 2–4 days. For complementation tests, Dia2 was expressed from pRS415 ADH1p. The FACS profile was obtained using a FACSCalibur instrument (BD Biosciences). To measure cell size, cells were photographed using an Axio Observer microscope (Carl Zeiss), and the lengths of more than 75 cells each were manually measured.

**Yeast two-hybrid screening**

Two-hybrid screening was performed using pLexA-Dia2 AF as bait in the L40 GAL–DIA2 strain, in which DIA2 expression is repressed in the presence of glucose, and the B42–Yeast ORF library as prey. Transformants were screened, and HIS3 and B42 were selected on YPD medium. The plates were incubated at 30°C for 2–4 days. For complementation tests, DIA2 was expressed from pRS415 ADH1p. The FACS profile was obtained using a FACSCalibur instrument (BD Biosciences). To measure cell size, cells were photographed using an Axio Observer microscope (Carl Zeiss), and the lengths of more than 75 cells each were manually measured.
Table 1  Yeast strains

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<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
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<td>YSM695</td>
<td>BY4741, trp1::hisG, CTF4-3HA (TRP) dia2A::kanMX MCM4-5FLAG(hph)</td>
<td>This study</td>
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Parental strains.
W303-1a MATA, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15.
BY4741 MATA his3A1 leu2ΔO met15ΔO ura3ΔO.
L40 MATA his3Δ200 trp1-901 leu2-3112 ade2LY2S::(4lexAop-HIS3) URA3::::(8lexAop-lacZ)GAL.

Cruz Biotechnology, sc-805; 1:2500 dilution)). α-Mouse IgG- HRP (Sigma; 1:5000 dilution) and Protein A–HRP (GE Healthcare; 1:2500 dilution) were used as secondary antibodies. Proteins were detected using either SuperSignal West Pico or Dura (Pierce) or Novex ECL (Invitrogen).

Protein turnover
To measure the protein turnover in vivo, cells were cultured in YPD medium to a growth of OD600 ¼ 0.6. Cells were collected during the time course after the addition of 0.2 mg/ml CHX. For in vitro ubiquitination assays, SCF Dia2 and substrates were purified from Sf21 cells co-expressing His6Myc-Rbx1, Cdc53, Skp1 and Dia2-LRR). The reactions were stopped by adding sample buffer, and ubiquitination was examined by immunoblotting with an anti-FLAG antibody.

ChIP-on-chip analyses
Chromatin immunoprecipitation and anti-BrdU immunoprecipitation on DNA chip (ChIP-on-chip) analyses were performed as previously described (Katou et al., 2006). To examine the whole region of chromosome VI and whole genomic region, the rikDACF (Affymetrix, CA, USA) DNA chip and S. cerevisiae whole-genome tiling array 1.0R (Affymetrix) were used, respectively.

Supplementary data
Supplementary data are available at The EMBO Journal Online (http://www.embojournal.org).

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Conflict of interest
The authors declare that they have no conflict of interest.

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