SCF^{Dia2} 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^{Dia2} 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 tetratricopeptide 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 hydroxyurea treatment. These results demonstrate that Dia2 is involved in the regulation of replisome activity through a direct interaction with replisome components.
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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 impedence 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 SCF^{Dia2} 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 SCF^{Dia2} would presumably be crucial for Dia2 function. We do not yet know what the SCF^{Dia2} substrates are and how SCF^{Dia2} 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 SCF^{Dia2}-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 ubiquitinated by E3 and degraded by proteasomes. A previous study overcame this problem by modifying conventional yeast two-hybrid screening to identify SCF^{Cdc4} substrates (Kishi *et al*, 2007, 2008). To identify SCF^{Dia2} 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, SCF^{Dia2} substrates were not degraded by endogenous Dia2. Second, we used Dia2 ΔF, which lacks the F-box, as the bait (Figure 1A). Dia2 ΔF 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 *MRC1* clones, three *CTF4* clones and one *MCM2* clone. The *MRC1* clone was truncated at its C-terminal region and consisted of the first 557 amino acids of Mrc1. We found that full-length Mrc1 also interacts with Dia2 ΔF (Figure 1B). If Mrc1 were the substrate for SCF^{Dia2}, one prediction would be that the introduction of wild-type (WT) Dia2 would decrease the two-hybrid interaction between Dia2 and Mrc1, as active SCF^{Dia2} could ubiquitinate and degrade Mrc1. Figure 1B shows that this is the case, as *MRC1* 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 *DIA2* ΔF as bait. A combination of these modifications resulted in the strongest two-hybrid interaction. These results are consistent with the hypothesis that Mrc1 is a substrate for SCF^{Dia2}.

Physical interaction between Dia2 and Mrc1

To examine the physical interaction between Dia2 and Mrc1 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 Mrc1, but not of Dia2 and Tof1, which were used as negative controls (Figure 1C). To test whether endogenous Dia2 and Mrc1 bind to each other in yeast, endogenous Mrc1 and Dia2 were FLAG- and Myc-tagged, respectively, at their C-termini. We considered Dia2-His10FLAG to be functional because Dia2-His10FLAG strains were resistant to MMS, unlike the *dia2Δ* strain (Supplementary Figure 1). We found co-immunoprecipitation of Mrc1 with Dia2 by using an anti-FLAG antibody, but not by using a control antibody (Figure 1D). These results suggested that endogenous Mrc1 binds to endogenous Dia2 *in vivo*. To test whether not only Mrc1 but also Ctf4 physically interacts with Dia2 in yeast, Dia2 ΔF was expressed from the *GAL1* promoter, and co-immunoprecipitation of endogenous Ctf4 was examined. Figure 1E shows that both Mrc1 and Ctf4 bind to FLAG-tagged Dia2 ΔF, consistent with a previous genome-wide protein interaction study (Ho *et al*, 2002). Thus, Dia2 physically interacts with Mrc1 and Ctf4 *in vivo*.

Regions required for Dia2–Mrc1 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 Mrc1, Ctf4 or Mcm2 by two-hybrid assay. Deletion of the TPR motif diminished the two-hybrid interactions between Dia2 and Mrc1, 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 Mrc1, Ctf4 and Mcm2. We also found that Mrc1 and Mcm2 co-immunoprecipitated with WT HA-Dia2 from yeast extracts,

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 with 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 ΔTPR (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 ΔF 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 ΔTPR 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 ($P < 0.001$, χ^2 -test). By contrast, DIA2 ΔF and DIA2 ΔLRR cells showed size distributions very similar to those of vector control cells ($P > 0.1$, χ^2 -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 αHA 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 αHA 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, ΔLRR lacks amino acids 347–746 and 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 χ^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/RTT101* 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 ΔTPR* 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 ΔTPR* 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 ΔTPR* could suppress the synthetic lethal/slow growth phenotype with *hst3Δ*, but not with *cul8Δ*. These results suggest that the TPR motif of Dia2 has an essential role in the absence of *Cul8*. Expression of *DIA2 ΔTPR* slightly affected the growth of *cul8Δ*, suggesting that *DIA2 ΔTPR* 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^{Dia2} 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 ubiquitinated by SCF^{Dia2}. Thus, we asked whether Mrc1 is destabilized *in vivo* in an SCF^{Dia2}- 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 monitored during the time course to examine the stability of the protein (Figure 4A, Supplementary Figure 3). In the absence of MG132, we found that the amount of Mrc1 decreased gradually after CHX addition, whereas in the presence of MG132, the amount of Mrc1 did not change. Similarly, Mrc1 was stabilized in the *dia2Δ* strain (Figure 4B, Supplementary Figure 3). These results suggested that Mrc1 is destabilized by Dia2 in a proteasome-dependent manner *in vivo*. The destabilization of Mrc1 is neither rapid nor dramatic like that for the known SCF targets Sic1 and Cdc6 (Schwob *et al*, 1994; Drury *et al*, 1997). We reasoned that this is because destabilization of Mrc1 by SCF^{Dia2} may be regulated both temporally and spatially. To test this hypothesis, we determined the cell cycle stage at which Mrc1 was the least stable. Cells were arrested by α -factor, HU or nocodazole at G1, S or G2/M phase, respectively, and the stability of Mrc1 was investigated in the presence of CHX (Figure 4C, Supplementary Figure 3). In G1 and G2/M phase cells, no significant difference in Mrc1 stability was observed between

DIA2 and *dia2Δ* cells. By contrast, in HU-arrested cells, Mrc1 was particularly unstable in *DIA2* cells but was stable in *dia2Δ* cells. These results suggest that SCF^{Dia2} destabilizes Mrc1 particularly during S phase. RPCs, which contain Mrc1 and Cdc45–MCM–GINS complex, is formed only during S phase (Gambus *et al*, 2006, 2009; Tanaka *et al*, 2009). We hypothesized that SCF^{Dia2} would recognize and ubiquitinate MRC1 bound to MCM. To test this, we examined the stability of Mrc1 bound to MCM after CHX addition (Figure 4D). The amount of Mrc1 bound to Mcm4 was dramatically reduced after CHX addition in *DIA2* cell extracts, but it changed by very little in *dia2Δ* cell extracts. The amount of Mcm2 bound to Mcm4 did not change during the CHX chase in *DIA2* or *dia2Δ* cells, implying that it is unlikely that Mcm2 is a substrate for SCF^{Dia2}. These results suggest that SCF^{Dia2} preferentially recognizes and ubiquitinates Mrc1 in RPC. To test whether SCF^{Dia2} is able to ubiquitinate Mrc1 directly, an *in vitro* ubiquitination assay was performed. Subunits of SCF^{Dia2} were co-expressed in Sf21 cells and purified with Ni²⁺-NTA agarose. Either His-tagged Mrc1 or Tof1 was also expressed in insect cells and purified using Ni²⁺-NTA agarose. We found that Mrc1, but not Tof1, was ubiquitinated *in vitro* by partially purified SCF^{Dia2} (Figure 4E). Figure 4F shows that both WT Mrc1 and Mrc1 deletion mutant (1–557 a.a.) were ubiquitinated by SCF^{Dia2} and that this ubiquitination required E1, E2, SCF^{Dia2}, ubiquitin and ATP. These results indicate that SCF^{Dia2} is able to ubiquitinate Mrc1 directly *in vitro*. Altogether, Mrc1 can be ubiquitinated by SCF^{Dia2}, which explains previous two-hybrid interactions (Figure 1B). We also found that not only Mrc1 bound to Mcm4, but also Ctf4 bound to Mcm4 was greatly reduced during the CHX chase in *DIA2* cells but not in *dia2Δ* cells (Figure 4G), and that Ctf4 was ubiquitinated by SCF^{Dia2} *in vitro* (Figure 4H). Thus, we concluded that Ctf4 is also likely to be a substrate for SCF^{Dia2}.

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 Δ LRR 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.

Figure 4 Destabilization of Mrc1 by SCF^{Dia2}. (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 αMyc. 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 αFLAG antibody and co-immunoprecipitated proteins were analysed by immunoblotting. (E) *In vitro* ubiquitination of Mrc1 using Ni²⁺-NTA agarose-purified SCF^{Dia2}. Baculoviruses expressing the four components of SCF^{Dia2} were co-transfected into Sf21 cells. Both Rbx1 and Dia2 were 6His tagged. At 3 days after transfection, cell extracts were prepared and SCF^{Dia2} was purified by Ni²⁺-NTA agarose chromatography. Purified SCF^{Dia2} was used as E3 for the *in vitro* ubiquitination assay. Either 6His3FLAG-Mrc1 or 6His3FLAG-Tof1 was purified from Sf21 cells by Ni²⁺-NTA agarose beads and used as a substrate. The amount of SCF^{Dia2} was titrated two-fold. After the reaction, the proteins were analysed by immunoblotting following SDS-PAGE. Mrc1 was detected by αFLAG. (F) Requirement of factors for Mrc1 ubiquitination. 6His3FLAG-Mrc1 (1–557 a.a.) was purified from Sf21 cells by Ni²⁺-NTA agarose beads and used as a substrate. Substrates were incubated either in the presence of E1, E2, E3, His-Ub and ATP (complete), or in the absence of one of these factors. After the reaction, the proteins were analysed as in (E). (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

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 μ g/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 α HA 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 μ g/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)**.

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 *dia2 Δ* 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 *dia2 Δ* 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 *dia2 Δ* cells. We also found that Cdc45 accumulated at inter-origin regions in *dia2 Δ* 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 *dia2 Δ* 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 *dia2 Δ* 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 *dia2 Δ* 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 *dia2 Δ* cells, suggesting that S-phase progresses at a similar rate in

Figure 6 Localization of Dia2, Mrc1 and Cdc45 on chromosome VI in the wild-type (WT) strain in the presence of 200 mM HU. Cells 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. Chromatin immunoprecipitation (ChIP) analysis was carried out as described previously (Katou *et al*, 2003). Each blue bar represents the binding ratio of Dia2 (upper panel), Mrc1 (middle panel) and Cdc45 (lower panel) at each locus, and shows a significant enrichment in some of the IP fractions. The yellow line represents the average signal ratio at loci that are not enriched (grey bars) in each IP fraction. The scale of the vertical axis is log2. Positions of the centromere (CEN6) and all previously mapped autonomous replicating sequences (ARSs) are shown. The horizontal axis indicates the length of chromosome VI in kb. Dia2, Mrc1 and Cdc45 were tagged by 10 $\times$ FLAG, 18 $\times$ Myc and 3 $\times$ HA, respectively.

the absence of Dia2. In the presence of 200 mM HU, the FACS peak did not move in *dia2* Δ 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 *dia2* Δ cells, however, the FACS peak gradually moved toward 2c content. These results demonstrated that *dia2* Δ 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 *dia2* Δ 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, *dia2* Δ 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.

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 *dia2Δ* mutant in S-phase under the presence of HU. Cells expressing HA-tagged version of Cdc45 and adenovirus thymidine 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 *dia2Δ* 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 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 *in vivo* in a

Dia2-dependent manner and is ubiquitinated by SCF^{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^{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^{Dia2} might be involved in the dissociation of SCF^{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 Δ TPR 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* Δ TPR could not suppress synthetic lethality with *rrm3 Δ* (Dr Karim Labib, personal communication), suggesting that the interaction with the replisome through the TPR motif is extremely important for dealing with DNA damage caused by *RRM3* deletion. Furthermore, we found that *DIA2* Δ TPR shows a synthetic growth defect with *cul8 Δ* . Thus, we presume that loading of SCF^{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^{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- ϵ ,

which is responsible for leading strand DNA synthesis (Lou *et al*, 2008). There are two separate polymerase- ϵ -binding sites in Mrc1. Interestingly, the N-terminal-binding site of Mrc1 dissociates from polymerase- ϵ on DNA damage. Similar to polymerase- ϵ , Dia2 binds to Mrc1 through two separate binding sites: 380–557 a.a. and 701–800 a.a. These regions partially overlap with polymerase- ϵ -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- ϵ 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 10 μ g/ml α -factor, 0.2 M HU and 10 μ g/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 to OD₆₀₀ = 1.0 and serially diluted in 10-fold increments, and 4- μ l volumes were spotted onto the indicated media. 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.

Yeast two-hybrid screening

Two-hybrid screening was performed using pLexA-Dia2 Δ F 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 HIS⁺ and β -galactosidase⁺ colonies were isolated. Seven clones for MRC1, three clones for CTF4 and one clone for MCM2 were obtained.

Immunoprecipitation and immunoblotting

Yeast extracts for immunoprecipitation were prepared as follows. Cells were collected from 25 ml of mid-log phase culture and lysed in IPLB (50 mM HEPES-KOH (pH 7.5), 150 mM NaCl, 5 mM MgCl₂, 5% glycerol, 0.1% NP-40, 0.1% Tween-20 and protease inhibitors) by bead beating using the Multi-Beads Shocker (MB400U, Yasui Kikai, Osaka, Japan). Clarified lysates were incubated at 4°C for 3 h with Protein G sepharose beads (GE Healthcare) to which 2 μ g of antibody was bound. The beads were washed, and bound proteins were solubilized by SDS-PAGE sample buffer. For immunoblotting, total cell lysates were prepared by either TCA precipitation or alkaline lysis. Proteins were transferred onto a nitrocellulose membrane and detected using either mouse monoclonal antibodies (α Myc 9E10 (1:1000), α FLAG M2 (1:5000) and α HA 12CA5 (1:1000)), goat polyclonal antibody (α Mcm2 (Santa Cruz Biotechnology, sc-6680; 1:2000 dilution) and α Cdc53 (Santa Cruz Biotechnology, sc-6716; 1:1000 dilution)), or rabbit polyclonal antibodies (α Myc (Santa Cruz Biotechnology, sc-789; 1:2500 dilution), α FLAG (Rockland 600-461-383; 1:1000 dilution) and α HA (Santa

Table 1 Yeast strains

Strain	Genotype	Source
YSM403	<i>W303-1a, deltapdr5::hphNT, DIA2::DIA2-HIS10FLAG(kan)</i>	This study
CDC45-3HA	<i>BY4741, trp1::hisG, CDC45::CDC45-3HA(TRP)</i>	Katou <i>et al</i> (2003)
YSM390	<i>BY4741, trp1::hisG, CDC45::CDC45-3HA(TRP), dia2Δ::HIS</i>	This study
YSM432	<i>W303-1a, dia2Δ::kanMX + pSM425 (pRS415-ADH1p-3HA-DIA2WT)</i>	This study
YSM431	<i>W303-1a, dia2Δ::kanMX + pSM424 (pRS415-ADH1p-3HA-DIA2ΔF)</i>	This study
YSM430	<i>W303-1a, dia2Δ::kanMX + pSM423 (pRS415-ADH1p-3HA-DIA2ΔTPR)</i>	This study
YSM429	<i>W303-1a, dia2Δ::kanMX + pSM422 (pRS415-ADH1p-3HA-DIA2ΔLRR)</i>	This study
YSM428	<i>W303-1a, dia2Δ::kanMX + pRS415-ADH1p-3HA</i>	This study
YSM215	<i>L40, dia2::GAL-DIA2 (kanMX)</i>	This study
YSM216	<i>L40, dia2Δ::kanMX</i>	This study
YSM404	<i>W303-1a, MRC1::MRC1-MYC18(URA), pdr5Δ::hphNT, DIA2::DIA2-HIS10FLAG(kanMX)</i>	This study
YSM396	<i>W303-1a, MRC1::MRC1-MYC18(URA), pdr5Δ::hphNT</i>	This study
YSM280	<i>W303-1a, MRC1::MRC1-MYC18(URA)</i>	This study
YSM368	<i>W303-1a, dia2Δ::kanMX, MRC1::MRC1-MYC18(URA)</i>	This study
YSM378	<i>W303-1a, ura3-52::GAL-DIA2ΔF3FLAG (URA3), MRC1::MRC1-3HA(LEU)</i>	This study
YSM379	<i>W303-1a, ura3-52::GAL-DIA2ΔF3FLAG (URA3), CTF4::CTF4-3HA(LEU)</i>	This study
YSM592	<i>W303-1a, DIA2::DIA2-HIS10FLAG(kan)</i>	This study
YSM593	<i>W303-1a, cdc34-2, DIA2::DIA2-HIS10FLAG(kan)</i>	This study
YSM620	<i>W303-1a, skp1-11, DIA2::DIA2-HIS10FLAG(kan)</i>	This study
YSM594	<i>W303-1a, cdc4-1, DIA2::DIA2-HIS10FLAG(kan)</i>	This study
YSM690	<i>W303-1a, MRC1-MYC18(URA) MCM4-5FLAG(hph)</i>	This study
YSM691	<i>W303-1a, MRC1-MYC18(URA) dia2Δ::kan MCM4-5FLAG(hph)</i>	This study
YSM694	<i>BY4741, trp1::hisG, CTF4-3HA (TRP) MCM4-5FLAG(hph)</i>	This study
YSM695	<i>BY4741, trp1::hisG, CTF4-3HA (TRP) dia2Δ::kan MCM4-5FLAG(hph)</i>	This study

Parental strains.

W303-1a MATa, leu2-3,112 trp1-1 can1-100 ura3-1 ade2-1 his3-11,15.

BY4741 MATa his3Δ1 leu2Δ0 met15Δ0 ura3Δ0.

L40 MATa his3Δ200 trp1-901 leu2-3112 ade2LYS2:: (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 $OD_{600} = 0.6$. 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 Dia2-3HAHis6, or expressing His63FLAG-Mrc1, by Ni²⁺-NTA agarose (Qiagen). After 60 min of incubation at 26°C, 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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