An FHA domain–mediated protein interaction network of Rad53 reveals its role in polarized cell growth

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The DNA damage checkpoint kinase Rad53 is important for the survival of budding yeast under genotoxic stresses. We performed a biochemical screen to identify proteins with specific affinity for the two Forkhead associated (FHA) domains of Rad53. The N-terminal FHA1 domain was found to coordinate a complex protein interaction network, which includes nuclear proteins involved in DNA damage checkpoints and transcriptional regulation. Unexpectedly, cytosolic proteins involved in cytokinesis, including septins, were also found as FHA1 binding proteins. Consistent with this interaction, a Rad53 mutant defective in its nuclear localization was found to localize to the bud neck. Abnormal morphology was observed in cells overexpressing the FHA1 domain and in rad53Δ cells under DNA replication stress. Further, septin Shs1 appears to have an important role in the response to DNA replication stress. Collectively, the results suggest a novel function of Rad53 in the regulation of polarized cell growth in response to DNA replication stress.

Introduction

The DNA damage response consists of a complex protein network that mediates the detection of damaged DNA and regulates multiple cellular processes (Zhou and Elledge, 2000; Kolodner et al., 2002; Nyberg et al., 2002; Rouse and Jackson, 2002; McGowan and Russell, 2004). In Saccharomyces cerevisiae, an evolutionarily conserved kinase cascade consisting of Mec1, Tel1, Rad53, and Dun1 is responsible for amplifying the DNA damage signal from DNA damage recognition enzymes and transducing such signal to downstream targets in the form of protein phosphorylation (Longhese et al., 1998; Foiani et al., 2000). In Saccharomyces cerevisiae, an evolutionarily conserved kinase cascade consisting of Mec1, Tel1, Rad53, and Dun1 is responsible for amplifying the DNA damage signal from DNA damage recognition enzymes and transducing such signal to downstream targets in the form of protein phosphorylation (Longhese et al., 1998; Foiani et al., 2000). Although Mec1 and Tel1 are involved in sensing DNA damage (Jackson, 1996), Rad53 and Dun1 appear to function as effector kinases to regulate multiple cellular processes, such as the cell cycle, DNA replication, chromosome segregation, histone turnover, gene transcription, and possibly DNA repair (Allen et al., 1994; de la Torre Ruiz and Lowndes, 2000; Zhao and Rothstein, 2002; Gunjan and Verreault, 2003; Tercero et al., 2003; Krishnan et al., 2004). Rad53 is critical for cells to cope with various DNA damage stresses, as cells with Rad53 deletion or kinase-dead mutations are hypersensitive to genotoxic stresses (Allen et al., 1994; Sun et al., 1996; Pellicioli et al., 1999). In response to DNA damage or stalled replication forks, Rad53 is hyperphosphorylated and activated in a Mec1-dependent manner (Sun et al., 1996; Emili, 1998; Vialard et al., 1998). The activation of Rad53 is accompanied by its autophosphorylation, induced by its association with the hyperphosphorylated forms of the adaptor proteins Rad9 or Mrc1 (Alcasabas et al., 2001; Gilbert et al., 2001; Schwartz et al., 2002). Rad53 appears to be directly involved in the regulation of cell cycle, as its overexpression leads to cell cycle arrest even in the absence of exogenous DNA damage stresses (Sun et al., 1996). Despite its importance, the targets of Rad53 in the DNA damage response are poorly known.

Rad53 consists of a central serine/threonine kinase domain, flanked by an N-terminal Forkhead associated 1 (FHA1) domain and a C-terminal FHA2 domain (Durocher et al., 1999). The FHA domain is found in a wide range of signaling proteins, with known roles in mediating protein–protein interactions through the binding of phosphorylated substrates (Hofmann and Bucher, 1995; Durocher and Jackson, 2002). DNA damage–induced interaction between Rad53 and hyperphosphorylated Rad9 is mediated by both the FHA1 and -2 domains of Rad53.
Although differences in the binding specificity of both domains were found using an oriented phosphopeptide library approach (Durocher et al., 2000), the presence of either domain alone is sufficient for Rad53 activation (Schwartz et al., 2002; Pike et al., 2003). Additionally, the FHA domains likely mediate the targeting of Rad53 to other proteins for their regulation by Rad53. For example, chromatin assembly protein Asf1 and phosphatase Ptc2 were shown to bind to the FHA1 domain of Rad53 in a phosphorylation-dependent manner (Emili et al., 2001; Hu et al., 2001; Leroy et al., 2003; Schwartz et al., 2003). Several other proteins have been shown to be regulated in a Rad53-dependent manner. Swi6 is an essential regulatory subunit of two different START-dependent transcription factors, Swi4 and Mbp1, which regulate the transcription of many genes involved in DNA replication (Koch and Nasmith, 1994). Swi6 appears to undergo Rad53-dependent phosphorylation in response to DNA damage stresses and was suggested to be a substrate of Rad53 (Sidoroava and Breeden, 2003). Dun1 was also shown to be phosphorylated and activated by Rad53 (Bashkirov et al., 2003). Furthermore, several other proteins were found to interact with Rad53, including Cdc7/Dbf4 (Duncker and Brown, 2003), Kap95 (Smolka et al., 2005), Yta7 (Smolka et al., 2005), Mdt1 (Pike et al., 2004), and others (Ho et al., 2002). Despite these studies, the mechanism by which Rad53 regulates cell growth in response to DNA damage and replication stress remains poorly understood.

The cell cycle of the budding yeast consists of highly coordinated events, including bud emergence, polarized cell growth, and protein trafficking, which are synchronized with the initiation and progression of DNA replication. After successful completion of DNA replication, mitosis and cytokinesis occur, and a new round of cell cycle begins (Lew et al., 1997). At the initiation of S-phase, bud emergence is accompanied by the localization of bud site selection proteins and septins (Gladfelter et al., 2001; Longtine and Bi, 2003). Septins are a family of conserved proteins that form filaments at the cortex of the mother-bud neck (Versele and Thorner, 2005). Localization of septins to the bud neck persists throughout the cell cycle except for disassembly and reassembly during G1 phase (Gladfelter et al., 2005). In budding yeast, genes encoding septins, i.e., Cdc3, Cdc10, Cdc11, and Cdc12, were identified through the isolation of temperature-sensitive mutations that prevented cytokinesis at restrictive temperature, resulting in the formation of chains of multinucleated and multibudded cells (Hartwell, 1971). More recently, a fifth member of the septin family was identified, namely, Shs1 (seventh homologue of septin) (Mino et al., 1998). Septins are known to perform important functions in spindle orientation, bud-site selection, the establishment and maintenance of polarized bud growth, the switch from polarized to isotropic bud growth, cell cycle, and morphogenesis checkpoints (Barral et al., 1999; Longtine et al., 2000; Segal and Bloom, 2001; Kusch et al., 2002; Gladfelter et al., 2005). Increasingly, the regulatory role of septins in coordinating multiple steps in cell cycle progression is being revealed (Field and Kellogg, 1999; Longtine and Bi, 2003; Gladfelter et al., 2005).

In this study, we performed a proteomic screen to identify proteins that bind to the FHA domains of Rad53. This led to the finding that the FHA1 domain of Rad53 coordinates the interaction between Rad53 and a wide variety of proteins. In contrast, the FHA2 domain appears to have a rather specialized role in binding to Rad9 after DNA damage treatment. Among the FHA1 binding proteins, septins and their associated proteins represent a major functional group. We present evidence that Rad53 may play a role in the regulation of polarized cell growth in response to DNA replication stress.

### Results

**Proteomic screening of Rad53 FHA binding proteins using a PATH approach**

A proteomic approach was developed to identify Rad53 FHA binding proteins present in yeast cell extracts. Various N-terminal **PATH** (protein A–TeV–His) (Fig. 1 A) fusion FHA domains bound to the IgG resin were used in the affinity purification. As shown in Fig. 1 B, the FHA binding proteins were purified using a tandem affinity purification (TAP) method (see Materials and methods). As a control, the same purification was performed using a mutant FHA protein carrying a point mutation previously shown to reduce its binding to phosphorylated substrates (R70A for FHA1 and R605A for FHA2; Durocher et al., 1999). Fig. 1 C shows the purified FHA binding proteins.

![Figure 1](image-url)
Although contaminant proteins, i.e., bands common in both wild type (WT) and mutant FHA purification, are still present, bands specific to the WT FHA1 can be seen. To identify the specific binding proteins of WT FHA domain, we used stable isotope labeling–based quantitative mass spectrometry, as illustrated in Fig. 1 B. Each purified sample was independently digested with trypsin and then labeled with a stable isotope containing N-isotag reagent (light N-isotag for WT sample and heavy N-isotag for mutant sample; see Materials and methods; Smolka et al., 2005). The labeled samples were combined and analyzed by mass spectrometry for identification and quantification of proteins (Fig. 1 B). Proteins identified in the WT FHA purification, but not the mutant FHA purification, as determined by their isotope labeling, were then considered specific FHA binding proteins for further validation studies.

In a separate experiment, to identify DNA damage–induced changes in the FHA binding proteins, WT PATH-FHA1 (or PATH-FHA2) resin was used to purify proteins from untreated cells and methyl methanesulfonate (MMS)–treated cells (the eluted proteins had similar patterns like Fig. 1 C and are not depicted). The purified proteins from untreated cells were digested by trypsin and labeled with the light N-isotag, and those from MMS-treated cells were labeled with the heavy N-isotag. Quantitative mass spectrometry was again used to identify any MMS-induced changes in the specific binding proteins of the FHA domains.

The Rad53 FHA1 domain mediates a complex protein interaction network that includes the five related septins

The specific binding proteins of FHA domains of Rad53 are summarized in Table I. Although most of them interact with the FHA1 domain of Rad53 independent of DNA damage, Rad9 and Mrcl interact with the FHA1 domain only after MMS treatment. Rad9 was also found to bind to the FHA2 domain of Rad53 after MMS treatment, consistent with previous findings (Sun et al., 1998; Vialard et al., 1998). Additionally, Asf1 and Ptc2, both known FHA1 binding proteins (Emili et al., 2001; Hu et al., 2001; Leroy et al., 2003), were identified. The identification of these known FHA binding proteins validates the PATH approach. Interestingly, Swi6 and its associated proteins, Swi4, Mbp1, and Whi5, were found to bind to the FHA1 domain of Rad53, further supporting a previously identified link between Rad53 and Swi6 (Sidorova and Breeden, 2003). Because the purification was performed under nondenaturing conditions, it is not surprising that protein complexes were purified and identified.

Although the FHA2 domain was found to bind only Rad9, the FHA1 domain of Rad53 binds to a wide variety of proteins, most of which are novel (Table I). Interestingly, several cytosolic proteins were found, including the septins (Cdc10, Cdc11, Cdc12, Cdc3, and Shs1) and proteins involved in bud site selection (Bud3, Bud4, and Bud14), all of which localize to the bud neck. Based on the number of identified peptides for each protein identified (Table I), a crude indicator of protein abundance, we deduce that the septins are among the more abundant FHA1 binding proteins. To confirm the specificity of FHA1 binding, protein extracts of strains containing TAP- or HA-tagged genes of interest were analyzed by affinity purification with either WT or mutant GST-FHA proteins immobilized on glutathione resins, and the bound protein was analyzed by Western blotting. Fig. 2 A confirms that in MMS-treated cells, Rad9 binds to both the FHA1 and -2 domains, whereas Mrcl binds to only the FHA1 domain. The specificity of binding between septins and the FHA1 domain was similarly confirmed by Western blot analysis (Fig. 2 B). Furthermore, as shown in Fig. 2 B, deletion of genes encoding any of the septin-associated proteins Bud3, Bud4, or Bud14, as well as deletion of SHS1, did not affect the binding of the FHA1 domain to Cdc11. Deletion of CDC10, which results in cells with abnormal cell morphology (not depicted), was found to impair the ability of FHA1 domain to bind Cdc11 (Fig. 2 B). Collectively, the results show that the FHA1 domain most likely binds to septins directly in a Cdc10-dependent manner.

Binding specificities of most other FHA1 binding proteins have also been confirmed using the same approach, and the results are shown in Fig. S1 (available at http://www.jcb.org/cgi/content/full/jcb.200605081/DC1). In all cases, the R70A mutation, which is known to diminish the binding of the FHA1 domain to phosphopeptides (Durocher et al., 1999), greatly reduces the binding of the identified FHA1 binding proteins.

**Rad53 can transiently localize to the bud neck in an FHA1-dependent manner**

The finding that the septin complex binds to the FHA1 domain was unexpected because Rad53 is a known nuclear protein and septins are known to localize at the bud neck. We next asked whether a localization of Rad53 at the bud neck could be detected. As shown in Fig. 3 A, the majority of GFP-tagged WT Rad53 is nuclear, although some heterogeneous GFP signal was detected in the cytoplasm upon close inspection. It is difficult to observe the localization of WT Rad53 at the bud neck, especially in late S-phase, when the GFP signal from nuclear Rad53 is within close proximity to the bud neck and overpowers any possible signal from the bud neck. To better visualize the localization of Rad53 outside of the nucleus, we generated a Rad53 mutant in which a putative nuclear localization signal (NLS) in the C-terminal region of Rad53 was removed (Smolka et al., 2005; see Materials and methods). Results in Fig. 3 A confirm the loss of predominant nuclear localization of the Rad53 NLS truncation mutant. In unsynchronized cells, the Rad53 NLS mutant was detected in the bud neck of a small percentage of the cells (~8%; Fig. 3 B). Interestingly, all of the cells showing positive Rad53 localization to the bud neck had large buds and a still undivided nucleus. We then examined the effect of DNA damage on the localization of the Rad53 NLS mutant. Exposure to MMS or hydroxyurea (HU) drastically increases the percentage of cells showing the localization of Rad53 to the bud neck, again in cells with large buds (Fig. 3 A). Closer inspection shows that the GFP signal of the Rad53 NLS mutant appears as a double ring–like pattern (Fig. 3 A, enlarged panel). Importantly, a Rad53 NLS mutant containing an additional FHA1 domain R70A mutation fails to localize to the septins, further indicating that the localization of Rad53 to bud neck is mediated...
through its FHA1 domain. We also analyzed cells at different times after the release from α-factor arrest and did not observe the same striking GFP signal at the septin ring as observed after HU or MMS treatment (unpublished data). Importantly, Rad53-GFP cells exhibited the same sensitivities to HU, MMS, or UV, compared with WT cells (unpublished data). Fig. 3 C shows the abundance levels of various GFP-tagged Rad53 mutant proteins, indicating that the lack of bud neck localization of Rad53 mutant containing both NLS truncation and R70A mutations was not due to its reduced protein abundance.

Table 1. Summary of the binding proteins of FHA1 and -2 domains of Rad53 from cells untreated or treated by MMS

| Function                          | Protein | Accession no. | Description                                      | FHA1 specific | FHA2 specific |
|-----------------------------------|---------|---------------|--------------------------------------------------|---------------|---------------|
|                                   |         |               | Detected in CTL | Detected in MMS | No. of peptides identified | Detected in CTL | Detected in MMS | No. of peptides identified | Validation | Known Rad53 binding protein |
| DNA damage                       | Rad9    | YDR217C       | Adaptor protein/Rad53 activation                  | X             | X             | 3                   | X             | 20                   | ✓           | Sun et al., 1998         |
| checkpoint                        | Mcr1    | YCL061C       |                                                   |               |               |                     |               |                      | ✓           |                        |
|                                   | Ptc2    | YER089C       | Phosphatase/checkpoint inactivation               | X             | X             | 3                   |               |                      | ✓           | Leroy et al., 2003       |
| Transcription factors and cofactors| Swi6    | YLR182W       | Regulation of cell cycle progression              | X             | X             | 4                   |               |                      | ✓           |                        |
|                                   | Mbp1    | YDIO56W       |                                                   | X             | X             | 4                   |               |                      | ✓           |                        |
|                                   | Swi4    | YER111C       |                                                   | X             | X             | 3                   |               |                      | ✓           |                        |
|                                   | Whi5    | YOR093W       |                                                   | X             | X             | 2                   |               |                      | ✓           |                        |
|                                   | Cst6    | YIL036W       | Basic leucine zipper                              | X             | X             | 4                   |               |                      | ✓           |                        |
|                                   | Gln3    | YER040W       | Nitrogen catabolite repression                    | X             | X             | 3                   |               |                      | ✓           |                        |
|                                   | Ih1     | YJR223C       | Ribosome gene transcription                       | X             | X             | 5                   |               |                      | ✓           |                        |
| Other functions                   | Esc1    | YMR219W       | Telomeric silencing                               | X             | X             | 6                   |               |                      | ✓           | Emil et al., 2001        |
|                                   | Adf1    | YJL115W       | Nucleosome assembly factor                         | X             | X             | 3                   |               |                      | ✓           |                        |
|                                   | Crp1    | YHR146W       | Binds to cruciform DNA structures                 | X             | X             | 10                  |               |                      | ✓           |                        |
|                                   | Src1    | YML034W       | Sister chromatid segregation [putative]          | X             | X             | 2                   |               |                      | ✓           |                        |
|                                   | Ecm16   | YMR126W       | RNA helicase                                      | X             | X             | 2                   |               |                      | ✓           |                        |
|                                   | Net1    | YL076W        | Exit from mitosis                                 | X             | X             | 7                   |               |                      | ✓           |                        |
|                                   | Yta7    | YGR270W       | ATPase/bromodomain                                | X             | X             | 3                   |               |                      | ✓           | Smolka et al., 2005      |
| Cytokinesis                       | Cdc3    | YJR314C       | Septins                                           | X             | X             | 23                  |               |                      | ✓           |                        |
|                                   | Cdc12   | YHR107C       |                                                   | X             | X             | 20                  |               |                      | ✓           |                        |
|                                   | Shs1    | YDL225W       |                                                   | X             | X             | 21                  |               |                      | ✓           |                        |
|                                   | Cdc11   | YJR076C       |                                                   | X             | X             | 11                  |               |                      | ✓           |                        |
|                                   | Cdc10   | YCR002C       |                                                   | X             | X             | 7                   |               |                      | ✓           |                        |
|                                   | Bud4    | YJR092W       | Bud site selection                                | X             | X             | 9                   |               |                      | ✓           |                        |
|                                   | Bud3    | YCL014C       |                                                   | X             | X             | 10                  |               |                      | ✓           |                        |
|                                   | Bud14   | YAR014C       |                                                   | X             | X             | 6                   |               |                      | ✓           |                        |
| Other functions                   | Sec2    | YNL272C       | Protein trafficking                               | X             | X             | 16                  |               |                      | ✓           |                        |
|                                   | Ubp1    | YDL122W       | Ubiquitin-specific protease                       | X             | X             | 20                  |               |                      | ✓           |                        |
|                                   | Rck2    | YJR248W       | Protein kinase                                    | X             | X             | 13                  |               |                      | ✓           |                        |
|                                   | Fyv8    | YGR196C       | Unknown                                          | X             | X             | 13                  |               |                      | ✓           |                        |
|                                   | Mnr2    | YKL064W       | Unknown                                          | X             | X             | 2                   |               |                      | ✓           |                        |

X indicates that the protein was detected in the condition used. Check marks indicate validation using FHA domain pull down and Western blot analysis, as described in the text. Some proteins were not validated because of technical problems in generating tagged strains.
Although Rad53-GFP still underwent MMS-induced hyperphosphorylation and slower gel shift, truncation of Rad53 NLS largely abolished its MMS-induced hyperphosphorylation (Fig. 3 C). Therefore, the majority of Rad53 needs to be nuclear for its proper activation.

Rad53 is involved in the regulation of polarized cell growth in response to DNA replication stress

Because the FHA1 domain of Rad53 interacts with many proteins, we asked what the role of the FHA1 domain of Rad53 might be. We examined the effect caused by overexpression of the FHA domains of Rad53 in yeast cells. Overexpression of the FHA1 domain was found to result in aberrant cell morphology, showing multiple elongated buds that fail to detach from the mother cell, even after zymolase treatment (Fig. 4 A). In contrast, overexpression of the R70A mutant FHA1 domain, or WT and the R605A mutant FHA2 domain, does not induce any such defect. Fig. 4 C shows the staining of a cell with overexpression of the FHA1 domain. It appears to be multinucleated and lacks any staining for septin Cdc11, suggesting that septin organization is disrupted by FHA1 overexpression.

We next asked whether any morphological defect could be observed in rad53Δ cells. No major morphological defect was detected in the untreated rad53Δ cells, although a closer analysis of rad53Δ cells did reveal various defects in budding pattern and cell wall integrity (see Enserink et al. on p. 729 of this issue). Upon HU treatment, a striking morphological defect characterized by an elongated bud was readily observed for rad53Δ cells (Fig. 4, D and E). Such phenotype was observed in ~30% of rad53Δ and rad53-KD cells after 14 h of chronic HU treatment. Together, the results suggest that Rad53 plays a role in the control of polarized cell growth in response to DNA replication stress.

Figure 2. Confirmation of the binding specificity of the FHA binding proteins of Rad53. (A) Protein extracts from MMS-treated (0.1% MMS for 3 h) Rad9-HA or Mrc1-TAP cells were divided into equal fractions and subjected to pull-down assays using different GST fusion FHA domains as indicated. The GST-FHA domain used in the pull-down assay was stained by Ponceau as a control. Protein extracts from untreated Shs1-TAP, Cdc11-TAP, or Cdc10-HA cells were subjected to the same FHA domain pull-down assays. (B) Binding of the FHA1 domain to septins is Cdc10 dependent. Single-deletion strains for different septins and septin-associated proteins were analyzed for the ability of the FHA1 domain to specifically bind TAP-tagged Cdc11.

Figure 3. Rad53 can transiently localize to the bud neck. (A) Removal of the C-terminal NLS of Rad53 abrogates its predominant nuclear localization (compare two left views). Additional MMS treatment (0.05% for 3 h) led to a high percentage of cells with bud neck localization of the NLS mutant Rad53-GFP. Closer inspection revealed a double ring-like localization pattern of the Rad53 NLS mutant, as indicated by the arrows. In contrast, the Rad53 NLS mutant containing an additional R70A mutation fails to localize to the bud neck. DIC, differential interference contrast. (B) Quantitative analysis of the cells showing localization of Rad53-NLS-GFP to the bud neck. Treatment of either 150 mM HU or 0.05% MMS for 3 h led to a substantial increase in the number of cells with a bud neck localization of the Rad53-NLS-GFP. In each case, 200 cells were counted. (C) Abundances of various GFP-tagged Rad53 were detected by anti-GFP Western blot (WB). Protein extracts were prepared from untreated and MMS-treated (0.01% for 4 h) cells. After detection, the membrane was stained with Ponceau for loading control.
to HU treatment, and its kinase activity appears to be essential for such regulation.

**Shs1 participates in the response to DNA replication stress**

The morphology defect caused by overexpression of the FHA1 domain was found to be largely suppressed by deletion of Shs1 (Fig. 5 A), suggesting that SHS1 may somehow function down-stream of the FHA1-mediated binding of Rad53 to the septins. We then examined whether the SHS1Δ cells have any morphological defect in response to HU treatment. Interestingly, HU treatment of shs1Δ cells induces multiple elongated buds that fail to detach (Fig. 5 B), even after zymolase treatment. Interestingly, Shs1 was efficiently phosphorylated by Rad53 in vitro (Fig. 5 C), raising the possibility that it may be a Rad53 substrate. Next, we asked whether shs1Δ cells are hypersensitive to chronic treatment with HU. As shown in Fig. 5 D, although the shs1Δ cells are not sensitive to chronic treatment with HU at 30°C, they are hypersensitive to HU treatment at 37°C. The shs1Δ cells are almost as sensitive as rad53Δ cells, and such loss of viability at 37°C is specific to HU treatment, because shs1Δ cells are not sensitive at 37°C in the absence of HU, or even in the presence of MMS (Fig. 5 D). Collectively, these results suggest that Shs1 may play an important role during the response to DNA replication stress, although the precise nature of such role is unknown.

**Discussion**

Numerous studies have suggested roles for Rad53 in the control of cell cycle arrest, histone turnover, control of late origin firing, stabilization of stalled replication forks, and control of chromosome segregation (Allen et al., 1994; Paulovich and Hartwell, 1995; Santocanale and Diffley, 1998; Shirahige et al., 1998; Lopes et al., 2001; Gunjan and Verreault, 2003; Tercero et al., 2003; Krishnan et al., 2004). Here, we identified a protein...
interaction network mediated by the FHA domains of Rad53 (Table I). It includes proteins involved in diverse cellular processes, such as DNA damage checkpoints, cell cycle control, transcriptional regulation, and cytokinesis. The interaction between septins and the FHA1 domain of Rad53 led us to the finding of a novel function for Rad53 in the regulation of polarized cell growth.

The FHA domain-mediated protein interaction network of Rad53 in the DNA damage response
The FHA domains of Rad53 appear to have distinct but overlapping functions in the DNA damage response (Pike et al., 2003; Schwartz et al., 2003). Here, we show that the binding proteins for the FHA1 and -2 domains of Rad53 are quite different, with the exception of Rad9 (Table I). Previously, Rad9 was known to interact with both the FHA1 and -2 domains of Rad53, as it was suggested to mediate the activation of Rad53 (Schwartz et al., 2002). Here, we further identified Mrc1, another adaptor protein in the DNA damage checkpoint, as a specific binding protein for the FHA1 domain, but not the FHA2 domain. As Mrc1 is known to function in the maintenance of the DNA replication fork (Alcasabas et al., 2001; Osborn and Elledge, 2003), our observation immediately suggests that the FHA1 domain may have a specialized function in mediating cellular responses to DNA replication stress. Consistent with this suggestion, mutations (R70A and N107A) in the FHA1 domain lead to elevated sensitivity to HU treatment and no detectable sensitivity to MMS treatment (unpublished data).

The FHA1 domain was found to also interact with a wide variety of proteins involved in several processes (Table I). Some of them have been identified previously, including Ptc2 and Asf1 (Leroy et al., 2003; Schwartz et al., 2003). However, most of these FHA1 binding proteins are novel. Because many of these FHA1 binding proteins are not known to form protein complexes (Gavin et al., 2002; Ho et al., 2002), it is likely that they interact with the FHA1 domain of Rad53 independently. Some of them do form protein complexes, including the septins and the Swi6 complex. In these cases, the direct FHA1 binding partner is unknown. Swi6 was previously found to be a Rad53 substrate (Sidorova and Breeden, 2003). Here, we found that the Swi6 complex binds to the FHA1 domain of Rad53, suggesting that the FHA1 domain of Rad53 may facilitate the phosphorylation of Swi6 by Rad53. Similar mechanisms may also...
exist for other FHA1 binding proteins. Several nuclear proteins were found to be FHA1 binding proteins, including Net1, which functions in ribosomal DNA silencing and the regulation of mitotic exit (Shou et al., 1999; Straight et al., 1999), Esc1, which is implicated in gene silencing (Andrulis et al., 2002) and suppression of gross chromosome rearrangements (Smith et al., 2004), and others (Table 1). Other known FHA1 binding proteins, including Sgs1 (Bjergbaek et al., 2005), Dbf4 (Duncker et al., 2002), and Mdt1 (Pike et al., 2004), were not found here, probably because of their low abundance. Likewise, other low-abundant proteins could exist and remain to be identified by more sensitive techniques.

It is interesting to note that after DNA damage treatment, Rad9 and Mrc1 are the only additional proteins found to interact with the FHA domains of Rad53, whereas binding of the other FHA1 binding proteins is independent of DNA damage treatment. This leads to the hypothesis that in the absence of DNA damage stresses, Rad53 is in a dynamic binding equilibrium with many FHA1 binding proteins, yet the kinase remains mostly inactive. In response to DNA damage or replication stress, Rad53 is temporarily recruited to Rad9 or Mrc1 via its FHA domains, for its activation (Alcasabas et al., 2001; Gilbert et al., 2001). Once activated, Rad53 may phosphorylate its downstream targets as facilitated by the FHA1 and -2 domains (Fig. 6).

**Rad53 is involved in the regulation of polarized cell growth under DNA replication stress**

Several lines of evidence support the idea that Rad53 may regulate polarized cell growth in response to DNA replication stress. First, the FHA1 domain of Rad53 binds specifically to septins. Second, consistent with the binding between Rad53 and septins, an NLS truncation mutant of Rad53 transiently localizes to the bud neck in an FHA1-dependent manner. We have attempted to visualize a localization of WT Rad53 to the bud neck in various mutants that fail to position the nucleus close to the bud neck (kar9Δ and dyn1Δ). However, because of the low abundance of WT Rad53 outside of the nucleus, we could not conclusively detect Rad53 at the bud neck. Third, overexpression of the FHA1 domain of Rad53 induces a morphological defect and defective septin organization. Fourth, rad53Δ and rad53-KD cells treated by HU exhibit elongated bud growth. Additionally, the role of Rad53 in the regulation of polarized cell growth is further supported by the observation of various morphological defects of rad53Δ cells (Ensenerk et al., 2006).

We further propose that Shs1 may function in the response to replication stress. Deletion of Shs1 results in a strong HU-induced morphological defect and abolishes the morphological defect caused by overexpression of the FHA1 domain. Besides being a good substrate for Rad53 phosphorylation in vitro, Shs1 was also found to be required for cell viability under DNA replication stress at 37°C. However, the precise role of Shs1 is still unclear. We are now characterizing the in vivo phosphorylation of Shs1 and, consistent with the in vitro results reported here, we have detected at least one in vivo Rad53-dependent phosphorylation site on Shs1 and are assessing its biological relevance.

Furthermore, because septins provide a site where many regulatory proteins are known to localize (Longtine and Bi, 2003), Rad53 may also regulate other septin-associated proteins under DNA replication stress. It is possible that a concerted action from multiple pathways contributes to how Rad53 regulates polarized cell growth in budding yeast. Collectively, we propose that Rad53 controls proper polarized cell growth during DNA replication.

In summary, a network of FHA binding proteins has been identified for Rad53. The FHA-mediated interaction of Rad53 with Rad9 and Mrc1 likely functions in the activation of Rad53 and targets Rad53 to the site of DNA damage or to the DNA replication fork. Further, a network of Rad53 FHA1 binding proteins suggests a role of Rad53 in coordinating a global cellular response, including the regulation of polarized cell growth in yeast. Understanding the role of each FHA binding protein of Rad53 in the DNA damage response should further shed light on its diverse functions.

**Materials and methods**

**Yeast strains, plasmids, and genetic methods**

Strains used in this work are listed in Table S1 (available at http://www.jcb.org/cgi/content/full/jcb.200605081/DC1). All strains of TAP-tagged proteins were obtained from the Open Biosystems collection. For epitope tagging of Rad53, the RDKY5763 cells (ura3-52, leu2Δ1, trp1Δ63, his3Δ200, lys2ΔBgl, hom3-10, ade2Δ1, ade8, YEL069C::URA3, and smd1::TRP1) were used. For deletion or epitope tagging of Bud3, Bud4, Bud14, Cdc10, and Shs1, BY4741 and RDKY5763 cells were used. To generate endogenous C-terminal–tagged Cdc10-3xHA, Rad9-3xHA, and Rad53-GFP, we used standard homologous recombination technique and the pFA6a plasmids (a gift from M. Longtine, Oklahoma State University, Stillwater, OK; Longtine et al., 1998). To make GFP-tagged Rad53 NLS truncation mutant, GFP was fused to the C-terminal end of endogenous Rad53 with concurrent removal of the amino acid 781 to the C terminus. To generate a kinase-dead mutant of Rad53, Rad53 was first cloned into the pFA6a and mutated and then the mutant Rad53 was reintroduced back into the endogenous RAD53 locus in rad53 cells. Correct integration and mutations were all verified by DNA sequencing.

**Figure 6. A network of Rad53 FHA domain binding proteins.** In the absence of exogenous DNA damage treatment, the FHA1 domain of Rad53 is in a dynamic binding equilibrium with many FHA1 binding proteins, yet the kinase remains mostly inactive. In response to DNA damage or replication stress, Rad53 is recruited to Rad9 or Mrc1 for activation, via both its FHA domains. We propose that the dynamic binding equilibrium of active Rad53 with its FHA domain binding proteins assists in substrate targeting.
Expression and purification of FHA-Domain–mediated protein interaction network of Rad53

The FHA domain was cleaved off by adding 100 units of TEV protease to the cleared cell extract. The FHA domain was then subcloned into the PATH plasmid using the same restriction sites. Mutant FHA was generated using site-directed mutagenesis. In each case, the sequence is confirmed by DNA sequencing.

PATH purification of FHA-interacting proteins

2 liters of yeast cells (BY4741) were grown inYPD medium to an OD600 of 1.5. Approximately 10 g of cells were broken in an ice-cooled bead beater with 40 ml lysis buffer containing 50 mM Tris-HCl, pH 8.0, 250 mM NaCl, 0.2% NP-40, 0.5 mM DTT, 5 mM NaF, 10 mM β-glycerophosphate, 1 mM sodium vanadate, 5 mM EDTA, 1 mM PMSF, 0.2 mM benzamidine, 1 μM leupeptin, and 1.5 μM pepstatin. Cell debris was removed by centrifugation at 30,000 g for 30 min. The cleared cell extract was incubated with IgG-Sepharose (GE Healthcare) for 2 h and washed extensively by TBS-N (20 mM Tris-HCl, pH 7.5, 150 mM NaCl, and 0.2% NP-40) with protease inhibitors (5 mM EDTA, 1 mM PMSF, 0.2 mM benzamidine, 1 μM leupeptin, and 1.5 μM pepstatin). Cell debris was removed by centrifugation at 30,000 g for 30 min. Protein extract was divided into two equal fractions, each incubated with 0.1 ml of WT or the mutant PATH-FHA containing IgG resin overnight at 4°C. The resins were then washed with 20 ml of lysis buffer and resuspended in 1.5 ml of lysis buffer without EDTA. The FHA domain was cleaved off by adding 100 units of TEV protease to the cleared cell extract. The FHA domain was then subcloned into the PATH plasmid using the same restriction sites. Mutant FHA was generated using site-directed mutagenesis. In each case, the sequence is confirmed by DNA sequencing.

Western blot analysis

To confirm the binding specificity of FHA binding proteins, 50 ml of yeast cells containing the TAP::PATH-FHA tagged gene of interest was grown in YPD to an OD of 1.0. Cells were harvested and broken by vortexing with glass beads. The cleared cell extract was then incubated with the same amount of WT or mutant GST-FHA proteins bound to glutathione resin. After binding, the resin was washed with 4 × 1 ml of TBS-N, boiled in SDS sample buffer, and subjected to Western blot analysis using anti-TAP antibody (Open Biosystems) or anti-HA antibody (Roche Applied Science).

In vitro phosphorylation assay of septins by Rad53

Recombinant 6×His-Rad53 was used to phosphorylate septins in vitro (Smolka et al., 2005). Approximately 5–10 μg of GST-fused Cdc3, Cdc10, Cdc11, Cdc12, or Shs1 were incubated with 100 ng of Rad53 in 20 μl of kinase buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.2% NP-40, 0.2 mM ATP, and 10 μg ATP) for 40 min at 30°C. After phosphorylation, the samples were boiled in SDS sample buffer with DTT for 5 min, and 10% of the sample was analyzed by SDS-PAGE and subjected to autoradiography.

Plasmids used in this work are listed in Table S2 (available at http://www.jcb.org/cgi/content/full/jcb.200605081/DC1). For overexpression studies, WT and mutant FHA1 (amino acid residues 2–279) and FHA2 (amino acid residues 523–821) domains of Rad53 were cloned into pYES2-NTC vector (Invitrogen) using BamHI and NotI restriction sites. For pull-down assays, the same FHA domains were subcloned into pGEX-4T1. To make the PATH tag, a sequence containing the protein A and TEV cleavage site was amplified from the plasmid pREP1 NT (gift from K. Gould, Vanderbilt University School of Medicine, Nashville, TN; Tasto et al., 2001) using a primer containing a 6×His tag sequence and then inserted into the pET21a plasmid (Novagen) using Ndel and BamHI, resulting in the PATH plasmid. Different FHA domains were then subcloned into the PATH plasmid using the same restriction sites. Mutant FHA was generated using site-directed mutagenesis. In each case, the sequence is confirmed by DNA sequencing.

Microscopy

Microscopy was performed on live cells resuspended in water. Images were taken with a microscope (Axiovert 200; Carl Zeiss Microimaging, Inc.), coupled to a camera (AxioCam HRc; Carl Zeiss Microimaging, Inc.), and the AxioVision software version 4.4.1.0 (Carl Zeiss Microimaging, Inc.). For confocal imaging, yeast were grown to log phase (OD600 ~0.1) and treated with various agents before imaging. Imaging was done on a spinning disk confocal (McBain Instruments) mounted live in minimal media on an inverted microscope (TE2000e; Nikon). Images were acquired using a 60× 1.4 NA Plan Apo objective lens with conventional mode optics. Differential interference contrast, DAPI, FITC, and Rhodamine images were acquired using the same settings, including laser intensity. Each fluorescence image presented is a maximum intensity projection of a z-series stack through the entire yeast cell (5–8 μm), whereas a single differential interference contrast image was acquired at the midpoint of the z stack. All imaging was conducted at room temperature (~23°C).

Immunofluorescence samples were processed and mounted as previously described (Cheeseman et al., 2002), and images were acquired using an upright microscope (E800; Nikon) with conventional mode optics. Differential interference contrast, DAPI, FITC, and Rhodamine images were acquired using a 60× 1.4 NA Plan Apo objective lens with conventional mode optics. Differential interference contrast, DAPI, FITC, and Rhodamine images were acquired using the same settings, including laser intensity. Each fluorescence image presented is a maximum intensity projection of a z-series stack through the entire yeast cell (5–8 μm), whereas a single differential interference contrast image was acquired at the midpoint of the z stack. All imaging was conducted at room temperature (~23°C). Images were processed in MetaMorph for brightness and contrast and minimal gamma adjustments.

Online supplemental material

Fig. S1 shows confirmation of the FHA1 binding proteins. Table S1 lists yeast strains used in this study. Table S2 lists plasmids used in this study. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200605081/DC1.

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