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). 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.

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Abbreviations used in this paper: FHA, Forkhead associated; HU, hydroxyurea; MMS, methyl methanesulfonate; NLS, nuclear localization signal; PATH, protein A–Tev–His; TAP, tandem affinity purification; WT, wild type.

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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 Nasmyth, 1994). Swi6 appears to undergo Rad53-dependent phosphorylation in response to DNA damage stresses and was suggested to be a substrate of Rad53 (Sidorova 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.

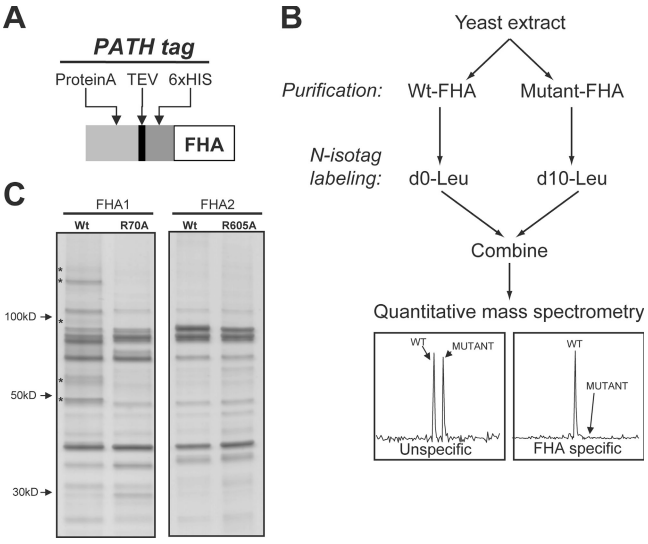

Figure 1. ***PATH* purification of the Rad53 FHA1 and -2 domain binding proteins.** (A) Schematics of the *PATH* tag, consisting of protein A, TEV site, and 6xHis tag. The *PATH* tag is fused to the N terminus of various FHA domains, which are expressed in bacteria. IgG-Sepharose resin is used for their purification. (B) WT and mutant *PATH*-FHA resins are used for a parallel purification of FHA binding proteins in an equal amount of yeast cell extract. After purification, the d0-leucine containing light N-isotag is used to label the WT FHA binding proteins, and the d10-leucine containing heavy N-isotag is used to label proteins from the mutant FHA purification. The labeled samples are combined and analyzed by mass spectrometry (Smolka et al., 2005). Contaminant proteins are expected to be common to both WT and mutant FHA purifications, thus showing similar abundance as detected by mass spectrometry. In contrast, the specific binding proteins of WT FHA domain are expected to be detected as a single peak containing only the light N-isotag. In this way, the specific FHA binding proteins will be determined. (C) Silver staining of 0.5% of the purified proteins of the FHA1 and -2 domains of Rad53 from untreated cells. Asterisks indicate the specific bands present in the sample of proteins purified with WT FHA1.

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 Mrc1 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 Mrc1 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 overwhelms 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

Table I. **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			Validation	Known Rad53 binding protein
				Detected in CTL	Detected in MMS	No. of peptides identified	Detected in CTL	Detected in MMS	No. of peptides identified		
Nuclear											
DNA damage checkpoint	Rad9	YDR217C	Adaptor protein/ Rad53 activation		X	3		X	20	✓	Sun et al., 1998
	Mrc1	YCL061C			X	4				✓	
	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	YDL056W		X	X	4				✓	
	Swi4	YER11C		X	X	3				✓	
	Whi5	YOR093W		X	X	2					
	Cst6	YIL036W	Basic leucine zipper	X	X	4					
	Gln3	YER040W	Nitrogen catabolite repression	X	X	3				✓	
	Ifh1	YLR223C	Ribosome gene transcription	X	X	5				✓	
Other functions	Esc1	YMR219W	Telomeric silencing	X	X	6					
	Asf1	YJL115W	Nucleosome assembly factor	X	X	3				✓	Emil et al., 2001; Hu et al., 2001
	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	YJL076W	Exit from mitosis	X	X	7				✓	
	Yta7	YGR270W	ATPase/ bromodomain	X	X	3				✓	Smolka et al., 2005
Cytosolic											
Cytokinesis	Cdc3	YLR314C	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	YLR248W	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.

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.

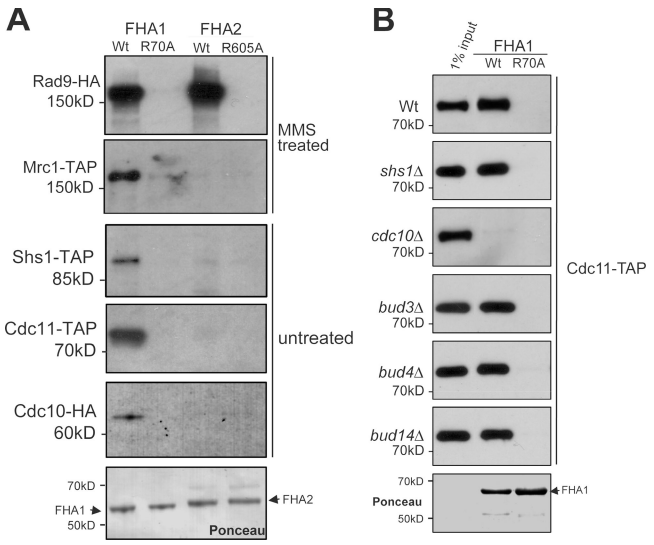

Figure 2. **Confirmation of the binding specificity of the FHA binding proteins of Rad53.** (A) Protein extracts from MMS-treated (0.1% MMS for 3 h) Rad9-HA or Mrc1-TAP cells were divided into equal fractions and subjected to pull-down assays using different GST fusion FHA domains as indicated. The GST-FHA 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.

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

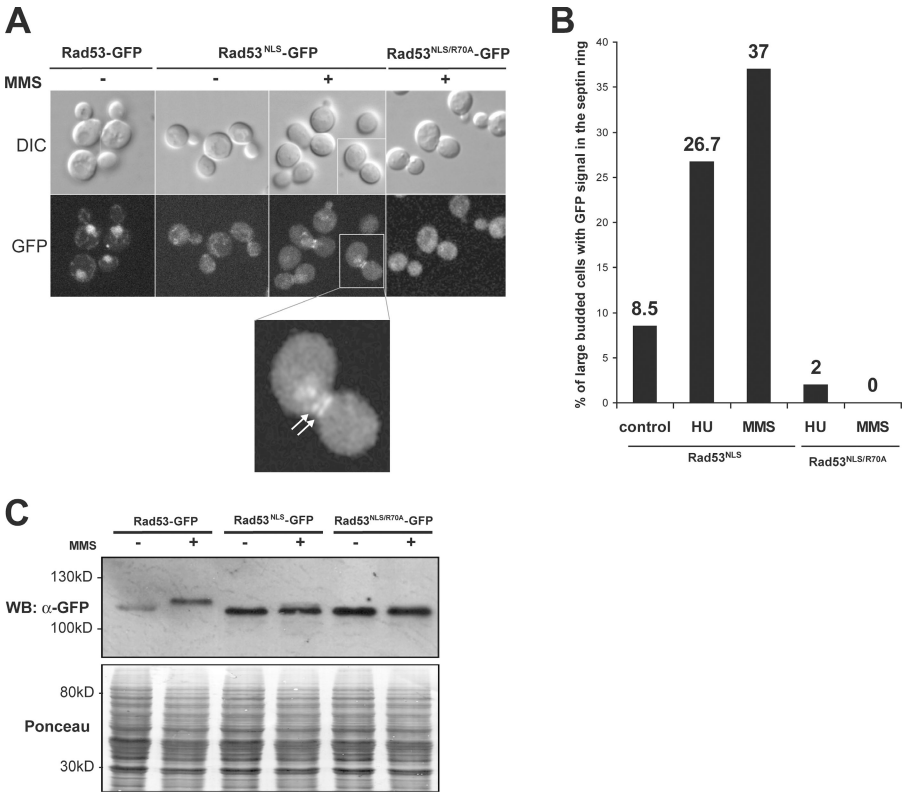

Figure 3. **Rad53 can transiently localize to the bud neck.** (A) Removal of the C-terminal NLS of Rad53 abrogates its predominant nuclear localization (compare two left views). Additional MMS treatment (0.05% for 3 h) led to a high percentage of cells with bud neck localization of the NLS mutant Rad53-GFP. 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.

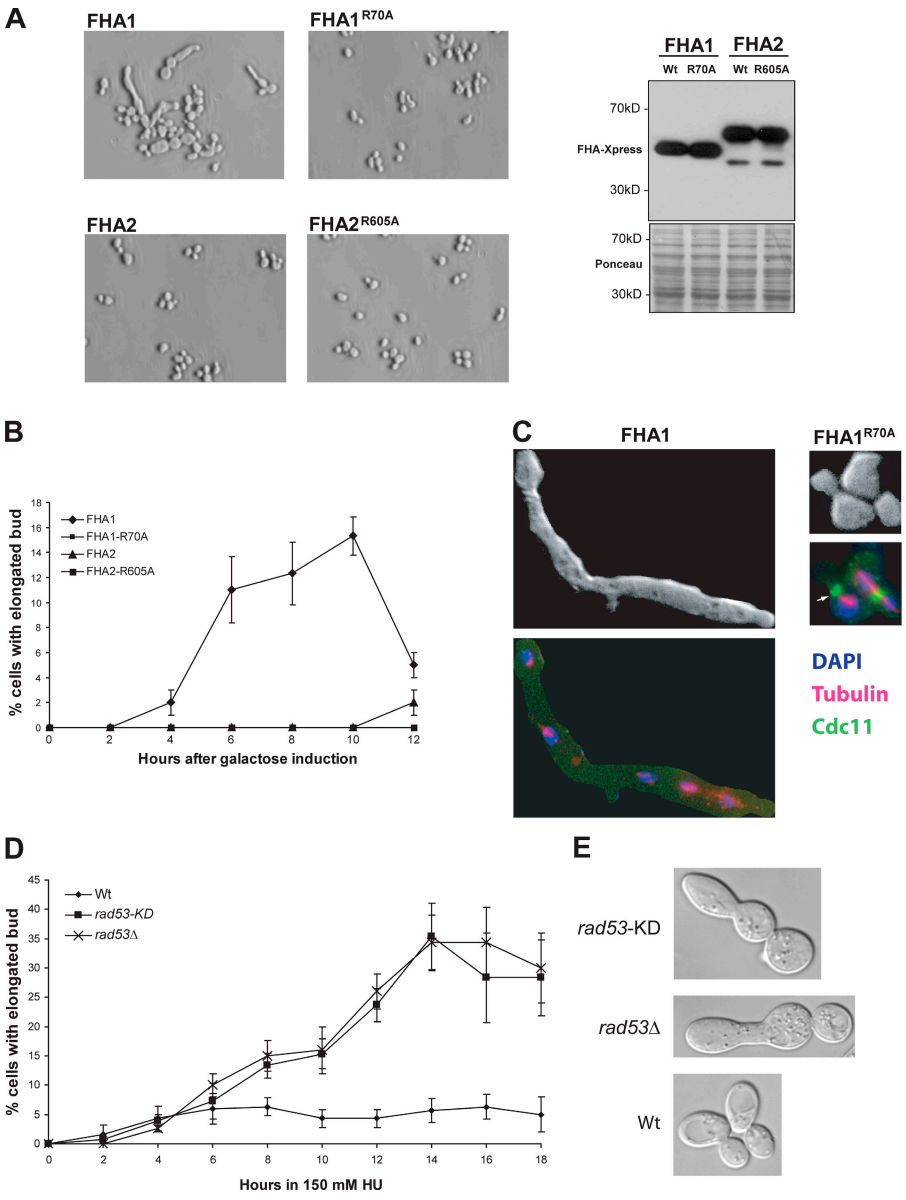

Figure 4. **Morphogenesis defects caused by impairing Rad53 function.** (A) Galactose-induced overexpression of the WT FHA1 domain induces elongated buds. In contrast, overexpression of the FHA1 R70A mutant or WT and R605 mutant FHA2 proteins does not induce any visible defect. Protein extract of the cell cultures were prepared 10 h after galactose induction, and the expression level of the FHA domains was monitored by anti-Xpress Western blot. The membrane was stained with Ponceau for loading control. (B) Quantitation of the elongated bud phenotype. For each time point, at least 200 cells were counted in triplicate experiments. (C) Example of a cell with an FHA1 domain–induced morphological defect, zymolase treated and stained for DAPI, tubulin, and Cdc11. Cells are multinucleated and lack proper septin organization. (right) Staining of a cell after overexpression of the FHA1 domain with an R70A mutation, where normal Cdc11 staining is observed. The arrow indicates the staining of a normal septin ring. (D) *RAD53* deletion or kinase-dead exhibit elongated bud phenotype after treatment with 150 mM HU. For each time point, at least 200 cells were counted in triplicate experiments. Error bars indicate mean ± SD. (E) Example of the elongated bud phenotype observed for *rad53Δ*, *rad53*-KD, and WT cells. All strains are in the RDKY5763 background.

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 downstream 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 *rad53* 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

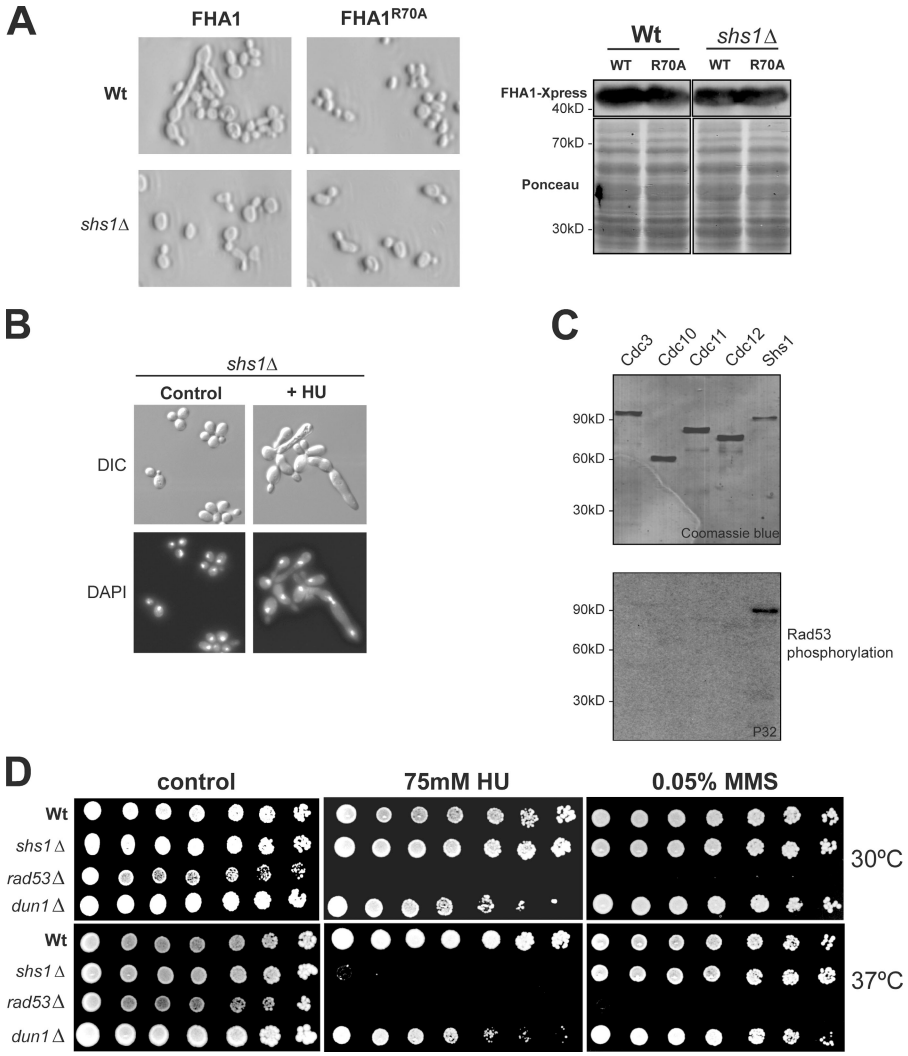

Figure 5. **Involvement of septin Shs1 in the response to DNA replication stress.** (A) Deletion of *SHS1* rescues the elongated bud phenotype induced by FHA1 domain overexpression. Protein extract of the cell cultures were prepared 10 h after galactose induction, and the expression level of the FHA domains was monitored by anti-Xpress Western blot. The membrane was stained with Ponceau for loading control. (B) *shs1Δ* cells exhibit elongated buds after treatment with 150 mM HU for 6 h. (C) In vitro phosphorylation of individual septin components by Rad53. Each septin was expressed and purified from *E. coli*. Among these septins, Shs1 is the only septin efficiently phosphorylated by Rad53. (D) *shs1Δ* cells are hypersensitive to replication stress at 37°C. Indicated cells (in RDKY5763 background) were plated on normal YPD plates or freshly prepared YPD plates containing 75 mM HU or 0.05% MMS. Cells were then grown at 30 or 37°C. Shs1 is required for cell viability at 37°C in the presence of HU. Note that *shs1Δ* cells are considerably more sensitive than *dun1Δ* cells under HU at 37°C. A serial dilution of threefold was used.

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 the FHA1 interacting 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 I). 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).

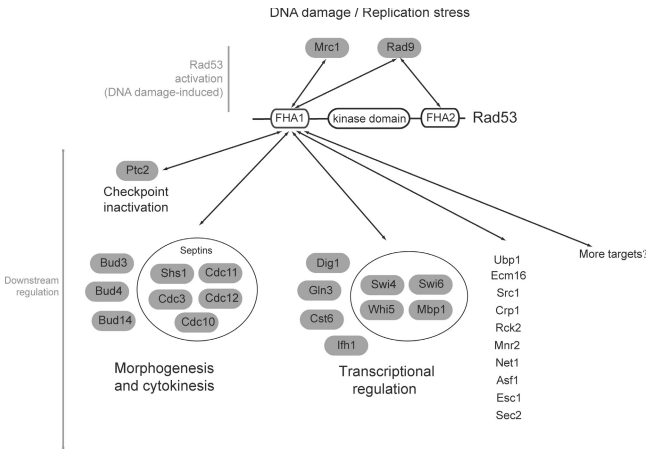

Figure 6. **A network of Rad53 FHA domain binding proteins.** In the absence of exogenous DNA damage treatment, the FHA1 domain of Rad53 is in a dynamic binding equilibrium with many FHA1 binding proteins, yet the kinase remains mostly inactive. In response to DNA damage or replication stress, Rad53 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.

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 (Enserink 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 sml1::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 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.

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/NT-C 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 first amplified from the plasmid pREP1 NT (a gift from K. Gould, Vanderbilt University School of Medicine, Nashville, TN; Tasto et al., 2001) using a primer containing a 6xHis tag sequence and then inserted into the pET21a plasmid (Novagen) using NdeI 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.

Expression and purification of *PATH*-FHA proteins and GST fusion proteins

The *PATH*-FHA1 (amino acid residues 2–279) and -FHA2 (amino acid residues 523–821) domains of Rad53 and their respective mutants (R70A and R605A) were expressed in BL21 cells and induced by 0.1 mM IPTG at 30°C for 3 h. Cells were harvested and lysed by sonication in 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. The cleared cell extract was incubated with IgG-Sepharose (GE Healthcare) for 2 h and washed extensively by TBS-N. Purity and abundance of bound *PATH*-FHA proteins on the IgG resin were examined by either TEV protease cleavage or boiling in SDS sample buffer and subsequent SDS-PAGE analysis. Typically, the procedure resulted in a concentration of 5 μg FHA protein per μl of IgG resin. To make GST fusion proteins, GST-FHA proteins or GST-tagged septins were similarly expressed in BL21 cells. Glutathione resin was used for their purification according to manufacturer's instruction (GE Healthcare).

***PATH* purification of FHA-interacting proteins**

2 liters of yeast cells (BY4741) were grown in YPD medium to an OD_{600} 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, 150 mM NaCl, 0.2% NP-40, 0.5 mM DTT, 5 mM NaF, 10 mM β-glycerolphosphate, 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. 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 (Invitrogen) for 2 h at room temperature. Supernatant containing 6xHis-tagged FHA protein and its interacting proteins was collected and was further incubated with 0.1 ml of Ni-NTA resin (QIAGEN) for 1 h at room temperature. The Ni-NTA resin was washed with 10 ml of lysis buffer and then with 5 ml of TBS buffer. To elute the FHA binding proteins (but not the FHA domain), the Ni-NTA resin was incubated for 5 min at 80°C in 400 μl of an elution buffer containing 8 M urea, 100 mM Tris-HCl, pH 8.0, and 500 mM NaCl. Eluted proteins were reduced and alkylated before trypsin digestion, as described previously (Smolka et al., 2005). For MMS treatment, 0.05% of MMS was added to cells for 2 h before harvesting.

Western blot analysis

To confirm the binding specificity of FHA binding proteins, 50 ml of yeast cells containing a TAP- or HA-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 resins were washed by 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 6xHis-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 μC ATP^{32}) 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 encoding GST fusion of septins were a gift from J. Thorner (University of California, Berkeley, Berkeley, CA).

N-isotag labeling of peptides

For labeling of the amino groups of peptides, we used an isotope-coded leucine-containing N-isotag reagents. The d0-tBoc-Leu-NHS reagent was purchased from Nova Biochem. The d10-tBoc-Leu-NHS was synthesized using the method described previously (Smolka et al., 2005). The light N-isotag, i.e., d0-tBoc-Leu-NHS, was used to label WT FHA binding proteins, whereas the d10-tBoc-leu-NHS was used to label mutant FHA binding proteins. The labeling procedure was performed as described previously (Smolka et al., 2005).

Mass spectrometry and data analysis

Samples were analyzed by μLC-ESI-MS/MS on a quadrupole ion trap mass spectrometer (Finnigan LCQ; Thermo Electron Corporation) as previously described (Smolka et al., 2005). For data analysis, SEQUEST was used for peptide identification, and the XPRESS and INTERACT software were used for quantitation as described previously (Smolka et al., 2005). The complete yeast database was used to analyze MS/MS spectra with no restriction on the enzyme used, and a variable modification of serine and threonine residues by phosphorylation was included in the database search. Only the top-matched, doubly tryptic peptides with high-quality MS/MS spectra were subsequently subjected to manual inspection. All proteins listed in Table I were identified with at least two different peptides, both containing the light N-isotag labeling reagent, which was used to label WT FHA binding proteins.

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 (OD_{600} ~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 1.5× auxiliary magnification (90× magnification total) using a charge-coupled device camera (Orca ER; Hamamatsu) with 2 × 2 binning. Acquisition parameters, shutters, and focus were controlled by MetaMorph software (Universal Imaging). All fluorescence 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 multimode optics. Differential interference contrast, DAPI, FITC, and Rhodamine images were acquired using a 60× 1.4 NA Plan Apo objective lens and MetaMorph software controlling the Orca ER charge-coupled device camera with 1 × 1 binning. 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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