NFBD1, a Novel Nuclear Protein with Signature Motifs of FHA and BRCT, and an Internal 41-Amino Acid Repeat Sequence, Is an Early Participant in DNA Damage Response*

Yong Lei Shang, Amanda J. Bodero, and Phang-Lang Chen‡

From the Department of Molecular Medicine and Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, San Antonio, Texas 78245

Efficient repair of DNA double-strand breaks depends on the intact signaling cascade, comprising molecules involved in DNA damage signal pathways and checkpoints. Budding yeast Rad9 (scRad9) is required for activation of scRad53 (mammalian homolog Chk2) and transduction of the signal further downstream in this pathway. In the search for a mammalian homolog, three proteins in the human data base, including BRCA1, 53BP1, and a nuclear factor with BRCT domains protein 1 (NFBD1), were found to share significant homology with the BRCT motifs of scRad9. Because BRCA1 and 53BP1 are involved in DNA damage responses, a similar role for NFBD1 was tested. We show that NFBD1 is a 250-kDa nuclear protein containing a forkhead-associated motif at its N terminus, two BRCT motifs at its C terminus, and 13 internal repetitions of a 41-amino acid sequence. Five minutes after γ-irradiation, NFBD1 formed nuclear foci that colocalized with the phosphorylated form of H2AX and Chk2, two phosphorylation events known to be involved in early DNA damage response. NFBD1 foci are also detected in response to camptothecin, etoposide, and methylmethanesulfonate treatments. Deletion of the BRCT motifs of NFBD1 has no effect on DNA damage-induced NFBD1 foci formation. Conversely, deletion of the BRCT motifs abrogates damage-induced NFBD1 foci. Ectopic expression of the BRCT motifs reduced damage-induced NFBD1 foci and compromised phosphorylated Chk2- and phosphorylated H2AX-containing foci. These results suggest that NFBD1, like BRCA1 and 53BP1, participates in the early response to DNA damage.

Genomic DNA in all living species is continuously assaulted by exogenous sources, such as environmental mutagens, and endogenous factors, such as reactive oxygen species, that arise during normal cellular metabolism (1). Cells have evolved surveillance mechanisms to promote faithful transmission of genetic information, which involves detection of DNA damage and a resulting signal transduction cascade leading to the induction of cell cycle checkpoints and initiation of DNA repair (2–5). Defects in DNA damage response pathways may result in inefficient repair and accumulation of mutations.

Participants in the DNA damage response pathways can be divided into sensors, proximal kinases, adaptors, transducer kinases, and effectors (4, 5). In budding yeast, a heterotrimeric complex, Rad17-Ddc1-Mec3, which shares homology with proliferating cell nuclear antigen and the replication factor c-like factor Rad24 are thought to be DNA damage sensors (6). Mec1 and Tel1, members of the phosphatidyl inositol-3 kinase-like kinase family, are classified as proximal kinases that activated in response to DNA damage (7, 8). Rad9, an adaptor protein, is phosphorylated by Mec1, which in turn activates the downstream transducer kinase Rad53, a serine/threonine kinase (9–12). Activated Rad53 then further transduces and amplifies signals to downstream effectors that regulate the cell cycle machinery (13–15).

Components of this signaling pathway are conserved in mammals (4, 5). In the initial step of checkpoint activation, DNA damage sensor(s) Rad9-Rad1-Hus1, which share homology with proliferating cell nuclear antigen and the reduced folate carrier protein-like factor Rad17, relay signals to the proximal kinases, ataxia telangietasia mutated (ATM) and ataxia telangiectasia and Rad3-related (ATR) (16–20). Signals are then amplified by the transducer kinases, Chk1 and Chk2 (21–25). Finally, activation of the downstream effectors, such as p53 and CDC25, leads to cell cycle arrest (26–27).

Budding yeast Rad9 (scRad9) was the first cell cycle checkpoint protein discovered. It is essential for arresting division upon DNA damage (28). When yeast is treated with DNA-damaging agents, scRad9 is phosphorylated, which promotes binding to the C-terminal forkhead homology-associated (FHA) motif of Rad53 (12). Therefore, scRad9 plays a critical role in this signaling pathway by ensuring that the damage signal is transduced downstream. A mammalian homologue of scRad9, however, has not been identified. The structural signature motifs of scRad9 are two BRCT repeats in its C terminus. Based on this structural similarity, it was predicted that three mammalian proteins, BRCA1, p53BP1, and an uncharacterized protein, KIAA0170, might be the candidates for the scRad9 homologue (29–31). Only BRCA1 and 53BP1 have been significantly characterized to date.

In addition to their structural similarity, BRCA1 and Rad9 both serve as substrates of ATM and ATR kinases in response

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† To whom correspondence should be addressed: Dept. of Molecular Medicine and Institute of Biotechnology, The University of Texas Health Science Center at San Antonio, 15355 Lambda Dr., San Antonio, TX 78245. Tel.: 210-567-7353; Fax: 210-567-7377; E-mail: chenp0@uthscsa.edu.

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The abbreviations used are: ATM, ataxia telangiectasia mutated; ATR, ataxia telangiectasia and Rad3-related; FHA, forkhead-associated; NFBD1, nuclear factor with BRCT domains protein 1; EGFP, enhanced green fluorescent protein; Chk2T68P, Chk2 phosphorylated at threonine 68; γ-H2AX, H2AX phosphorylated at serine 139; GFP, green fluorescence protein; GST, glutathione S-transferase; IR, ionizing radiation; DSB, double-strand break; Gy, gray; 53BP1, tumor suppressor p53-binding protein 1.
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Fig. 1. Protein structure of NFBD1 and characterization of anti-NFBD1 antibodies. A, human NFBD1 protein has a single FHA motif, 13 internal repeats, and two BRCT motifs. B, alignment of 13 internal repeats shows high homology of the 41 amino acid repeats. C, [35S]Methionine-labeled cell extracts were used for immunoprecipitation (IP) in the presence of mouse anti-NFBD1 serum (lane 2), serum preincubated with GST protein (lane 3), or serum preincubated with GST protein fused antigen (lane 4). Pre-immune serum was used as a negative control (lane 1). To remove nonspecific binding, the immunoprecipitation products were dissociated and immunoprecipitated a second time with mouse anti-NFBD1 serum (lane 5). Rabbit NFBD1 anti-serum was also used for immunoprecipitation (lane 6). Affinity-purified mouse (lane 7) and rabbit (lane 8) anti-NFBD1 were used for immunoblotting.

Cell Culture and Treatments with DNA-Damaging Agents—HeLa, a human cervical carcinoma cell line; IMR90, a human diploid fibroblast; and LEM, an Epstein-Barr virus immortalized human B cell line, were cultured in Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum, 2 mM l-glutamine, 50 units of penicillin, and 50 μg/ml of streptomycin at 37°C with 10% CO2. Cells grown in log-phase were irradiated in a 137Cs radiation source (Mark I, Model 68A Irradiator; JL Shepherd & Associates). UV irradiation was performed using a Stratalinker UV source (Stratagene). Before UV irradiation, the culture medium was removed and cells were washed once with phosphate-buffered saline. The medium was replaced immediately after irradiation. All the cells were then cultured at 37°C and harvested at the indicated time points. For treatment with genotoxic agents, the cells were exposed for 1 h at the indicated dose, washed with phosphate-buffered saline, and cultured in fresh medium for another hour before being fixed for immunostaining. Untreated or mock-treated cells were treated identically with phosphate-buffered saline washes and medium changes but no irradiation.

Antibodies—The fragment of the NFBD1 gene encoding amino acids 2–224 was amplified by polymerase chain reaction using Pfu polymerase (Stratagene) and was cloned into the pGEX vector (Amersham Biosciences). The recombinant protein was expressed in BL21-LysS cells and purified with glutathione S-transferase beads. The purified recombinant protein was resolved by SDS-polyacrylamide gel electrophoresis and stained with Coomassie blue. The polyepitope band was excised, electroeluted from the gel, and subsequently used as an antigen to immunize mice and rabbits. Rabbit antibodies were purified using antigen-coupled affinity chromatography. Rabbit α-γ-H2AX was purchased from Upstate Biotechnology (Lake Placid, NY), rabbit α-Chk2T68P from Cell Signaling Technology (Beverly, MA), and mouse α-GFP from Roche Applied Science.

Immunoprecipitations and Western Blotting—[35S]Methionine labeling, immunoprecipitations, and immunoblotting were performed as described previously (50, 51). α-NFBD1 and α-GFP monoclonal antibodies were used for antigen detection in the immunoblots.

Immunostaining—Procedures for immunostaining were adapted from Durfe et al. (52). Briefly, cells grown on coverslips to 60–70% confluence were treated with various DNA-damaging agents and fixed in 3% formaldehyde with 0.1% Triton X-100. Cells on coverslips were permeabilized by 0.05% saponin and blocked with 10% normal goat serum. All primary antibodies were used at a dilution of 1:100 to 1:1000 in 10% goat serum. The secondary antibodies, including anti-rabbit or mouse Alexa 555 and anti-rabbit or anti-mouse Alexa 488 (Molecular Probes, Eugene, OR) were used at a dilution of 1:1000. Cells were further stained with 4’,6-diamidino-2-phenylindole for detection of DNA and mounted in Permafluor (Lipshaw-Immunonon, Pittsburgh, PA). Immunofluorescence images were captured using a Zeiss Axioplan2 fluorescence microscope.

Transfections—Transfections were performed using Lipofectin (Invitrogen) according to the manufacturer’s instructions. The transfected cells were supplemented with fresh medium and irradiated at 36–38 h after transfection.

MATERIALS AND METHODS

Construction of Plasmids—The NFBD1 full-length cDNA (kindly provided by T. Nagase, Kansai DNA Research Institute, Chiba, Japan) was cloned into the EGFP-C2 expression vector (Clontech, Palo Alto, CA). Subsequent motif deletions were made in the EGFP-NFBD1 construct using the QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) and confirmed by DNA sequencing. Primers encoding the FHA domain of NFBD1 were synthesized, annealed, and cloned into EGFP-C2. The fragment containing the FHA repeats was ligated into pGEX (codon 1839–2089) was obtained by polymerase chain reaction using Pfu polymerase (Stratagene) and cloned in-frame with nuclear localization signal in EGFP-C2.

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RESULTS

Identification of Cellular NFBD1 Protein with Anti-NFBD1 Antibodies—We found that NFBD1, in addition to its N-terminal FHA motif and two C-terminal BRCT motifs, had 13 unique internal repeats, each containing 41 amino acids enriched with serine, threonine, and proline residues (Fig. 1). Interestingly, potential ATM/ATR and cyclin-dependent kinase phosphorylation sites are scattered throughout these repeats. To identify the encoded protein of NFBD1, we used the recombinant GST fusion protein containing the N-terminal 223 amino acids of NFBD1 as an antigen (named GST-FHA) to raise mouse and rabbit polyclonal antibodies. Cell extracts prepared from [35S]methionine-labeled lymphoid cells (LEM) were used for immunoprecipitation with either mouse NFBD1 antisera or preimmune serum. As shown in Fig. 1C, a protein with an apparent molecular mass of 250 kDa, which is near the predicted molecular mass, was specifically immunoprecipitated by anti-NFBD1 antisera but not pre-immune sera (lanes 1 and 2). Addition of the original GST-FHA antigen (lane 4), but not GST alone (lane 3), blocked the immunoprecipitation of 250-kDa protein. To further ensure the specificity of the antibodies, the anti-NFBD1 immunoprecipitates were released and reprefi-
response pathway, including 53BP1 and BRCA1, form subnuclear foci after DNA damage (42, 43, 53). To determine whether NFBD1 has similar properties, we examined its subcellular localization by immunostaining with anti-NFBD1 antibodies. In actively growing HeLa cells, purified rabbit NFBD1 antisera but neither pre-immune nor GST-FHA antigen blocked demonstrated immunoreactivity confined to nuclei (Fig. 2A). HeLa cells treated with ionizing radiation (IR) showed distinct nuclear foci positive for anti-NFBD1 immunoreactivity (Fig. 2B). To confirm that these signals are attributed to NFBD1, full-length NFBD1 protein was tagged with GFP and expressed in the same HeLa cells. GFP-NFBD1, like endogenous NFBD1, localized exclusively in nuclei and was present in subnuclear foci after IR (Fig. 2C). Furthermore, when HeLa cells expressing the GFP-NFBD1 were immunostained with anti-NFBD1 antibodies, the IR-induced nuclear foci derived from GFP signals (exogenous) colocalized with the foci detected by immunostaining with anti-NFBD1 antibodies (endogenous) (Fig. 3D). These results suggest that NFBD1 is present in nuclear foci upon IR.

Next we tried to determine whether the foci that form in response to other DNA-damaging agents contain NFBD1. Camptothecin, a DNA topoisomerase I inhibitor, and etoposide, a topoisomerase II inhibitor, produced single-strand breaks and double-strand breaks (DSBs), respectively. Treatment with these two drugs induced prominent formation of foci containing NFBD1. Similar results were also observed in cells treated with the alkylating agent methylmethanesulfonate or the cross-linking agent mitomycin C, which both produce synthetic strand breaks (Fig. 3). Cells treated with UV light, which causes pyrimidine dimers but not DSBs, showed only a few foci positive for NFBD1 (Fig. 3). These results suggest damage-specific recruitment of NFBD1 to subnuclear foci during cellular responses.

NFBD1 Forms Foci Very Early in Response to IR—DNA DSBs induce formation of nuclear foci at the sites of breaks containing several damage response proteins. First, we characterized the kinetics of NFBD1 foci formation induced by IR in a normal human fibroblast line, IMR90. Cells were fixed at different time points after 1 Gy of γ-irradiation and immunostained against NFBD1 immunoreactivity (Fig. 2B). To confirm that these signals are attributed to NFBD1, full-length NFBD1 protein was tagged with GFP and expressed in the same HeLa cells. GFP-NFBD1, like endogenous NFBD1, localized exclusively in nuclei and was present in subnuclear foci after IR (Fig. 2C). Furthermore, when HeLa cells expressing the GFP-NFBD1 were immunostained with anti-NFBD1 antibodies, the IR-induced nuclear foci derived from GFP signals (exogenous) colocalized with the foci detected by immunostaining with anti-NFBD1 antibodies (endogenous) (Fig. 3D). These results suggest that NFBD1 is present in nuclear foci upon IR.

FIG. 5. Colocalization of NFBD1 with γ-H2AX and Chk2T68P in response to γ-radiation. A, HeLa cells untreated or treated with γ-radiation (8 Gy) were fixed 30 min after irradiation and costained with anti-NFBD1 (red) and anti-γ-H2AX antibody (green). B, after mock treatment or γ-radiation (8 Gy), cells were fixed and co-stained with anti-NFBD1 (red) and anti-Chk2T68P (green). Yellow dots indicate colocalization.

Colocalization of NFBD1, γ-H2AX, and Phosphorylated Chk2 at IR-induced Foci—DNA DSBs form nuclear foci at the sites of DNA DSBs upon IR (54, 55). H2AX is a variant of H2A that undergoes phosphorylation at serine 139. γ-H2AX appears in discrete nuclear foci at sites of DNA breaks within 1–3 min after irradiation. The kinetics of NFBD1-posi-
treated or treated with transiently transfected into HeLa cells. 24 h later, cells were left untreated or GFP. anti-GFP (Roche Applied Science), and then immunoblotted using anti-wild-type or mutant NFBD1 plasmid constructs were used for IP with γH2AX (54, 56), prompting us to ask whether NFBD1 and co-localize in IR-induced foci. Indeed, NFBD1 and γH2AX immunoreactivity colocalize in foci that formed within 15 min to 4 h after irradiation (Fig. 5A).

In addition to γH2AX, Chk2, a mammalian homologue of the transducing kinase Rad53, was also examined. In response to IR, ATM rapidly phosphorylates Chk2 at threonine 68, which activates Chk2 kinase activity (23, 57, 58) and its relocation to sites of DNA DSBs (55). To test whether NFBD1 also co-localizes with Chk2T68P after IR, we immunostained irradiated cells using antibodies specific for T68-phosphorylated Chk2. As shown in Fig. 5B, immunoreactivity to both Chk2T68P and NFBD1 antibodies colocalized at the IR-induced foci at all time points from 15 min to 4 h after irradiation. Collectively, these results indicate that NFBD1, like γH2AX and Chk2T68P, is recruited to sites of DNA breaks during the early stage of the response to DNA damage.

Localization of NFBD1 at IR-induced Foci Requires BRCT Motifs—NFBD1 contains three significant signature motifs. Each of them can be important in mediating the function it plays in DNA damage response. To determine which of the three motifs of NFBD1 was essential for the IR-induced foci formation, we generated deletion mutants of NFBD1 that removed them individually (Fig. 6A). The mutated proteins were N-terminally tagged with GFP, and their expression was assayed in transfected HeLa cells by immunoprecipitation with anti-GFP antibodies followed by Western blotting. As shown in Fig. 6B, each of the mutated proteins was detected in transfected HeLa cells. Interestingly, all the mutated fusion proteins were localized in nuclei, suggesting that none are important for nuclear localization of NFBD1 (Fig. 6C).

Expression of the BRCT Motif of NFBD1 Abolishes IR-induced Foci Formation—The major function of the BRCT motifs is to establish protein-protein interactions. Their limited sequence homology indicates that each BRCT motif has a distinct binding partner. To determine the effect of expression of the BRCT motifs on NFBD1 foci formation, we generated deletion mutants of NFBD1 that removed them individually (Fig. 6A). These results indicate that the BRCT motifs have a dominant-negative effect on NFBD1 localization.

Because NFBD1 colocalizes with γH2AX and Chk2T68P at IR-induced foci upon irradiation, we tested whether γH2AX- and Chk2T68P-containing foci would be altered in cells expressing the NFBD1-derived GFP-BRCT fusion protein. We immunostained the transfected cells with anti-γH2AX and found that the intensity of γH2AX staining was similar to that of the control, indicating that the phosphorylation of H2AX remained intact (Fig. 7A). IR-induced foci were substantially reduced, however, in cells expressing the dominant-negative GFP-BRCT fusion protein (Fig. 7D). These results suggest that NFBD1 may have a role in recruitment of γH2AX to sites of DNA breaks but no obvious role in H2AX phosphorylation at serine 139. Similarly, when we immunostained the transfected cells with anti-Chk2T68P antibodies, IR-induced Chk2T68P foci were diminished in the presence of GFP-BRCT (Fig. 7F), suggesting that NFBD1 is also required for recruitment of Chk2T68P to sites of DNA breaks.

DISCUSSION

In a search for a mammalian homolog of scRad9, a critical adaptor protein in DNA damage signal pathway, we characterized a novel nuclear protein, NFBD1, the BRCT motifs of which share significant homology with those of scRad9. NFBD1 was identified as a 250-kDa nuclear protein with specific antibodies. Within 5 min after IR, NFBD1 was found in nuclear foci, which also seemed to contain phosphorylated forms of H2AX (γ-H2AX) and Chk2 (Chk2T68P), two proteins known to be involved in early DNA damage response. NFBD1 foci formation was also detected in response to camptothecin, etoposide, and methylmethanesulfonate treatment. Deletion of the BRCT motif of NFBD1 had significant effects on the formation of IR-
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induced foci containing NFBD1. Moreover, ectopic expression of the NFBD1-derived BRCT motif alone does not greatly reduce IR-induced formation of foci containing NFBD1 but also reduced formation of foci containing either Chk2T68P or γ-H2AX. These results suggest that NFBD1 participates in the early stage of cellular response to DNA damage, a functional property similar to that of scRad9.

In the DNA damage signal cascade of budding yeast, Rad9 is required for the activation of the Chk2 homolog Rad53 (10). Phosphorylation of Rad9 by Mecl upon DNA damage results in the formation of an epitope that binds to the FHA motif of Rad53. Rad9 self-associates (59) and forms oligomers to bring two Rad53 molecules into close proximity to facilitate in trans autophosphorylation of the bound Rad53 molecules (60). Thus, Rad9 serves as an adaptor to recruit Rad53.

In mammalian cells, Chk2 activation in response to IR requires initial phosphorylation of Thr-68 by ATM and subsequent autophosphorylation (57, 58, 61). How Thr-68 phosphorylation leads to Chk2 autophosphorylation and activation is currently unclear. Because the DNA damage signaling pathway is highly conserved among different eukaryotes and because ATM can only phosphorylate Chk2 but fails to activate it in vitro (58), it is likely that an adaptor protein for Chk2 activation exists in mammals. 53BP1 and BRCA1 were speculated as the adaptors for Chk2 activation, but in the absence of functional BRCA1, Chk2 nonetheless can be efficiently activated upon IR. On the other hand, depletion of 53BP1 can partially affect the phosphorylation of Chk2 at T68 (46). Thus, 53BP1, but not BRCA1, may serve as an adaptor protein to facilitate Chk2 activation. Our immunostaining results have shown that NFBD1 colocalizes with Chk2T68P at the site of DNA breaks, and overexpression of the NFBD1-derived BRCT motif diminished the IR-induced foci positive for Chk2T68P. This finding, combined with structural clues, suggests an intriguing possibility that NFBD1 may also function as an adaptor protein.

NFBD1 has a dominant-negative effect on NFBD1 function. This result plays a role in the recruitment of γ-H2AX to sites of DNA breaks but very little role in the phosphorylation of H2AX at serine 139. Although further confirmation is needed, our data suggest that NFBD1 may play little role in sensing DNA damage.

The results presented in this study suggest strongly that NFBD1 may serve as an adaptor protein in the DNA damage response pathway in mammalian cells. Because mutation of either BRCA1 (75) or Chk2 accounts for some hereditary cases of breast cancer (76), mutation of NFBD1 may also be a potential contributing factor to the formation of breast or other cancers. Further elucidation of the precise role(s) that NFBD1 plays in DNA damage response and in tumor formation will address this intriguing possibility.

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**REFERENCES**

1. Hartwell, L. (1992) Cell 71, 543–546

2. Nurse, P. (1997) Cell 91, 855–867

3. Melo, J., and Toczyski, D. (2002) Curr. Opin. Cell Biol. 14, 237–245

4. Rouse, J., and Jackson, S. P. (2002) Science 297, 537–540

5. Zhou, B., and Elledge, S. J. (2002) Nature 408, 433–439

6. Melo, J. A., Cohen, J., and Toczyski, D. P. (2001) Genes Dev. 15, 2809–2821

7. Morrow, D. M., Tagle, D. A., Shiloh, Y., Collins, F. S., and Hieter, P. (1995) Cell 82, 811–840

8. Jackson, S. P. (1996) Curr. Opin. Genet. Dev. 6, 19–25

9. Sun, Z., Hsiao, J., Fay, D. S., and Stern, D. F. (1998) Science 281, 272–274

10. Vialard, J., Ebert, C. S., Green, M. C., and Lowndes, N. F. (1998) EMBO J. 17, 5679–5688

11. Emili, A. (1998) Mol. Cell 2, 183–189

12. Schwartz, M. F., Duong, J. K., Sun, Z., Morrow, J. S., Pradhan, D., and Stern, D. F. (2002) Mol. Cell 9, 1055–1065

13. Pelliccio, A., Lucca, C., Liberri, G., Marinii, F., Lopes, M., Plevani, P., Romano, A., Di Fiore, P. P., and Poiani, M. (1999) EMBO J. 18, 6561–6572

14. Schramaek, V., Neecke, H., Brevet, V., Corda, Y., Lucchini, G., Longhese, M. P., Gilson, E., and Veli, G. (2001) Genes Dev. 15, 1845–1858

15. Zhao, X., Chabes, A., Deng, Y., Vahelander, L., and Rothstein, R. (2001) EMBO J. 20, 3544–3553

16. Rasen, M., Burtelow, M. A., Dufault, V. M., and Karnitz, L. M. (2000) J. Biol. Chem. 275, 29761–29767

17. Venzelovas, C., and Thelen, M. P. (2000) Nucleic Acids Res. 28, 2481–2493

18. Post, S., Weng, Y. C., Cimprich, K., Chen, L. B., Xu, Y., and Lee, E. Y. (2001) Proc. Natl. Acad. Sci. U. S. A. 98, 13102–13107

19. Hao, S., Tibbet, R. S., Brumbaugh, K. M., Fang, Y., Richardson, D. A., Ali, A., Chen, S. M., Abraham, R. T., and Wang, X. F. (2001) Nature 411, 969–974

20. Zou, L., Cortez, D., and Elledge, S. J. (2002) Genes Dev. 15, 198–208

21. Sanchez, Y., Wong, C., Thomas, R. B., Richman, B., Wu, Z., Pigna-Worms, H., Elledge, S. J. (1999) Cell 97, 47–57

22. Brown, A. L., Lee, C. H., Schwartz, J. K., Minoku, N., Pigna-Worms, H., and Chant, J. H. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 3745–3750

23. Matsuoka, S., Huang, M., and Elledge, S. J. (1998) Science 282, 1893–1897

24. Liu, Q., Guntuku, S., Cui, X. S., Matsuoka, S., Cortez, D., Tamai, K., Luo, G., Carattini-Rivera, S., DeMayo, F., Bradley, A., Donohew, L. A., and Elledge, S. J. (2000) Genes Dev. 14, 1448–1459

25. Zhao, H., and Pigna-Worms, H. (2001) Mol. Cell. Biol. 21, 4129–4139

26. Bartek, J., and Lukas, J. (2001) Curr. Opin. Cell Biol. 13, 738–747

27. Taylor, W. R., and Stark, G. R. (2001) Science 290, 1805–1815

28. Weinert, T. A., and Hartwell, L. H. (1988) Science 241, 317–322

29. Koonin, E. V., Altschul, S. F., and Bork, P. (1996) Nat. Genet. 13, 266–268

30. Bork, P., Hofmann, K., Bucher, P., Neuwald, A. F., Altschul, S. F., and Koonin, E. V. (1997) FEBS Lett. 419, 61–66

31. Callebaut, I., and Mornon, J. P. (1997) FEBS Lett. 400, 25–30

32. Cortez, D., Wang, Y., Qin, J., and Elledge, S. J. (1999) Science 286, 1162–1166

33. Tibbetts, R. S., Cortez, D., Brumbaugh, K. M., Scully, R., Livingston, D., Elledge, S. J., and Abraham, R. T. (2000) Genes Dev. 14, 2889–3002

34. Xu, B., Kim, S. T., and Kastan, M. B. (2001) Mol. Cell 21, 3445–3450

35. Xu, X., Weaver, Z. L., Link, S. P., Li, C., Getay, J., Wang, X. W., Harris, C. C., Ried, T., and Deng, C. X. (1999) Mol. Cell 3, 389–395

36. Lee, J. S., Collins, K. M., Brown, A. L., Lee, C. H., and Chung, J. H. (2000) Mol. Cell 5679–5688
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Yong Lei Shang, Amanda J. Bodero and Phang-Lang Chen

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