21. ECFP (*14*) is *Aequorea victoria* GFP with mammalian codons and the following additional mutations: K26R, F64L, S65T, Y66W, N146I, M153T, V163A, and N164H (*8*). A gene encoding a fusion of the peptide Ala-Glu-Ala-Ala-Ala-Arg-Glu-Ala-Cys-Cys-Arg-Glu-Cys-Cys-Ala-Arg-Ala to the COOH-terminus of ECFP was created with the following primer in a polymerase chain reaction (PCR): 5′-GCCGAATTCTTAGGCCCTGGCGCAGCACTCCCTGCAGCAGGCCTCCCTGGCGGCGGCCTCGGCCTTGTACAGCTCGTCCATGCCG-3′. The resulting gene was inserted into the pcDNA3 vector (Invitrogen) at Hind III and Eco RI restriction sites. After amplification in DH5 bacteria, it was transfected into HeLa cells with Lipofectin (Gibco-BRL). The gene for *Xenopus* calmodulin was mutated to encode cysteines at residues 6, 7, 10, and 11 by PCR with the following primer: 5′-CGCGGATCCGCCACCATGCATGACCAACTGACATGCTGCCAGATTTGCTGCTTCAAAGAAGCCTTCTCATTATTC-3′, and inserted into pcDNA3 as described above.

22. Images of cells at room temperature were acquired with a cooled charge-coupled device camera (Photometrics, Tucson, AZ) controlled by Metafluor software (Universal Imaging, West Chester, PA).

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Rad53 FHA Domain Associated with Phosphorylated Rad9 in the DNA Damage Checkpoint

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The Rad53 protein kinase of *Saccharomyces cerevisiae* is required for checkpoints that prevent cell division in cells with damaged or incompletely replicated DNA. The Rad9 protein was phosphorylated in response to DNA damage, and phosphorylated Rad9 interacted with the COOH-terminal forkhead homology–associated (FHA) domain of Rad53. Inactivation of this domain abolished DNA damage–dependent Rad53 phosphorylation, G_2/M cell cycle phase arrest, and increase of *RNR3* transcription but did not affect replication inhibition–dependent Rad53 phosphorylation. Thus, Rad53 integrates DNA damage signals by coupling with phosphorylated Rad9. The hitherto uncharacterized FHA domain appears to be a modular protein-binding domain.

The consequences of DNA damage to eukaryotic cells are minimized by simultaneous activation of DNA repair mechanisms and cell cycle arrest at DNA damage checkpoints. In *Saccharomyces cerevisiae*, the proteins encoded by the genes *POL2*, *RFC5*, and *DPB11* are required for arrest in response to inhibition of DNA replication (*1*), whereas the proteins encoded by the genes *RAD9*, *RAD17*, *RAD24*, *DDC1*, and *MEC3* operate in response to DNA damage (*2*, *3*). *MEC1* and *RAD53* are required for both checkpoints (*3*, *4*). Rad53 (also called Spk1, Sad1, and Mec2) is an essential Ser/Thr/Tyr protein kinase (*5*, *6*). Mec1 belongs to a subgroup of the phosphatidylinositol-(3)-phosphate kinase family that includes known protein kinases (*7*). Signals generated by replication inhibition or by DNA damage apparently activate a protein kinase cascade, in which Mec1 is required for phosphorylation of Rad53 (*8*, *9*), which is required for phosphorylation and activation of protein kinase Dun1 (*10*).

A catalytically hypoactive Rad53, in which Ala^{208} was changed to Pro (*11*), was used to search for interacting proteins expressed from a *S. cerevisiae* cDNA library (S. J. Elledge, Baylor College of Medicine) in a yeast two-hybrid screen. The strongest interactor was a region of Rad9 (amino acids 553 to 1056) followed by polyadenylate. Authentic Rad9 and Rad53 also interacted with one another (Fig. 1A).

Rad9-FLAG (*12*) overexpressed with Rad53 migrated on gels as a heterogeneous polypeptide. The fastest migrating form (estimated molecular size just below 200 kD) predominated (Fig. 1, B and C) and was larger than the 149-kD size of the predicted sequence (*13*). These bands were only present in galactose-treated samples from strains carrying the tagged expression plasmid. The fastest migrating form of Rad9 migrated at a similar position to that of the in vitro transcription-translation product (*14*). Antibody to FLAG (anti-FLAG) cross-precipitated Rad53. Anti-Rad53 preferentially precipitated slower migrating forms of Rad9 (Fig. 1B). Bacterially produced glutathione S-transferase (GST)–Rad53 COOH-terminus also interacted with slow migrating forms of Rad9 from yeast lysates overexpressing both Rad9 and Rad53 (Fig. 1C). Calf intestine alkaline phosphatase (CIP) converted the slow migrating forms to the fast migrating form, indicating that the mobility shift of Rad9 is mostly

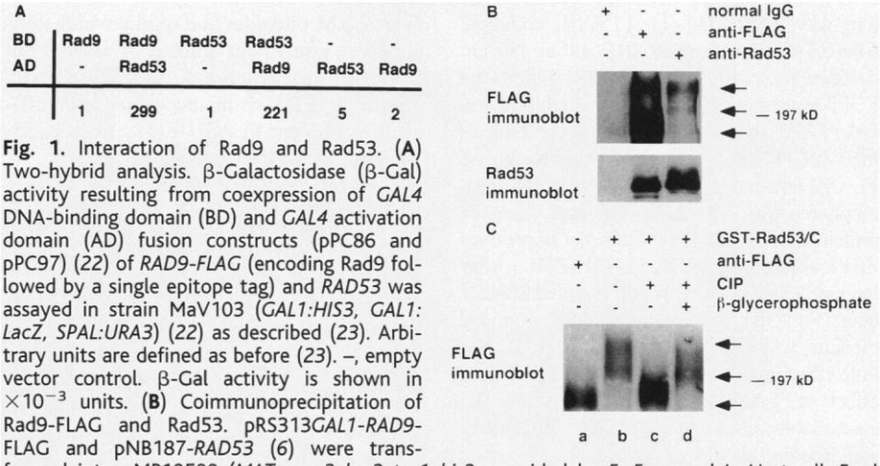

Fig. 1. Interaction of Rad9 and Rad53. (**A**) Two-hybrid analysis. β-Galactosidase (β-Gal) activity resulting from coexpression of *GAL4* DNA-binding domain (BD) and *GAL4* activation domain (AD) fusion constructs (pPC86 and pPC97) (*22*) of *RAD9-FLAG* (encoding Rad9 followed by a single epitope tag) and *RAD53* was assayed in strain MaV103 (*GAL1:HIS3, GAL1:LacZ, SPAL:URA3*) (*22*) as described (*23*). Arbitrary units are defined as before (*23*). –, empty vector control. β-Gal activity is shown in $\times 10^{-3}$ units. (**B**) Coimmunoprecipitation of Rad9-FLAG and Rad53. pRS313*GAL1-RAD9*-FLAG and pNB187-*RAD53* (*6*) were transformed into yMP10500 (*MATa ura3 leu2 trp1 his3*, provided by E. Foss and L. Hartwell, Fred Hutchinson Cancer Research Center). Expression of Rad9-FLAG and Rad53 was induced by incubating log phase cells in galactose (2%) for 3 hours. Equal portions of extracts from 100-ml cultures were immunoprecipitated and immunoblotted by means of detection with enhanced chemiluminesence. Arrows mark positions of forms of Rad9-FLAG precipitated with anti-Rad53. Bar marks position of a 197-kD marker. (**C**) Rad9 is phosphorylated. GST-Rad53/450-821 fusions were produced in *Escherichia coli* and bound to glutathione-Sepharose beads. Equivalent portions of yeast lysates prepared as in (B) were used for GST-affinity purification. One-fifth equivalent amount of lysate was precipitated with anti-FLAG. Beads were incubated with 50 units of CIP at 37°C for 10 min in the presence or absence of β-glycerophosphate, a CIP inhibitor (*24*). Samples were then analyzed by immunoblotting. Arrows mark forms of Rad9-FLAG. Bar indicates the position of a 197-kD marker.

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caused by phosphorylation (Fig. 1C).

The apparent selective recruitment of Rad53 to phosphorylated Rad9 suggested that the phosphorylation of Rad9 might be regulated. Cells expressing *RAD9-FLAG* under the control of the *RAD9* promoter were incubated with methyl methane sulfonate (MMS), which damages DNA, or with hydroxyurea (HU), which blocks DNA replication. MMS treatment, but not HU treatment, induced a mobility shift of Rad9 (Fig. 2A), indicating that Rad9 phosphorylation might be selectively regulated by DNA damage but not by inhibition of replication. Damage-regulated mobility shift of Rad9 was also detected in cells harboring *RAD9-HA* integrated at its chromosomal locus (Fig. 2B). In this strain, which expresses amounts of Rad9 and Rad53 similar to those in wild-type cells, anti-Rad53 selectively immunoprecipitated the slowly migrating form of Rad9 induced by MMS treatment (Fig. 2B, lane l). Thus, Rad53 appears to interact with phosphorylated Rad9 induced by DNA damage.

Rad53 deletion mutants were used to localize sequences required for Rad9-Rad53 interaction (Fig. 3A). Rad53 has two forkhead homology–associated (FHA) domains located before (FHA1) and after (FHA2) the kinase domain (*15*). The smallest mutant with full binding activity (amino

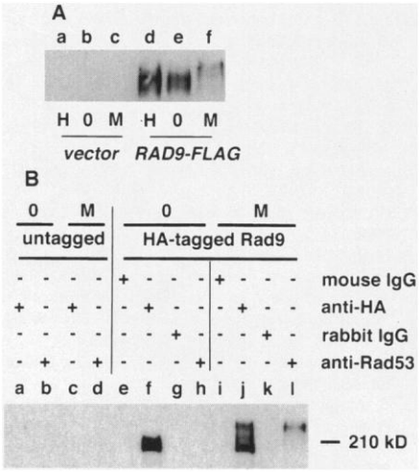

Fig. 2. Regulation of interaction between Rad9 and Rad53. (**A**) Regulation of Rad9 phosphorylation. TWY397 cells (*3*) carrying a centromeric plasmid (pRS316) with RAD9-FLAG under the control of the RAD9 promoter were mock-treated (0) or treated with 100 mM HU (H) or 0.02% MMS (M) for 0.5 hour. Cells were boiled in sample buffer, agitated with glass beads, and then analyzed by immunoblotting. Lanes a to c, cells with empty vector; lanes d to f, cells with FLAG-tagged *RAD9*. (**B**) Coimmunoprecipitation of endogenous Rad9 and Rad53. The protease-deficient strain *(BJ5460; MATa ura3 trp1 lys2 leu2 pep4::HIS3 prb1d1.6R)* and an isogenic strain with hemagglutinin (HA)–tagged endogenous *RAD9* (*RAD9-HA$_2$::TRP1*) were provided by A. Emili, Fred Hutchinson Cancer Research Center (*19*). Log phase cells (200 ml) were treated with 0.05% MMS (M) or mock-treated (0). Equal portions of lysates were immunoprecipitated with anti-HA (12CA5, Babco), anti-Rad53, or control antibodies [mouse or rabbit immunoglobulin G (IgG)]. Samples were immunoblotted with anti-HA. Bar indicates the position of the 210-kD marker.

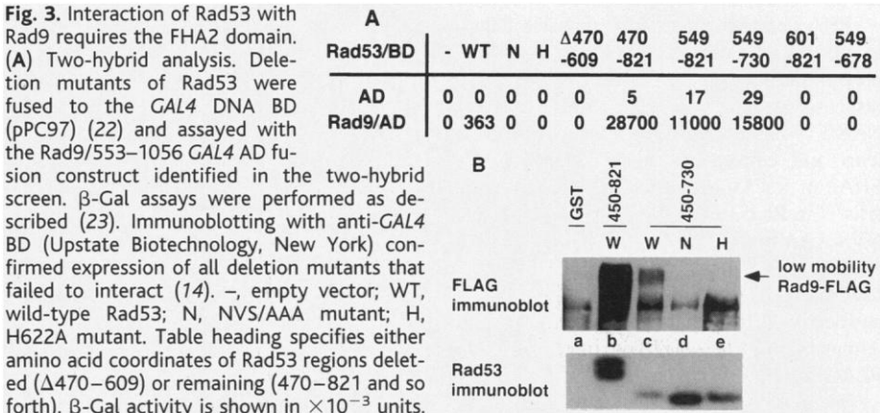

Fig. 3. Interaction of Rad53 with Rad9 requires the FHA2 domain. (**A**) Two-hybrid analysis. Deletion mutants of Rad53 were fused to the *GAL4* DNA BD (pPC97) (*22*) and assayed with the Rad9/553–1056 *GAL4* AD fusion construct identified in the two-hybrid screen. β-Gal assays were performed as described (*23*). Immunoblotting with anti-*GAL4* BD (Upstate Biotechnology, New York) confirmed expression of all deletion mutants that failed to interact (*14*). –, empty vector; WT, wild-type Rad53; N, NVS/AAA mutant; H, H622A mutant. Table heading specifies either amino acid coordinates of Rad53 regions deleted (Δ470–609) or remaining (470–821 and so forth). β-Gal activity is shown in $\times 10^{-3}$ units. (**B**) In vitro binding assay. GST-Rad53 fusions (produced as in Fig. 1C) were used to bind proteins from equal portions of yeast lysates and analyzed by immunoblotting. The fast migrating band in the lower panel of lane b is a degradation product of GST-Rad53/450–821. GST, GST alone; W, GST fusion with wild-type Rad53 fragment; N, GST fusion with NVS/AAA mutant fragment; H, GST fusion with H622A mutant fragment. Note differences in the amount of GST-Rad53 in lanes b and c, as indicated by the immunoblot in the lower panel. Numbers are amino acid coordinates of Rad53 regions fused to GST.

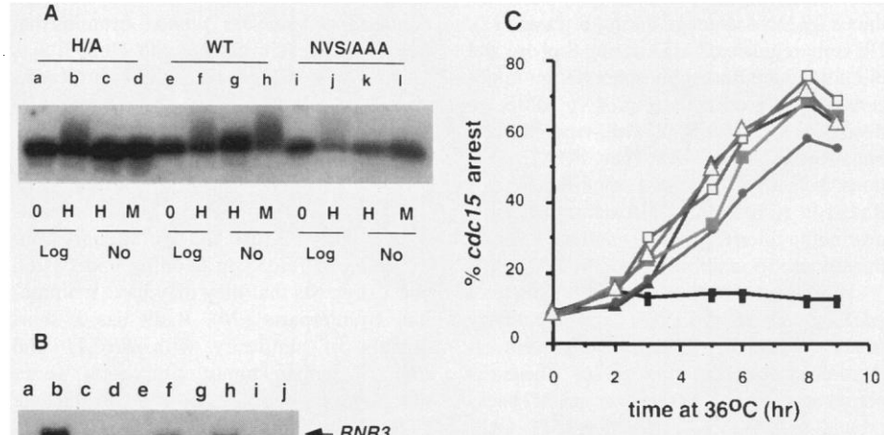

Fig. 4. Phenotypes of *rad53* FHA2 mutant alleles. (**A**) Regulated phosphorylation. Centromeric plasmids pRS316 carrying alleles of *RAD53* under the *RAD53* promoter were assayed in DZ424 cells (*rad53Δ::HIS3*) (*6*). Logarithmic phase (Log) or nocodazole-arrested (No) cells (*25*) were mock-treated (0) or treated with 100 mM HU (H) or 0.1% MMS (M) for 1 hour. Rad53 was then analyzed by anti-Rad53 immunoprecipitation and immunoblotting (*9*). WT, wild type; H/A and NVS/AAA, FHA2 mutants tested. (**B** and **C**) G_2/M phase DNA damage checkpoint function and transcriptional induction of *RNR3* in FHA2 mutants. DLY554 cells *(MATa, bar1::hisg, mec2-1, cdc13-1, cdc15-2, ade2-1, trp1-1, can1-100, leu2-3,112, his3-11,15, ura3, GAL+, psi+, ssd1-d2,* provided by T. Weinert, University of Arizona) (*26*) transformed with pRS316 carrying alleles of *RAD53* under its own promoter were grown to log phase at 23°C and arrested with α factor (10^{-4} μg/μl for 4 hours at 23°C). α factor was then washed out, and the incubation temperature was shifted to 36°C. After 0 or 3 hours, samples were removed for Northern (RNA) blot analysis of *RNR3* and actin as described (*18*). Samples were also removed at the indicated times and stained with 4′,6′-diamidino-2-phenylindole and observed by fluorescence microscopy. Cells with large buds and two nuclei were scored as *cdc15* arrested cells in late nuclear division (*18*). (B) *RNR3* transcription. (C) Accumulation of *cdc15*-arrested cells. WT (■), wild type; Spk1-1 (▲), A208P (*11*); □, empty vector. FHA2 mutants tested: H/A (●), NVS (■), and Δ611–776 (△).

acids 549 to 730; Fig. 3A) contains FHA2. GST-Rad53 fusions encompassing amino acids 450 to 821 and 450 to 730 interacted with shifted Rad9 from yeast lysates (Fig. 3B). Thus, a portion of the Rad53 COOH-terminus is sufficient for interaction with the phosphorylated subpopulation of Rad9.

FHA domains are modular domains found in proteins with divergent functions, including protein kinases involved in the DNA damage response and meiosis (*15*). The tripeptide NXS/T is highly conserved among FHA proteins and corresponds to NVS655–657 in FHA2 of Rad53 (*16*). In addition, a histidine (His^{622} in Rad53 FHA2) is invariant. Both the NVS/AAA triple substitution and the H/A (*16*) single substitution prevented the two-hybrid interaction between Rad53 and Rad9 (Fig. 3A) and nearly eliminated interaction of the COOH-terminal GST-Rad53 with low-mobility Rad9-FLAG from cell extracts (Fig. 3B).

We also investigated the effects of FHA2 mutations on Rad53 phosphorylation, which is correlated with Rad53 function (*8*, *9*). Rad9 is required for regulation of Rad53 in the DNA damage checkpoint (*17*). In nonsynchronized cells, HU induced phosphorylation of wild-type and mutant Rad53 proteins (Fig. 4A). The DNA damage response was assayed in the presence of nocodazole to prevent Rad9-independent activation of Rad53 induced by DNA damage during S phase (*17*). HU only regulates Rad53 during S phase and did not induce Rad53 phosphorylation in nocodazole-arrested cells (Fig. 4A). MMS induced mobility shift of wild-type but not mutant Rad53 (Fig. 4A). Thus, FHA2 mutations selectively prevent modification of Rad53 in response to DNA damage but appear not to interfere with regulation of Rad53 in response to inhibition of replication.

We used a temperature-sensitive allele of *cdc13* to activate the DNA damage pathway to test whether FHA2 mutations prevent activation of downstream processes. These experiments were performed in *cdc15* background to prevent cell division (*18*). Cells harboring Rad53 with the H/A mutation, NVS mutations, or a deletion covering most of FHA2 (Δ611–776) showed diminished *cdc13*-induced *RNR3* transcription (Fig. 4B) and G_2/M phase checkpoint arrest (Fig. 4C). We had previously failed to detect ultraviolet (UV) sensitivity associated with the Δ611–766 allele (*11*), but the assay conditions were less stringent and measured different end points. Hence, FHA2 is required for Rad53 to activate these processes in response to DNA damage.

FHA domains have no known function. The FHA2 domain in Rad53 evidently functions as a specific modular protein-binding unit, the function of which is modified by phosphorylation. This modulation may be achieved by a structural alteration of Rad9 secondarily induced by phosphorylation or by direct recognition of specific phospho-peptides.

DNA damage induces Rad9 phosphorylation, which enables an interaction with the effector protein Rad53. The identity of the Rad9 kinase or kinases is not certain. Mec1 is a candidate Rad9 kinase, because damage-induced phosphorylation of Rad9 is partially reduced in *mec1* cells (*14*, *19*) and absent in *mec1 tel1* double mutants (*19*). Rad53 itself appears to integrate incoming signals, because Rad53 mutations discriminate between the HU and MMS pathways. Rad53 may be activated by replication inhibition through a second protein analogous to Rad9. This regulation does not require FHA2. Cds1, a *Schizosaccharomyces pombe* protein that has sequence similarity with Rad53, has only a single FHA domain similar to Rad53 FHA1 and is only involved in the replication checkpoint (*20*). The Rad53 FHA1 domain is required for resistance to HU and UV light (*11*) and is therefore a candidate site for interaction with a replication pathway analog of Rad9. The evolutionary conservation of Rad53 in budding and fission yeast suggests that they may have mammalian counterparts (*20*). Rad9 has a short domain of similarity with *BRCA1* and *BRCA2*, human tumor suppressor genes that may function in meiotic or damage checkpoint responses (*21*). This conservation of mammalian and yeast checkpoint proteins may extend to FHA-containing protein kinases.

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