

EDD Mediates DNA Damage-induced Activation of CHK2*

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Michelle J. Henderson^{‡1,2}, Marcia A. Munoz^{†1}, Darren N. Saunders[‡], Jennifer L. Clancy[‡], Amanda J. Russell[‡], Brandi Williams[§], Darryl Pappin[¶], Kum Kum Khanna^{||}, Stephen P. Jackson[§], Robert L. Sutherland^{‡3}, and Colin K. W. Watts[‡]

From the [†]Cancer Research Program, Garvan Institute of Medical Research, 384 Victoria St., Darlinghurst, New South Wales 2010, Australia, [§]Gurdon Institute and Department of Zoology, University of Cambridge, Cambridge, United Kingdom, ^{||}Queensland Institute of Medical Research, Herston, Queensland 4029, Australia, and [¶]Advanced Research and Technology Group, Applied Biosystems, Framingham, Massachusetts 01701

EDD, the human orthologue of *Drosophila melanogaster* “hyperplastic discs,” is overexpressed or mutated in a number of common human cancers. Although EDD has been implicated in DNA damage signaling, a definitive role has yet to be demonstrated. Here we report a novel interaction between EDD and the DNA damage checkpoint kinase CHK2. EDD and CHK2 associate through a phospho-dependent interaction involving the CHK2 Forkhead-associated domain and a region of EDD spanning a number of putative Forkhead-associated domain-binding threonines. Using RNA interference, we demonstrate a critical role for EDD upstream of CHK2 in the DNA damage signaling pathway. EDD is necessary for the efficient activating phosphorylation of CHK2 in response to DNA damage following exposure to ionizing radiation or the radiomimetic, phleomycin. Cells depleted of EDD display impaired CHK2 kinase activity and an inability to respond to DNA damage. These results identify EDD as a novel mediator in DNA damage signal transduction via CHK2 and emphasize the potential importance of EDD in cancer.

EDD is the mammalian orthologue of the *Drosophila melanogaster* “hyperplastic discs” gene (*hyd*) (1) and encodes a large, predominantly nuclear protein with a number of putative functional domains (1, 2). The tumor suppressor properties of *hyd* and several studies on human cancer suggest the potential involvement of EDD in cancer. The EDD locus at 8q22.3 is frequently mutated in microsatellite-unstable gastric and colorectal cancers (3), and reduced expression of EDD was observed in invasive breast carcinomas when compared with normal breast or early stage disease (4). In another study, we identified

the EDD locus as a specific area of frequent amplification in breast, ovarian, hepatocellular and tongue carcinoma and melanoma. EDD protein was also overexpressed in a high proportion of breast and ovarian cancers (5).

The presence of ubiquitin-associated, UBR box, and homologous to E6-AP carboxyl terminus (HECT)⁴ domains strongly support a role for EDD as an E3 ubiquitin ligase (6–8). Indeed, a number of EDD-associating proteins have recently been described that interact via the HECT domain. One such protein, CIB1/KIP (calcium- and integrin-binding protein) was identified as a binding partner in our laboratory (2) and interacts with a number of DNA damage response proteins, including the catalytic subunit of the DNA-dependent protein kinase (9) and the polo-like kinases PLK-1 and PLK-3 (10). Another report identified TopBP1 (DNA topoisomerase IIβ-binding protein 1) as an EDD HECT domain-interacting protein, linking modulation of TopBP1 ubiquitination to the DNA damage response (11).

The cellular response to DNA damage is crucial for the maintenance of genomic and cellular integrity, and consequently breaches in this pathway often contribute to tumorigenesis. Cancer-associated deleterious aberrations often involve key early DNA damage signaling components, such as ATM (ataxia telangiectasia-mutated) and NBS1 or molecules critical for downstream transduction and repair, such as BRCA1/2, p53, and CHK2 (12). More recently, reports of tumor-associated up-regulation of DNA damage signaling have begun to emerge that might help explain the prevalence of mutation of downstream effectors in diverse human cancers (13–15).

Many studies have underscored the importance of CHK2 in transduction of DNA damage signaling in mammalian cells. In response to DNA damage, CHK2 phosphorylates numerous substrates, leading to arrest in G₁, S, and G₂/M phases of the cell cycle, activation of DNA repair, and in some cases apoptosis (16–23). CHK2-deficient mouse embryonic stem cells fail to maintain arrest in G₂ phase after DNA damage induced by ionizing radiation (IR) (24). Germ line and somatic mutations of CHK2 have been identified in hereditary and sporadic cancers in humans as well as in cases of Li-Fraumeni syndrome, a disorder characterized by the high incidence of a wide range of cancers (25–30).

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[†] These authors contributed equally to this work.

[‡] To whom correspondence may be addressed: Experimental Therapeutics Program, Children’s Cancer Institute Australia, P.O. Box 81 (High St.), Randwick, NSW 2031, Australia. Tel.: 61-2-9382-8556; Fax: 61-2-9382-1850; E-mail: mhenderson@ccia.unsw.edu.au.

[¶] To whom correspondence may be addressed. Tel.: 61-2-9295-8322; Fax: 61-2-9295-8321; E-mail: r.sutherland@garvan.org.au.

^{||} The abbreviations used are: HECT, homologous to E6-AP carboxyl terminus; E3, ubiquitin-protein isopeptide ligase; IR, ionizing radiation; FHA, Forkhead-associated; siRNA, small interfering RNA; aa, amino acids; GST, glutathione S-transferase; GFP, green fluorescent protein; Gy, Grays; PBS, phosphate-buffered saline; DSB, double-stranded DNA break(s).

The human CHK2 protein consists of three well defined domains: the (S/T)Q-rich region, the Forkhead-associated (FHA) domain, and the kinase domain. The (S/T)Q motif at the amino terminus of the protein is a regulatory domain containing multiple sites for ATM/ATR (ATM- and Rad3-related) phosphorylation (16). The FHA domain functions as a phosphopeptide-binding module, with a preference for phosphothreonine (31, 32). Li-Fraumeni syndrome-associated CHK2 missense mutations R145W and I157T lie within the FHA domain (33), as do R117G and K131N mutations found in familial breast and ovarian cancer, respectively. The kinase domain of CHK2 occupies much of the carboxyl-terminal half of the protein and is often the site of cancer-associated CHK2 mutations (25, 34).

In this report, we identify the checkpoint kinase CHK2 as an interacting partner for EDD and demonstrate a role for EDD in CHK2 activation. These findings highlight EDD as an important and novel mediator of the DNA damage signaling pathway in response to DNA double-stranded breaks.

EXPERIMENTAL PROCEDURES

Plasmids and siRNA Sequences—GST fusion proteins were expressed from pGEX plasmids in *Escherichia coli* BL21 Codon Plus strain (Stratagene, La Jolla, CA). GST-CHK2(N) comprised amino acids (aa) 2–225 of CHK2, and GST-Cdc25C comprised aa 200–256 of Cdc25C. Plasmid constructs for *in vitro* translation of EDD fragments 1–889, 889–1877, and 889–2799 have been previously described (2). Other EDD *in vitro* translation constructs were subcloned from EDD-(889–2799). The sequence of the siRNA oligoribonucleotides (synthesized by Ambion Inc., Austin, TX) used for the silencing of EDD expression was 5'-GCA GUG UUC CUG CCU UCUD-TdT-3'. siRNA directed against GFP, 5'-CUG GAG UUG UCC CAA UUC UdTdT-3', was used as a negative control.

Antibodies and Peptides—The antibodies used for immunoblotting were against GST (Santa Cruz Biotechnology, Inc., Santa Cruz, CA), CHK2 (N17; Santa Cruz Biotechnology), p53 (DAKO Australia), ATM (2C1; GeneTex), EDD (1), Rb (BD Biosciences Pharmingen), phospho-ATM (Ser¹⁹⁸¹) (Rockland Immunochemicals), phospho-BRCA1 (Ser¹⁵²⁴) (35), phospho-NBS1 (Ser³⁴³) (Cell Signaling Technology), phospho-CHK2 (Thr⁶⁸) (Santa Cruz Biotechnology), and phospho-p53 (Ser¹⁵) (New England Biolabs). TopBP1 antibody (anti-TopBP1.2) was a gift from Juhani Syvaoja (36). Peptides were synthesized by Mimotopes (Victoria, Australia).

Assay for CHK2 Binding Partners—Sepharose-bound GST-CHK2(N) fusion protein was incubated with HeLa cell nuclear extracts. Following thorough washing in 20 mM HEPES buffer (pH 7.4) containing 0.2 mM EDTA, 150 mM NaCl, 1 mM dithiothreitol, 1% Nonidet P-40, 1 mM sodium orthovanadate, 1 μM microcystin-LR, and EDTA-free protease inhibitor mixture, bound proteins were separated by SDS-PAGE and stained with silver. Proteins were identified by mass spectrometry as previously described (37).

Protein Interactions in Cell Lysates—For GST fusion protein pull-down of EDD from cell extracts, 0.5 mg of nuclear protein was incubated with 5 μg of GST or GST-CHK2(N) bound to glutathione beads for 1–2 h at 4 °C. Beads were washed exten-

sively in lysis buffer, and bound proteins were resolved by SDS-PAGE and transferred to polyvinylidene difluoride membrane for immunoblotting. For immunoprecipitation, 0.5–1 mg of nuclear or total cellular protein was incubated with 4 μg of N17 CHK2 antibody or goat IgG at 4 °C for 2 h, and antibody conjugates were captured on protein G-Sepharose beads (Zymed Laboratories Inc.).

Recombinant Protein Binding Assays—EDD protein fragments were synthesized with Promega T3 TNT coupled reticulocyte lysate or T7 TNT Quick Coupled Transcription/Translation systems using ³⁵S-labeled cysteine/methionine (PerkinElmer Life Sciences). Synthesized protein (15 μl) was diluted to 100 μl with lysis buffer and incubated with 5 μg of GST or GST-CHK2(N) at 4 °C for 2 h. Beads were collected by centrifugation and washed twice with lysis buffer and twice with radioimmune precipitation buffer, and bound proteins were visualized following SDS-PAGE and autoradiography. For peptide inhibition assays, peptide was incubated with the reaction mixture on ice for 5 min prior to the addition of *in vitro* translated protein. *In vitro* translated EDD or cell extracts were dephosphorylated with 15 units/μl λ protein phosphatase (New England Biolabs), and phosphatase was inactivated by the addition of 50 mM EDTA prior to the addition to other assay components.

Cell Culture, Transfection, and DNA Damage—HEK293 and MCF-7 lines were maintained as previously described (1, 2). HeLa cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum (Invitrogen). For siRNA transfection, cells were transfected with 2.8 pmol cm⁻² of siRNA oligoribonucleotides using Oligofectamine (Invitrogen). For siRNA complementation experiments, cells were transfected with 80 ng cm⁻² cDNA using Genejuice (Novagen) 8 h after siRNA transfection. RNA interference-immune EDD cDNA mutant (EDD^{R1}) was generated by site-directed mutagenesis using the following primer sequences: EDD^{MUTF}, 5'-GTTCAAGCAGGAGCAAGCTCAGTACCAAGCTT-CTTTCTGAAGATGATTCTC-3'; EDD^{MUTR}, 5'-GAGAACATCTTCAGAAAAGAAAGCTGGTACTGAGCTTGC-TCTGCTTCTGAAC-3'.

IR was delivered at a rate of 2 Gy min⁻¹ by a linear accelerator. HeLa and MCF-7 cells were treated with 65 μM phleomycin (InvivoGen) in serum-free medium for 1–4 h. Cells were harvested immediately, or medium was replaced as indicated. Total cellular protein was extracted in 1% Triton X-100 lysis buffer as described (1). Extraction of nuclear proteins in the presence of EDTA-free protease inhibitor mixture (Roche Applied Science), 0.5 mM sodium orthovanadate, and 20 mM sodium fluoride was carried out according to published methods (38) without dialysis. RNA extraction and Northern blot analysis were carried out as described (5).

In Vivo [³²P]Phosphate Labeling of EDD—*In vivo* labeling of COS7 cells expressing FLAG-tagged EDD, followed by phosphoamino acid analysis, was carried out as previously described (39).

CHK2 Kinase Assay—CHK2 was immunoprecipitated from 0.5–1 mg of MCF-7 protein extract using 1 μg of N17 CHK2 antibody (or 1 μg of goat IgG (Zymed Laboratories Inc.) for control). Antibody conjugates on protein G-Sepharose beads (Zymed Laboratories Inc.) were washed four times with radio-

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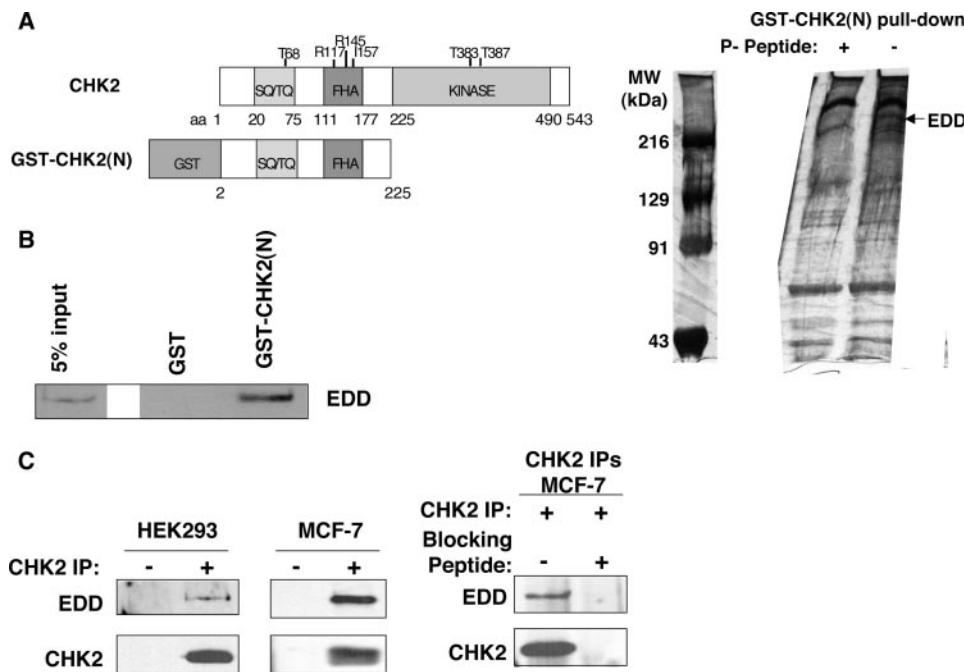


FIGURE 1. Physical association between EDD and CHK2. *A*, identification of EDD as a CHK2 binding partner. Schematic diagrams of CHK2 and the fragment of CHK2 used in binding assays (GST-CHK2(N)) are shown. The (S/T)Q-rich domain, the FHA domain, and the kinase domain are indicated along with key residues important for CHK2 function. Amino acid numbers defining domain boundaries are indicated below. *Right*, GST-CHK2(N) bound to glutathione-Sepharose beads was incubated with HeLa cell nuclear extract in the presence or absence of FHA-binding phosphopeptide, RWFDpTYLIRR. After washing, bound proteins were separated on 5–15% gradient gels and visualized by silver staining. Protein bands of interest were sequenced, and one of these was identified as EDD (arrow). *B*, EDD interacts with CHK2(N) in nuclear extracts. Nuclear extracts from MCF-7 cells were incubated with either GST or GST-CHK2(N) fusion protein bound to glutathione-Sepharose beads, and bound proteins were immunoblotted for EDD. *C*, EDD and CHK2 associate constitutively *in vivo*. Cell lysates from HEK293 or MCF-7 cells were incubated with CHK2 antibody (+) or nonspecific goat IgG (−). Following precipitation of immune complexes, bound proteins were immunoblotted for EDD. *Right*, immunoprecipitation following preincubation of CHK2 antibody with a 10-fold molar excess of antigenic peptide (sc-8812P).

immune precipitation buffer (0.5% (w/v) sodium deoxycholate, 150 mM NaCl, 1% (v/v) Nonidet P-40, 50 mM Tris-HCl, pH 8.0, 0.1% (w/v) SDS, 10% (v/v) glycerol, 5 mM EDTA) and twice with kinase buffer (50 mM Hepes, 50 mM β -glycerophosphate, 10 mM MgCl₂, 1 mM dithiothreitol). Immunoprecipitates were incubated in kinase buffer with 100 μ M ATP, 10 μ Ci [γ -³²P]ATP, and 4 μ g of GST-Cdc25C substrate for 5 min at 30 °C. Reactions were stopped by the addition of 30 μ l of Laemmli sample buffer and boiled for 5 min at 95 °C. Following SDS-PAGE, samples were transferred to polyvinylidene difluoride membrane (Millipore, New South Wales, Australia) and subjected to autoradiography, followed by immunoblotting for phospho-CHK2 and total CHK2.

Cell Viability Assay, G₂/M Checkpoint Analysis, and Microscopy—For the colony-forming assay, at 72 h post-transfection, cells were treated with 13 μ M (HeLa) or 32.5 μ M (MCF7) phleomycin for 1 h. Following replacement of medium, colonies were allowed to grow for 10 days (HeLa) or 14 days (MCF7). Following fixing and staining with the Diff Quik Stain kit (Lab Aids), colonies were quantitated with Quantity One software (Bio-Rad).

For the mitotic entry assay, cells were treated with phleomycin at 72 h post-transfection and harvested 24 h later. Cells were harvested by trypsinization, washed in PBS, and fixed in cold 80% ethanol for at least 1 h at –20 °C. Fixed cells were permeabilized in PBS, 0.2% Triton X-100 for 15 min at room temper-

ature. Following further centrifugation, cells were incubated with anti-phosphohistone-3 antibody (rabbit polyclonal antibody, catalog number 06-570; Upstate Biotechnology, Inc.) for 2 h at room temperature. Cells were washed in PBS, 1% bovine serum albumin and incubated with secondary antibody linked to Cy2 (Jackson Immunoresearch Laboratories) for 1 h in the dark; washed again in PBS, 1% bovine serum albumin; simultaneously treated with RNase A (0.5 mg ml^{–1}); and stained with propidium iodide (10 μ g ml^{–1} in PBS, 1% bovine serum albumin) for 45 min at 37 °C. The labeled cells were analyzed by fluorescence-activated cell sorting using a Becton Dickinson FACScalibur.

Transfected HeLa cells were seeded on chamber slides ~24 h before phleomycin exposure at a cell density of 2.4 \times 10⁴ cells cm^{–2}. Cells were treated with 65 μ M phleomycin for 1 h; fixed in 4% paraformaldehyde, PBS at various times after treatment; and permeabilized in 0.2% Triton X-100, PBS. By staining nuclei with 4',6-diamidino-2-phenylindole and counterstaining the cytoplasm for actin with phalloidin, it was possible to visualize a subpopulation of enlarged cells containing between two and four aberrant nuclei.

RESULTS

EDD Is a Novel Binding Partner of CHK2—CHK2 has three well defined domains: the (S/T)Q-rich regulatory domain containing multiple sites for ATM/ATR phosphorylation, the FHA phosphopeptide-binding domain, and the kinase domain (Fig. 1A) (16, 31, 32). In experiments aimed at identifying CHK2 binding partners, GST-CHK2(N) (aa 2–225) (Fig. 1A), which contains the (S/T)Q-rich and FHA domains of CHK2 fused to GST, was incubated with HeLa cell nuclear extract in the presence or absence of an optimal CHK2 FHA domain-binding phosphopeptide, RWFDpTYLIRR (32). Several co-purifying proteins bound only in the absence of competing peptide, and one of these was identified by mass spectrometry as EDD (Fig. 1A). This interaction was confirmed by GST-CHK2(N) pull-downs from MCF-7 lysates, followed by immunoblotting to detect EDD protein (Fig. 1B). Immunoprecipitation of endogenous CHK2 from whole cell extracts prepared from either HEK293 or MCF-7 cells followed by immunoblotting for EDD demonstrated an *in vivo* association between EDD and CHK2 (Fig. 1C). No association was seen when CHK2 immunoprecipitation was inhibited by preincubation of CHK2 antibody with the antigenic peptide. We were unable to detect CHK2 protein

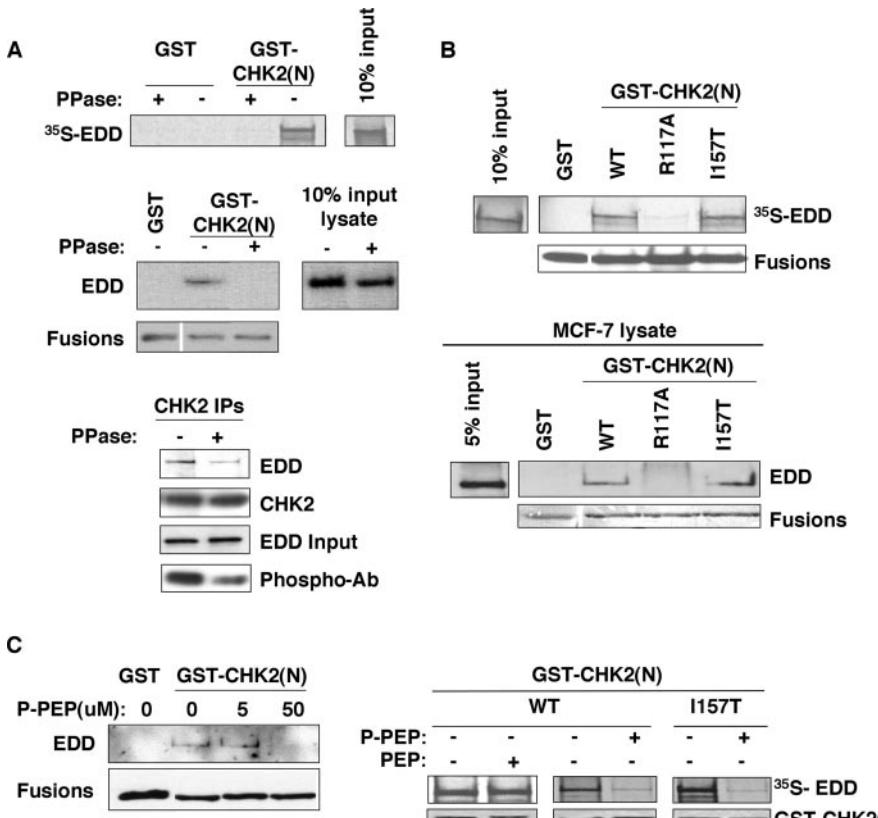


FIGURE 2. The phosphopeptide-binding interface of the CHK2 FHA domain is required for EDD binding. *A*, dephosphorylation of EDD inhibits CHK2 binding. *In vitro* synthesized ³⁵S-labeled EDD (top) or HeLa whole cell extracts (middle) were preincubated with λ protein phosphatase (PPase +) and then incubated with Sepharose-bound GST-CHK2(N). Bottom, MCF-7 cell extracts were incubated with protein phosphatase prior to CHK2 immunoprecipitation. A phospho-specific antibody (Phospho-Ab) was used to confirm dephosphorylation of extract. Bound EDD was detected by autoradiography (top) or immunoblotting. *B*, CHK2 FHA domain binds EDD. *In vitro* synthesized ³⁵S-labeled EDD (top) or MCF-7 whole cell extracts (bottom) were incubated with Sepharose-bound GST-CHK2(N) derivatives containing R117A or I157T substitutions. Bound EDD was detected by autoradiography (top) or immunoblotting (bottom), and fusion proteins were detected by Coomassie staining. *C*, an FHA-binding phosphopeptide inhibits the EDD-CHK2 interaction. *Left*, MCF-7 protein extracts were incubated with Sepharose-bound GST-CHK2(N) in the presence of FHA-binding phosphopeptide at the indicated concentrations, and bound EDD was detected by immunoblotting. *Right*, *in vitro* synthesized ³⁵S-labeled EDD was incubated with Sepharose-bound GST-CHK2(N) (WT) or GST-CHK2(N) (I157T) in the presence of FHA-binding phosphopeptide at 50 μ M or with non-phosphorylated peptide. Bound EDD was detected by autoradiography.

in EDD immunoprecipitates. This may be due to the location of the peptide epitope recognized by the EDD antibody, preventing antibody access when CHK2 is bound to EDD, since antibody and CHK2 require nearby regions of EDD for binding (see below). Thus, under normal physiological conditions, CHK2 and EDD appear to occur in the same protein complex, most likely through a direct interaction with the NH₂-terminal region of CHK2.

EDD Interacts with the FHA Domain of CHK2—The region of CHK2 that binds EDD spans both the (S/T)Q and FHA domains. Inhibition of binding by a CHK2 FHA-binding phosphopeptide (Fig. 1A) supports an FHA-mediated interaction. The CHK2 FHA domain has a preference for binding phosphothreonine (32), and EDD is known to be constitutively phosphorylated on both serine and threonine (40).⁵ If the EDD-CHK2 interaction is mediated through the FHA domain, prior

dephosphorylation of EDD should also inhibit association between EDD and CHK2. Indeed, λ protein phosphatase treatment of either *in vitro* translated EDD or whole cell extracts prior to incubation with GST-CHK2(N) or immunoprecipitation of CHK2, respectively, greatly impaired binding (Fig. 2A).

Mutations in the CHK2 FHA domain have been detected in a number of cancers, and several of these perturb binding between CHK2 and its interacting partners (25, 32–34). To assess the requirement for an intact FHA domain in the EDD-CHK2 interaction, GST-CHK2(N) fusion constructs containing specific single amino acid substitutions were tested for binding to *in vitro* translated EDD. The R117A mutant involves a residue within the FHA domain that is directly required for phosphothreonine binding (32), whereas the I157T Li-Fraumeni-associated mutant retains the ability to bind phosphothreonine but is unable to bind substrates of CHK2, such as p53, Cdc25A, BRCA1, and Cdc25C (20, 41, 42). The R117A mutation prevented efficient EDD binding (Fig. 2B). In contrast, the I157T substitution had no pronounced effect on EDD binding (Fig. 2B). Similar results were obtained when GST-CHK2(N) pull-down experiments were performed using MCF-7 whole cell extracts

(Fig. 2B, lower panel). These results support binding of the CHK2 FHA domain to a phosphorylated threonine residue in EDD.

Competition between EDD and the FHA-binding phosphopeptide was used to examine the interaction further. Preincubation of the GST-CHK2(N) fusion protein with the phosphopeptide at a concentration of 50 μ M inhibited binding between endogenous or *in vitro* translated EDD and GST-CHK2(N) (Fig. 2C). Similar competition was observed previously for the interaction between BRCA1 and CHK2 (32). Phosphopeptide also inhibited binding to the GST-CHK2(N) I157T mutant, suggesting that this mutant binds EDD in a similar fashion to wild type CHK2. These data further support an interaction between EDD and CHK2 that involves the phosphopeptide binding interface of the FHA domain and most likely requires phosphorylation of EDD.

A Large Region of EDD Is Required for CHK2 Binding—A consensus CHK2 FHA-binding sequence has been derived as pTXX(I/L/V), with a preference for aspartic acid at the -1-po-

⁵ M. J. Henderson, M. A. Munoz, D. N. Saunders, J. L. Clancy, A. J. Russell, B. Williams, D. Pappin, K. K. Khanna, S. P. Jackson, R. L. Sutherland, and C. K. W. Watts, unpublished results.

Role of EDD in CHK2 Activation

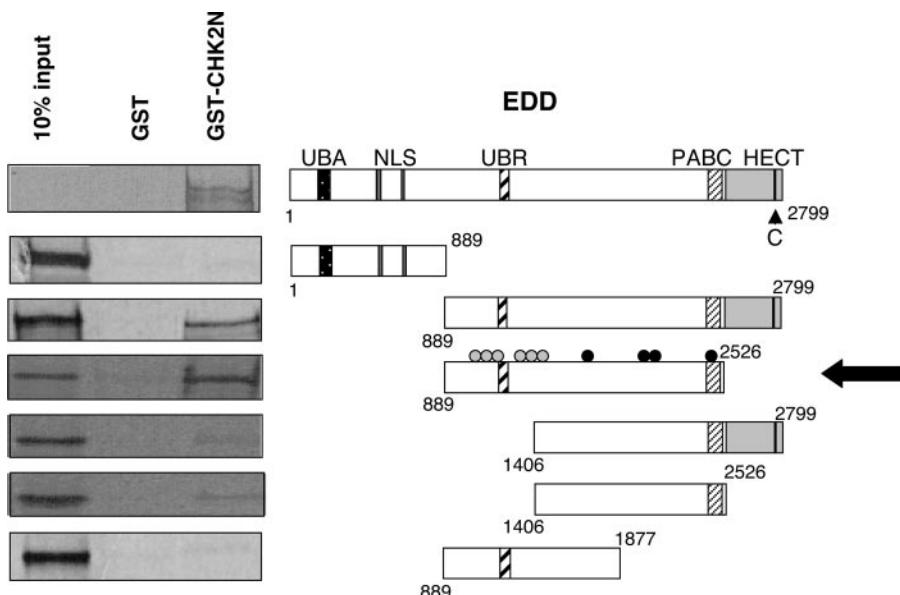


FIGURE 3. Mapping the region of EDD that interacts with CHK2. Schematic diagrams of EDD and fragments used for mapping showing major domains, including ubiquitin-associated domain (UBA), nuclear localization sequences (NLS), a HECT domain with ubiquitin-binding cysteine residue (C), a UBR box, and a poly(A)-binding protein carboxyl terminal motif homology domain (PABC). The numbers indicate amino acid positions of fragment boundaries. The circles indicate positions of potential FHA-binding threonines within the minimal binding fragment of EDD (large arrow; see "Experimental Procedures"). *In vitro* translated ^{35}S -labeled EDD fragments were incubated with Sepharose-bound GST-CHK2(N), and bound EDD was detected by autoradiography.

(1406–2799)), but, significantly, this domain alone was not sufficient for CHK2 interaction (EDD-(889–1877)). Thus, the minimal region of EDD able to bind CHK2 comprised amino acids 889–2526 and included the UBR box, the PABC homology region, and 10 consensus FHA-binding sequences (Fig. 3, *circles*). One of these sites has the preferred aspartic acid at –1. However, this and three other potential phosphorylated threonines in EDD (Fig. 3, *solid circles*) were mutated and had no effect on EDD-CHK2 association, even when all four residues were simultaneously mutated to allow for the possibility of more than one site being involved in binding.⁵ These findings suggest that if EDD-CHK2 interaction is direct, the FHA binding site may lie within the central region of EDD.

DNA Damage Induces Nuclear Accumulation of EDD—Agents that induce double-stranded DNA

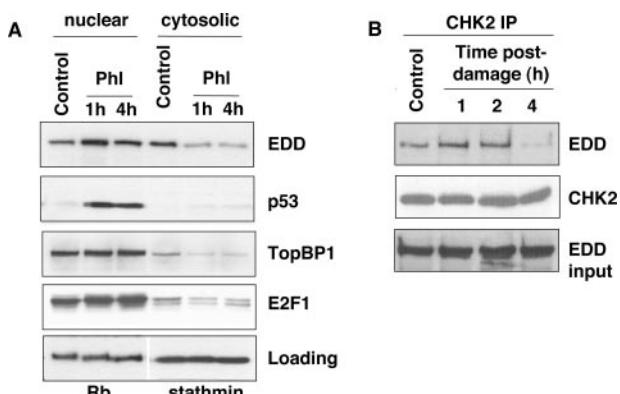


FIGURE 4. Modulation of EDD in response to radiomimetic-induced DNA damage. *A*, nuclear accumulation of EDD protein following exposure to phleomycin (Phl). Nuclear and cytosolic extracts were prepared from MCF-7 cells following 1- or 4-h exposure to phleomycin and analyzed by immunoblotting as indicated. pRb and stathmin were loading controls for nuclear and cytosolic fractions, respectively. *B*, dissociation of EDD and CHK2 following DNA damage. Nuclear extracts from MCF-7 cells exposed to phleomycin for 1 h and either harvested immediately (1 h) or at various times thereafter (2 or 4 h) or from mock-treated MCF-7 cells (control) were immunoprecipitated for CHK2. Bound proteins and 5% input protein were immunoblotted for EDD.

sition (32). The EDD protein contains 15 threonine residues that fit this consensus for FHA binding when phosphorylated. To examine which region of EDD is important for the EDD-CHK2 interaction, various ^{35}S -labeled *in vitro* translated EDD subfragments were tested for binding to GST-CHK2(N) (Fig. 3). Removal of the NH₂-terminal third of EDD did not prevent binding (EDD-(889–2799)), and further deletion of the HECT domain slightly enhanced the interaction (EDD-(889–2526)). Therefore, it is unlikely that EDD and CHK2 associate through threonine residues within the NH₂-terminal or HECT regions of EDD. Removal of the UBR box (7) abolished binding (EDD-

breaks (DSB), such as IR or radiomimetic drugs, disrupt the constitutive interaction between EDD and CIB (2), whereas TopBP1 is phosphorylated after DNA damage and is a probable ubiquitination target of EDD (11, 43). Together with the newly identified interaction with CHK2 (described above), these observations suggest that EDD may be involved in execution of the cellular response to DSB. To examine this possibility, MCF-7 human breast cancer cells were exposed to phleomycin and harvested for nuclear and cytoplasmic proteins 1–4 h after drug exposure. As expected, a pronounced stabilization of nuclear p53 was observed following phleomycin exposure (Fig. 4A). Levels of EDD in nuclear extracts were enhanced several-fold in response to DNA damage, and this was accompanied by depletion of EDD from the cytosolic fraction (1 h postdamage; Fig. 4A). As seen in Fig. 4A and other studies (44–46), this response is reminiscent of other DNA damage response proteins, such as p53, TopBP1, and E2F1, supporting a role for EDD in the DNA damage response.

To investigate whether the observed constitutive EDD-CHK2 interaction is modulated during the course of the DNA damage response, CHK2 was immunoprecipitated in parallel from nuclear extracts of MCF-7 cells that had been treated with phleomycin or vehicle. Although the constitutive interaction between EDD and CHK2 was initially maintained upon DNA damage, the two proteins dissociated at later times (between 2 and 4 h) after radiomimetic exposure (Fig. 4B). These changes in the EDD-CHK2 association were independent of fluctuating EDD or CHK2 levels, since input EDD levels were equalized in these experiments, and the amounts of immunoprecipitated CHK2 did not vary significantly. Using *in vivo* labeling and phosphopeptide mapping, we detected no change in the proportion of phosphothreonine in EDD in the presence and

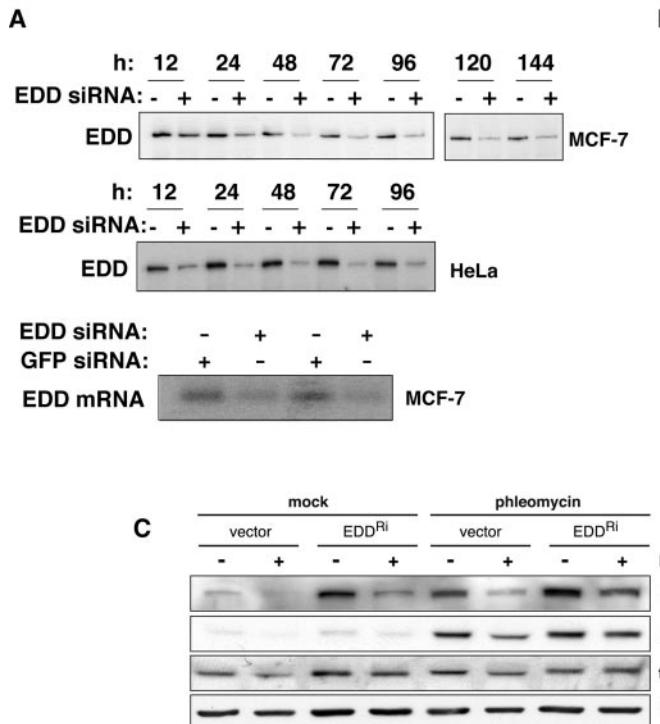


FIGURE 5. EDD is required for efficient CHK2 activation after DNA damage. *A*, a depletion of EDD protein and mRNA following siRNA transfection. *Top panels*, immunoblotting of MCF-7 or HeLa whole cell extracts prepared at 12 hourly intervals following transfection of siRNA against EDD or control siRNA (GFP). *Bottom*, Northern blot analysis of EDD mRNA transcript in duplicate experiments at 48 h post-transfection. *B*, effect of EDD depletion on the ATM-mediated response to IR. Following siRNA transfection for 96 h, MCF-7 cells were exposed to 12 Gy of IR and harvested 90 min later. Protein extracts were analyzed by immunoblotting for EDD, activated ATM (P-S1981), and phosphorylated ATM substrates. *C*, effect of EDD depletion on radiomimetic-induced CHK2 activation. 8 h following siRNA transfection, HeLa cells were transfected with EDD RNA interference-immune mutant cDNA (EDD^{Ri}) or vector control. 72 h post-RNA interference transfection, cells were exposed to phleomycin for 1 h and then harvested on ice. Protein extracts were analyzed for levels of EDD, P-T68 CHK2, total CHK2, and β -actin (*b*-actin).

absence of DNA damage (data not shown). Other experiments using exogenously expressed subfragments of EDD or immunoblotting of EDD immunoprecipitates in the presence or absence of phleomycin also revealed no change in phosphorylation status (data not shown). Nevertheless, it is still possible that a subtle change in the phosphorylation status of EDD leads to dissociation from CHK2 by direct or indirect means.

EDD Is a Mediator of CHK2 Activation—The observed association between EDD and CHK2 raised the possibility of a role for EDD in activation of CHK2. To examine this, RNA interference was used to down-regulate EDD in MCF-7 or HeLa cells, which were then exposed to IR or phleomycin. Fig. 5A (*top*) illustrates that EDD levels had declined to <50% of control levels by 24 h post-transfection in MCF-7 cells or by 12 h in HeLa cells. EDD protein levels continued to decline until 72 h (<20% of controls) in both MCF-7 and HeLa cells and remained at low levels for 6 days post-transfection (Fig. 5A, *top*). Decreased EDD protein was accompanied by a 4–5-fold decrease in EDD mRNA (Fig. 5A, *bottom*). Transfections utilized the lowest possible concentration of siRNA required to give adequate protein depletion, and microarray transcript profiling experiments confirmed no induction of interferon γ responses (data not shown).

In response to DNA damage, CHK2 is phosphorylated by ATM or ATR within the (S/T)Q-rich domain (47). In partic-

ular, ionizing radiation-induced DNA double-stranded breaks lead to ATM-dependent phosphorylation of CHK2 primarily on threonine 68 (48–51). This event is a prerequisite for the autophosphorylation of the CHK2 activation loop, leading to full kinase activity (33). Notably, DNA damage-induced phosphorylation of CHK2 on Thr⁶⁸ was significantly impaired in EDD-depleted cells compared with the strong induction seen in control (GFP siRNA) cells (Fig. 5B). Importantly, complementation with an EDD cDNA that is immune to EDD siRNA (EDD^{Ri}) restored both EDD expression and CHK2 activation to similar levels observed in non-siRNA-treated cells, despite the presence of EDD siRNA (Fig. 5C, compare lanes 5 and 8). These data demonstrate a specific requirement for EDD in phosphorylation of CHK2 Thr⁶⁸ and effectively exclude the possibility of off target effects of the EDD siRNA.

We also investigated the involvement of EDD in other components of the ATM-mediated response. Upon DNA damage, ATM undergoes autophosphorylation at serine 1981 (52). Activated ATM in turn

phosphorylates and activates a number of effector proteins including CHK2, BRCA1, NBS1, and p53 (53). ATM-dependent phosphorylation of the EDD-interacting protein, TopBP1, has also been reported (43). No difference in induction of ATM activation or phosphorylation of the ATM targets BRCA1(Ser¹⁵²⁴), NBS1(Ser³⁴³), or p53(Ser¹⁵) was observed between control (GFP siRNA) and EDD-depleted cells following exposure to 12 Gy of IR (Fig. 5B). Similarly, hyperphosphorylation of TopBP1 following IR treatment was not affected by decreased levels of EDD (Fig. 5B).

ATM-dependent phosphorylation of CHK2 on Thr⁶⁸ is a prerequisite for the autophosphorylation of the activation loop, leading to full kinase activity (33). To assess the requirement for EDD in induction of CHK2 kinase activity following DNA damage, *in vitro* kinase assays were performed. Impairment of Thr⁶⁸ phosphorylation in cells with low levels of EDD is evident as early as 15 min after DNA damage. In control (GFP siRNA) cells, we observed optimal induction of CHK2 kinase activity when cells were harvested 15–30 min after exposure to 4 Gy of IR (Fig. 6). Significantly, a 40–50% reduction in CHK2 kinase activity at all time points was observed in EDD-depleted cells when compared with control (GFP siRNA) cells, with the greatest deficit seen at 15–30 min post-IR (Fig. 6). As a further indicator of reduced CHK2 kinase activity, CHK2 autophosphorylation after DNA damage was considerably lower in cells

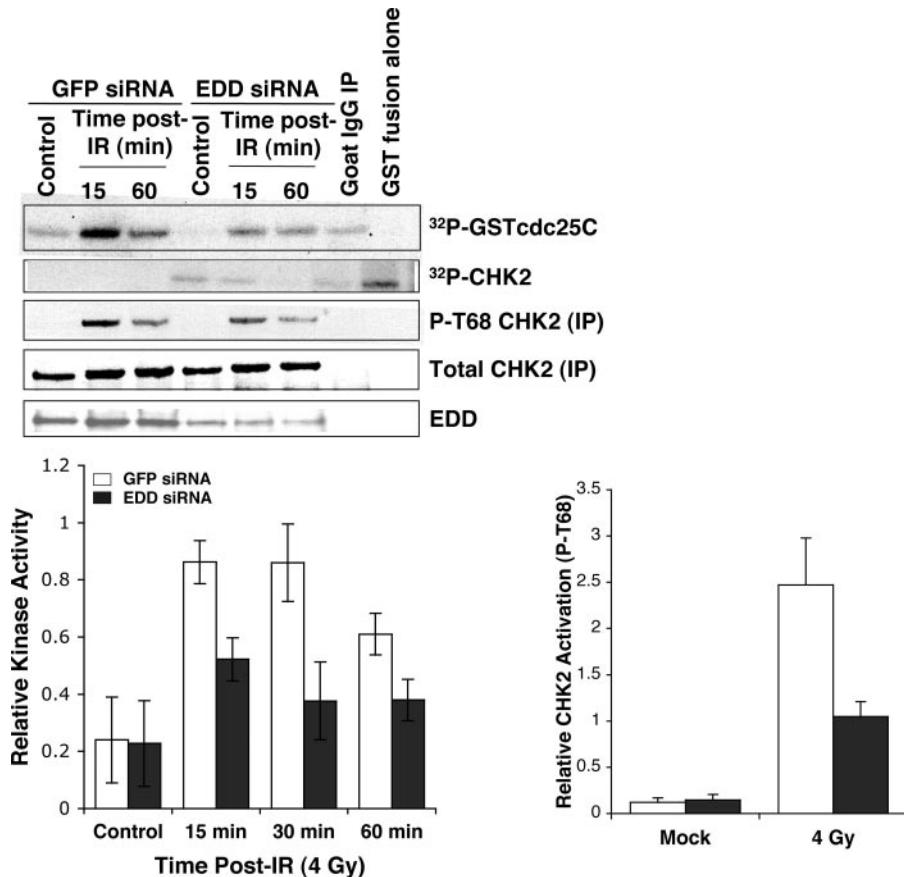


FIGURE 6. EDD is required for full CHK2 kinase activity after DNA damage. Effect of EDD depletion on IR-induced CHK2 kinase activity. *Top*, kinase assay of CHK2 immunoprecipitated from irradiated cell lysates using GST-Cdc25C as a substrate. MCF-7 cells were harvested at the indicated times after 4 Gy of IR exposure. Incorporation of ^{32}P into GST-Cdc25C substrate or CHK2 was detected by autoradiography. Levels of immunoprecipitated CHK2 and phospho-CHK2 (P-T68) were monitored by immunoblotting. *Lower panels*, kinase activity data are expressed as the means \pm S.E. relative to the maximal kinase activity seen for GFP siRNA cells in each experiment. Where necessary, kinase activity was corrected for total CHK2 protein. Impaired kinase activity was seen in EDD-depleted cells at 15, 30, and 60 min after IR ($p < 0.05, n = 4$). Quantitation of Thr⁶⁸ phosphorylation 15 min post-IR in these experiments is also presented ($p = 0.02$).

depleted of EDD than in control (GFP siRNA) cells. The reduction in CHK2 kinase activity is consistent with the ~60% reduction in the activating CHK2 Thr⁶⁸ phosphorylation observed in these experiments (Fig. 6). Together, these data provide compelling evidence that EDD is necessary for efficient Thr⁶⁸ phosphorylation and full kinase activation of CHK2 after IR-induced DNA damage and that this effect is critical in the early stages of the DNA damage response. Furthermore, the effect is specific to CHK2, since ATM and other substrates are unaffected.

Depletion of EDD Enhances Radiosensitivity and Impairs G₂/M Checkpoint Function—CHK2 is required for several pathways leading to an appropriate cellular response to DSB that in turn enables DNA repair and cell survival. Hypersensitivity to IR exposure, or radiosensitivity, is a common feature of cells with impairment of DNA damage signaling (53–55). To examine further the role of EDD in the response to DNA damage, cell survival after DNA damage induction was compared in control and EDD-depleted cells. EDD-depleted cells (72 h post-transfection) were treated with phleomycin, and cell viability was measured by a colony-forming assay (Fig. 7A). In line with previous observations of cells deficient in other DNA damage response proteins (56, 57), EDD-depleted cells showed significantly reduced viability after DNA damage.

These data demonstrate that EDD is required for an adequate cellular response to radiomimetic-induced DSB. The inability of EDD-depleted cells to efficiently activate CHK2 most likely results in compromised S phase and G₂/M checkpoints and apoptosis. Cells then enter mitosis carrying DNA damage. This is consistent with recent work showing that failure to activate CHK2 sensitizes cells to DNA damage-induced mitotic catastrophe and cell death as a final measure to prevent the survival of cells with major genomic aberrations (58). Indeed, as seen in CHK2-depleted cells (24), depletion of EDD impaired the ability of cells to maintain arrest in G₂ phase after DNA damage. Although there was a slight increase in the proportion of cells entering mitosis prior to the addition of phleomycin, EDD-depleted cells were able to initiate an efficient G₂/M arrest (3 h post-phleomycin treatment). Critically, EDD-depleted cells failed to maintain this arrest, with a significantly larger proportion of cells reentering mitosis 24 h post-phleomycin treatment compared with control (GFP siRNA) cells (Fig. 7B). Further evidence for premature mitotic reentry in EDD-depleted cells was the observation of a significantly increased subpopulation of multinucleated cells compared with control (GFP siRNA) cells 48 h after phleomycin treatment (Fig. 7C).

DISCUSSION

Correct activation and execution of DNA damage repair is essential for the maintenance of genomic stability and integrity, thus protecting cells against cancer-promoting mutations. The CHK2 tumor suppressor protein is a key mediator of cellular responses to genotoxic stress. We found that EDD, through an interaction with CHK2, is an important and novel mediator of the DNA damage signaling pathway in response to DNA double-stranded breaks. EDD binds to the FHA domain of CHK2 and is required for optimal CHK2 Thr⁶⁸ phosphorylation and kinase activity and for cell survival after DNA damage.

Consistent with a role for EDD in the DNA damage response, we observed accumulation of EDD in the nucleus following radiomimetic exposure. Several proteins involved in early transduction of the DNA damage signal form distinct foci on exposure to DNA-damaging agents (59). We were unable to detect EDD as part of any such DNA damage-induced foci when observing distribution of exogenously expressed GFP-

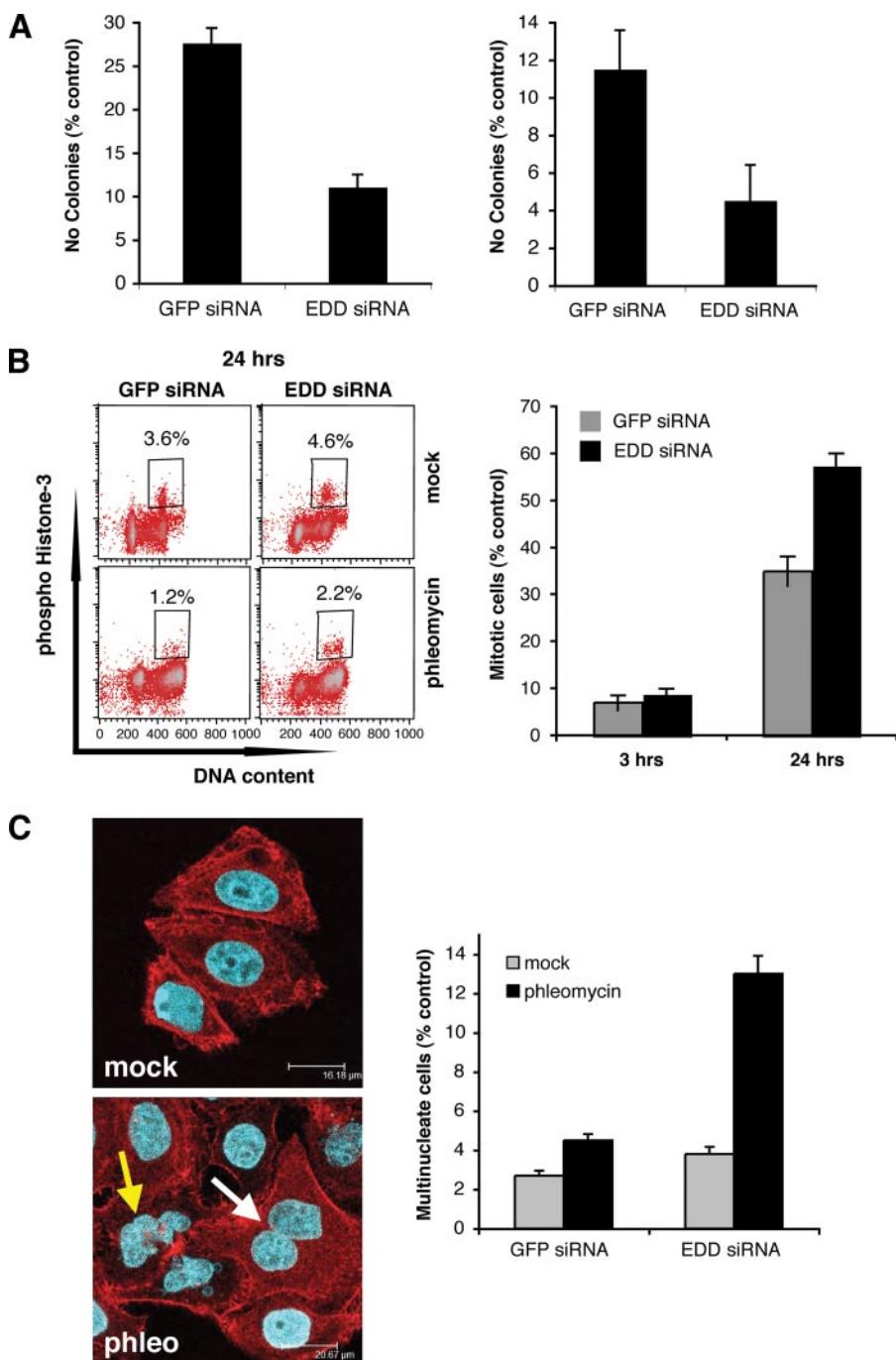


FIGURE 7. EDD mediates the cellular response to radiomimetic-induced DNA damage. *A*, effect of EDD depletion on colony formation following phleomycin treatment. Cells were exposed to phleomycin 72 h after siRNA transfection. Viability is expressed relative to untreated cells and represents the means \pm S.E. (MCF-7, $p = 0.02$, $n = 3$; HeLa, $p < 0.001$, $n = 12$). *B*, effect of EDD depletion on G₂/M checkpoint in HeLa cells following phleomycin treatment. Cells transfected with siRNA for EDD or control (GFP) were harvested 3 or 24 h after phleomycin (or mock) treatment, stained with anti-phosphohistone 3 antibody and propidium iodide, and analyzed by fluorescence-activated cell sorting. Representative fluorescence-activated cell sorting density plots from cells collected 24 h after treatment are shown with the mitotic population (anti-phosphohistone 3-positive) denoted within the polygon. Mitotic entry is expressed as percentage of anti-phosphohistone 3-positive cells after phleomycin treatment relative to untreated (mock) cells (means of triplicate experiments \pm S.E.). Significant differences were observed only at the later time point (#, $p < 0.005$, *t* test). *C*, EDD-depleted HeLa cells were seeded on microscopy slides, mock-treated or treated with phleomycin. 48 h after treatment, cells were fixed and stained with 4',6-diamidino-2-phenylindole (blue, nuclei), counterstained with phalloidin (red, cytoplasm), and visualized by confocal microscopy. The graph shows quantification of cells with >1 nuclei (as depicted by arrows), expressed as a percentage of total cells counted ($n = 200$). Results are presented as the mean of triplicate experiments \pm S.E. ($p < 0.0005$, *t* test).

tagged EDD.⁵ However, more consistent with complex formation between EDD and CHK2, we have observed a diffuse nuclear staining pattern similar to that of CHK2 (60).

Down-regulation or mutation of DNA damage repair proteins commonly promotes tumorigenesis. In the current study, down-regulation of EDD led to greatly diminished activating phosphorylation of CHK2 on Thr⁶⁸ accompanied by reduced CHK2 kinase activity. These observations are reminiscent of the effects of deficiency in other key mediators of CHK2 and support a role for EDD in this important component of the response to DSB (61–63). The decrease in survival observed in EDD-depleted cells is consistent with the degree of reduced viability observed upon down-regulation of other mediators of CHK2 activation, such as BRCA1 (56) and MDC1/NFBD1 (57). CHK2 activation contributes to initiation of apoptotic signaling upon DSB through p53 stabilization, and therefore in several models, CHK2 depletion results in enhanced cell survival due to defective apoptosis induction (23, 64, 65). However, CHK2 deficiency has also been associated with sensitization to death by mitotic catastrophe (58) and enhanced apoptosis in the absence of p53 (66). HeLa cells have a compromised p53 response, and indeed we observed that cells depleted of CHK2 via siRNA transfection displayed reduced cell survival following phleomycin treatment (data not shown). Our observations that depletion of EDD compromises G₂/M checkpoint maintenance and leads to aberrant mitosis also clearly support a role for EDD in optimal CHK2 function after DNA damage induction. Furthermore, the ability of EDD-depleted cells to initiate but not maintain a G₂/M arrest following DNA damage closely mimics the phenotype observed in cells from Chk2-null mice (24).

Several other recently described mediators of CHK2 activation are necessary for cell survival following exposure to IR, including 53BP1,

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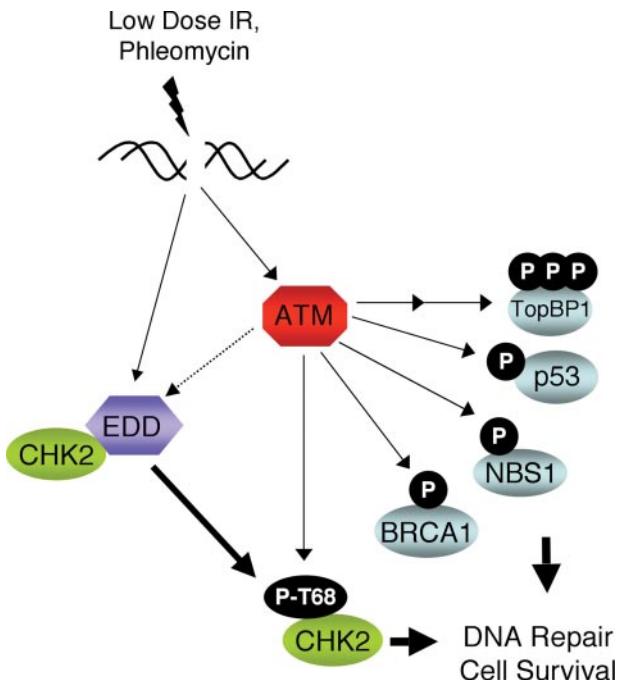


FIGURE 8. Role of EDD in the ATM-CHK2 DNA damage response pathway. Upon DNA damage causing double-stranded DNA breaks, activation of ATM leads to phosphorylation of CHK2 Thr⁶⁸ and other substrates, facilitating cell cycle arrest, DNA repair, and consequent cell survival. EDD interacts with CHK2 and is required to facilitate Thr⁶⁸ phosphorylation of CHK2 and full kinase activity. EDD could mediate CHK2 activation via a number of mechanisms, such as increasing the pool of newly synthesized CHK2 available for ATM phosphorylation, maintaining Thr⁶⁸ phosphorylation of CHK2 molecules by reducing phosphatase activity, or enhancing ATM-CHK2 interaction either by ubiquitination or by providing a molecular scaffold. EDD is modulated by DNA damage and could also be a target of ATM (dotted arrow).

NFBD1/MDC1, BRCA1, and NBS1 (55–57, 63, 67). Although these proteins appear to be required for phosphorylation of a number of additional ATM substrates, we found that phosphorylation of several ATM targets, such as BRCA1, NBS1, and p53, were unaffected in EDD-depleted cells. This excludes a role for EDD upstream of ATM and suggests that EDD works downstream of ATM in facilitating the phosphorylation and activation of CHK2 but not other substrates (Fig. 8).

We observed a constitutive association between EDD and CHK2 that, although initially maintained, was disrupted at later times after DNA damage. Similar dynamic interactions with CHK2 have been observed for the 53BP1 and BRCA1 proteins, and depletion of either protein can impair CHK2 Thr⁶⁸ phosphorylation in response to DNA damage (19, 55, 62). Cells with mutated BRCA1 are also impaired in their ability to phosphorylate other ATM substrates after IR (56, 68). Foray *et al.* showed that, unlike 53BP1, depletion of BRCA1 did not affect ATM kinase activity and suggested a scaffolding role for BRCA1 in ATM target phosphorylation in the presence of DNA double-stranded breaks. Similarly, EDD-deprived cells were still capable of ATM activation upon DNA damage, as measured by activating phosphorylation on Ser¹⁹⁸¹ of ATM, suggesting that rather than acting upstream of ATM, both EDD and BRCA1 facilitate phosphorylation of its substrates. EDD therefore joins an expanding list of proteins reported to facilitate CHK2 activation via ATM. These mediators are thought to rep-

resent mammalian counterparts of yeast Rad9 (scRad9), which, like several of these proteins, contains two BRCT motifs (69, 70). scRad9 serves as a scaffolding platform facilitating phosphorylation of Rad53 (the yeast counterpart of human CHK2) upon activation of Mec1/Tel1p (ATM orthologs in yeast) after DNA damage. It is increasingly apparent that transduction of the DNA damage signal through CHK2 is far more complex in mammals than in yeast, and in this respect it is of interest that no yeast homologues of EDD exist. It appears that the recently described mammalian mediator proteins, alongside others such as EDD, have overlapping and/or complementary functions in the activation of CHK2 after DNA damage by providing a molecular platform for ATM phosphorylation. However, the extraordinary degree of specificity with which EDD facilitates CHK2 phosphorylation might suggest that EDD may influence CHK2 activation by means other than a scaffolding role (see the legend to Fig. 8).

The CHK2 FHA domain has a critical role in the oligomerization, consequent autophosphorylation, and full activation of CHK2 that allows transduction of the DNA damage signal (33, 71). Interestingly, mutation of Arg¹¹⁷ in familial breast cancer has been reported (25), reinforcing the importance of interactions mediated by this residue in coordination of the DNA damage response. The interaction between EDD and CHK2 is mediated through the FHA domain of CHK2 and dependent upon phosphorylation of EDD. However, using site-directed mutants, it was shown that the EDD/CHK2 association does not require the Ile¹⁵⁷ residue at the distal interface of the FHA domain that is necessary for phosphoindependent interactions between CHK2 and its substrates (Fig. 4B) (*i.e.* EDD acts a modulator of CHK2 activation rather than being a target of CHK2 kinase activity). Conflicting reports exist on the precise role of the Arg¹¹⁷ residue in CHK2 activation (32, 71). The findings presented here concur with those of Ahn *et al.* (71), showing that, although DNA damage-induced activation of CHK2 containing the I157T mutation remained unaffected, the R117A mutation resulted in impairment of Thr⁶⁸ phosphorylation to a similar degree as that seen in EDD-depleted cells. These results therefore further support that phospho-dependent EDD binding to the FHA domain of CHK2 is necessary for its efficient Thr⁶⁸ phosphorylation and full kinase activation.

In pull-down experiments using HeLa cell extracts or *in vitro* synthesized EDD protein, specific binding was observed between EDD and a GST fusion construct of the NH₂-terminal region of CHK2 spanning the (S/T)Q and FHA domains. The minimal region of EDD required for CHK2 binding consisted of 1637 amino acids, including a UBR box similar to that found in the non-HECT yeast N-end rule ubiquitin ligase UBR1 and family members and a domain with homology to the poly(A)-binding protein carboxyl terminal motif, in addition to a number of potential CHK2 FHA binding motifs. The requirement of such a large region of the EDD protein for binding suggests that conformation of the protein may be important for exposure of the correct binding interface(s). Several recent findings indicate that FHA-target interactions are more complex than first thought, requiring an extended binding interface (72, 73). Furthermore, FHA domains may recognize several similar but nonidentical acidic target peptides and may bind to multiply phosphorylated

targets (*i.e.* clusters of phosphorylated residues in these targets) (74). The HECT domain, which mediates ubiquitin transfer to substrates, was not necessary for interaction. Indeed, binding was enhanced when this region was absent, suggesting a possible regulatory role for HECT domain-mediated ubiquitination in CHK2 activation.

In common with several other genes involved in DNA repair pathways, it is interesting to note that mice deficient in EDD display an early embryonic lethal phenotype (75). However, this is unlikely to relate to the role of EDD in CHK2 activation, since CHK2 null mice survive to adulthood (64), and the EDD-null phenotype is not rescued on a p53-null background, indicating involvement of EDD in additional important processes. Disruption of EDD is a feature of many cancers, and overexpression of EDD protein in serous ovarian carcinomas is independently predictive of disease relapse.⁶ In a study of CHK2 aberration in breast cancer, CHK2 was constitutively phosphorylated on Thr⁶⁸ in 50% of tumors, and it was proposed that such a state, if an early event, could promote selection of cells with a mutation in the downstream effector p53 (15). This raises the question of whether breast cancers with constitutive phospho-Thr⁶⁸ also exhibit high EDD levels and whether this correlates with p53 status. If alterations in EDD expression levels were an early event, this might indicate an inadequate DNA damage signaling cascade. CHK2-inactivating mutations are found in cancers, such as sporadic and familial breast and colon carcinoma (25, 28, 29). In these cancers, mutation of EDD might be expected to exacerbate the effects of partially functional CHK2 or reduced CHK2 gene dosage in the case of heterozygous mutants. In this respect, a truncating mutation in EDD in a subset of gastric and colorectal carcinomas was recently reported (3), and it would be interesting to correlate the incidence of aberrations in EDD and CHK2 in these tumor types. Further, the enhanced sensitivity to DNA-damaging agents that we observe in EDD-depleted cells predicts that EDD expression could represent an important marker of tumor susceptibility to genotoxic chemotherapeutic agents.

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