rent, and several different pharmacological agents have very similar effects on the native M-current and the KCNQ2+KCNQ3 channel. In particular, the compound XE991 is highly selective for both the M-current and KCNQ channels. Finally, the *KCNQ2* gene is the only known potassium channel gene that is expressed in a pattern that parallels the distribution of the M-current in peripheral sympathetic ganglia. These data make a compelling case for the hypothesis that the KCNQ2+KCNQ3 channel is a molecular correlate of the M-current in sympathetic neurons.

The *KCNQ2* and *KCNQ3* genes are also abundantly expressed in the CNS, and it is likely that the KCNQ2+KCNQ3 subunits contribute to the M-current in central neurons. This conclusion is consistent with the observation that mutations in either the *KCNQ2* or *KCNQ3* genes result in an inherited autosomal dominant epilepsy (*10–12*). The very similar phenotypes produced by mutations in either of these two distinct genes (*27*) can be explained by the observation that both gene products are required to produce full expression of functional channels. Identification of the physiological function of the channel encoded by the *KCNQ2* and *KCNQ3* genes may facilitate the development of symptomatic treatments for these epilepsies.

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30. The *KCNQ3* gene was initially identified as an expressed sequence tag in a search of GenBank (accession number AA001392). On the basis of this sequence, primers were designed and used to amplify partial KCNQ3 cDNA clones from rat brain and SCG cDNA by polymerase chain reaction (PCR). We determined an initial sequence encompassing the entire open reading frame of the *KCNQ3* gene by performing several rounds of 5′ and 3′ RACE (rapid amplification of cDNA ends) PCR using initial anchor oligonucleotides complementary to the partial cDNA clone and SCG cDNA as a template for amplification. Once cDNAs were obtained that extended beyond both the 5′ and 3′ ends of the open reading frame, oligonucleotides complementary to noncoding regions at either end of the coding sequence were designed. We amplified multiple full-length cDNA clones in independent PCR reactions from rat SCG cDNA using Expand Long Template PCR (Boehringer Mannheim, Indianapolis, IN) with several combinations of the following oligonucleotides: TTGACTCCCCATCCGACCT and GCCTTTGCCTTCTTTTGGG (forward reaction), and ACCGCGCACATGCATG and GTGACATGGGGAGGAAGAA (reverse reaction). Four independent clones were sequenced in their entirety in both directions by automatic sequencing (GenBank accession number AF091247). The deduced amino acid sequence was 95% identical to a recently described partial human KCNQ3 cDNA clone (*12*).
31. We amplified full-length KCNQ2 cDNAs from adult human brain cDNA using primers CCCCGCTGAGCCTGAG and TGTAAAAGGTCACTGCCAGG with the Expand High Fidelity enzyme mixture (Boehringer Mannheim). The KCNQ2 cDNA clone used in the biophysical studies was identical to the KCNQ2 cDNA isolated previously from a fetal brain cDNA library (*10*) with the exception of a small deletion in the carboxy intracellular domain (30 amino acids from residues 417 to 446). This region is also alternatively spliced in the KCNQ2 cDNA clone described by Biervert *et al.* (*11*). Preparation, injection of complementary RNA, and recording from oocytes were done as described (*28*). The standard extracellular recording solution contained 82 mM NaCl, 2 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2$, and 5 mM Na-Hepes (pH 7.6). Data collection and analysis were done with pClamp software (Axon Instruments, Foster City, CA).
32. Recordings of the M-current in sympathetic neurons in intact ganglia were done at room temperature as described (*3*). The standard extracellular recording solution contained NaCl (133 mM), KCl (4.7 mM), NaH_2PO_4 (1.3 mM), $NaHCO_3$ (16.3 mM), $CaCl_2$ (2 mM), $MgCl_2$ (1.2 mM), and glucose (1.4 g/liter) in an atmosphere of 95% O_2–5% CO_2 to give pH 7.2 to 7.4. Linopirdine and XE991 were from DuPont Pharmaceuticals (Wilmington, DE).
33. Preparation of RNA, RNase protection assays, and isolation of a specific rat KCNQ2 and KCNQ3 probes were done as described (*25*). RNA expression was quantitated directly from dried gels with a PhosphorImager (Molecular Dynamics, Sunnyvale, CA).

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Linkage of ATM to Cell Cycle Regulation by the Chk2 Protein Kinase

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In response to DNA damage and replication blocks, cells prevent cell cycle progression through the control of critical cell cycle regulators. We identified Chk2, the mammalian homolog of the *Saccharomyces cerevisiae* Rad53 and *Schizosaccharomyces pombe* Cds1 protein kinases required for the DNA damage and replication checkpoints. Chk2 was rapidly phosphorylated and activated in response to replication blocks and DNA damage; the response to DNA damage occurred in an ataxia telangiectasia mutated (ATM)–dependent manner. In vitro, Chk2 phosphorylated Cdc25C on serine-216, a site known to be involved in negative regulation of Cdc25C. This is the same site phosphorylated by the protein kinase Chk1, which suggests that, in response to DNA damage and DNA replicational stress, Chk1 and Chk2 may phosphorylate Cdc25C to prevent entry into mitosis.

When DNA is damaged, cells activate a response pathway that arrests the cell cycle and induces the transcription of genes that facilitate repair. The failure of this response results in genomic instability, a mutagenic condition that predisposes organisms to cancer. In eukaryotes, this checkpoint pathway initiated by DNA damage consists of several protein kinases, including the phosphoinositide kinase (PIK) homologs ATM, ATR, Mec1, and Rad3 and the protein kinases Rad53, Cds1, Chk1, and Dun1 (*1*). In mammals, in response to DNA damage, ATM controls cell cycle arrest in G_1 and G_2 and also prevents ongoing DNA synthesis (*1*). ATM controls G_1 arrest by activation of p53 (*2*), which induces transcription of the Cdk

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inhibitor p21 CIP1/WAF1, resulting in G_1 arrest (*3*). G_2 arrest is thought to involve maintenance of Cdc2 in a tyrosine-phosphorylated state, an ability that is important for preventing mitotic entry when DNA is damaged (*1*). In *S. pombe*, this is accomplished by activation of the Chk1 kinase, which can phosphorylate the Cdc25 tyrosine phosphatase, an activator of the cyclin-dependent kinase Cdc2 (*4–6*). Human Chk1 phosphorylates Cdc25C on Ser^{216}, which interferes with Cdc25C's ability to promote mitotic entry (*7*). Cdc25C is phosphorylated on Ser^{216} throughout interphase and is dephosphorylated directly before mitotic entry (*7*).

Although *S. pombe* Chk1 prevents mitosis in response to DNA damage, it is not required to prevent mitosis when replication is blocked. A second pathway is required during replication blocks, possibly acting through inhibition of Cdc25. Candidates include the *S. cerevisiae* Rad53 and *S. pombe* Cds1 protein kinases that are required for S phase checkpoint responses (*8–11*). These kinases are activated in a Mec1/Rad3-dependent manner in response to replication interference or DNA damage (*10, 12*). Rad53 is required for prevention of initiation of late origins of replication and slowing of DNA synthesis when DNA is damaged (*13*), a property shared with ATM in mammals. Rad53 is also required for preventing mitotic entry before the completion of DNA replication (*8*). In *S. pombe*, *cds1chk1* double mutants, but neither single mutant, enter mitosis when DNA replication is blocked (*10*), which indicates overlapping roles.

To investigate checkpoint conservation, we used polymerase chain reaction (PCR) and database analysis to identify Chk2, the mammalian homolog of *S. cerevisiae* Rad53 and *S. pombe* Cds1 (Fig. 1) (*14*). The longest human cDNA (1731 base pairs) encodes a 60-kD translation product of 543 amino acids (Fig. 1). Mouse *CHK2*, which encodes a 546–amino acid protein with 83% identity to human Chk2, begins at approximately the same methionine and has an in-frame upstream stop codon. Human CHK2 is most related to the *Drosophila melanogaster* Dmnk protein (34% identical and 45% similar) (Fig. 1, B and C), which is highly expressed in ovaries and might function in meiosis (*15*). *Caenorhabditis elegans* CHK2 was also identified (*16*). Human CHK2 is 26% identical and 37% similar to Rad53 and 26% identical and 34% similar to Cds1. Sequence analysis reveals a single forked head–associated (FHA) domain contained in the Rad53, Cds1, and Dun1 family of kinases (Fig. 1, A and B) (*17*). Rad53 has a second FHA domain that is not conserved in Chk2. Chk2 has a potential regulatory region rich in SQ and TQ (*18*) amino acid pairs. Northern (RNA) blot analysis revealed wide expression of low amounts of Chk2 mRNA with larger amounts in human testis, spleen, colon, and peripheral blood leukocytes (*16*).

We tested whether human CHK2 could complement the lethality of a *RAD53* deletion. Y324, a *rad53* deletion mutant kept alive by a copy of *RAD53* on a *URA3* plasmid, failed to grow on medium containing 5-fluoro-orotic acid (5-FOA), a chemical toxic to Ura^+ yeast cells. Plasmids expressing CHK2 or a kinase-defective mutant CHK2 $Asp^{347} \rightarrow Ala^{347}$ (D347A) under *GAL* promoter control were introduced into Y324 and tested for growth on synthetic complete (SC) medium containing 5-FOA and galactose. Cells bearing GAL-CHK2 (D347A) failed to produce colonies, but the presence of GAL-CHK2 allowed growth of 5-FOA–resistant colonies (*16*). The viability of these cells depended on CHK2 because they failed to form colonies when grown on glucose, which represses the *GAL* promoter (Fig. 1D). Furthermore, *rad53* mutants expressing CHK2 were more resistant to replication interference by hydroxyurea (HU) than were mutants kept alive by *RNR1* expression, further demonstrating functional conservation (*16*).

Affinity-purified Chk2 antibodies made to the COOH-terminal 18 amino acids of the human CHK2 (EAEGAETTKRPAVCAAVL) (*18*) recognized a 60-kD protein (Fig. 2A) in both HeLa and 293T cells that comigrated with Chk2 expressed by in vitro translation of the human CHK2 cDNA, and antibody binding was blocked by addition of excess antigenic peptide (*19*). These results indicate that the cDNA is full length and that the antibodies recognize the Chk2 protein. Two separate sera recognized the same sized polypeptide (*16*). Hemagglutinin (HA)-Chk2 expressed from the cytomegalovirus (CMV) promoter in 293T cells was detected as a 62-kD protein with antibodies to the COOH-terminal peptide of Chk2 or the HA epitope tag (Fig. 2B). Indirect immunofluorescence revealed diffuse nuclear staining of Chk2 in HeLa cells with brightly staining dots that did not change in response to DNA damage (*16*).

We examined whether Chk2 is modified in response to DNA damage as Rad53 is (*12*). Chk2 from extracts of cells exposed to ultra-

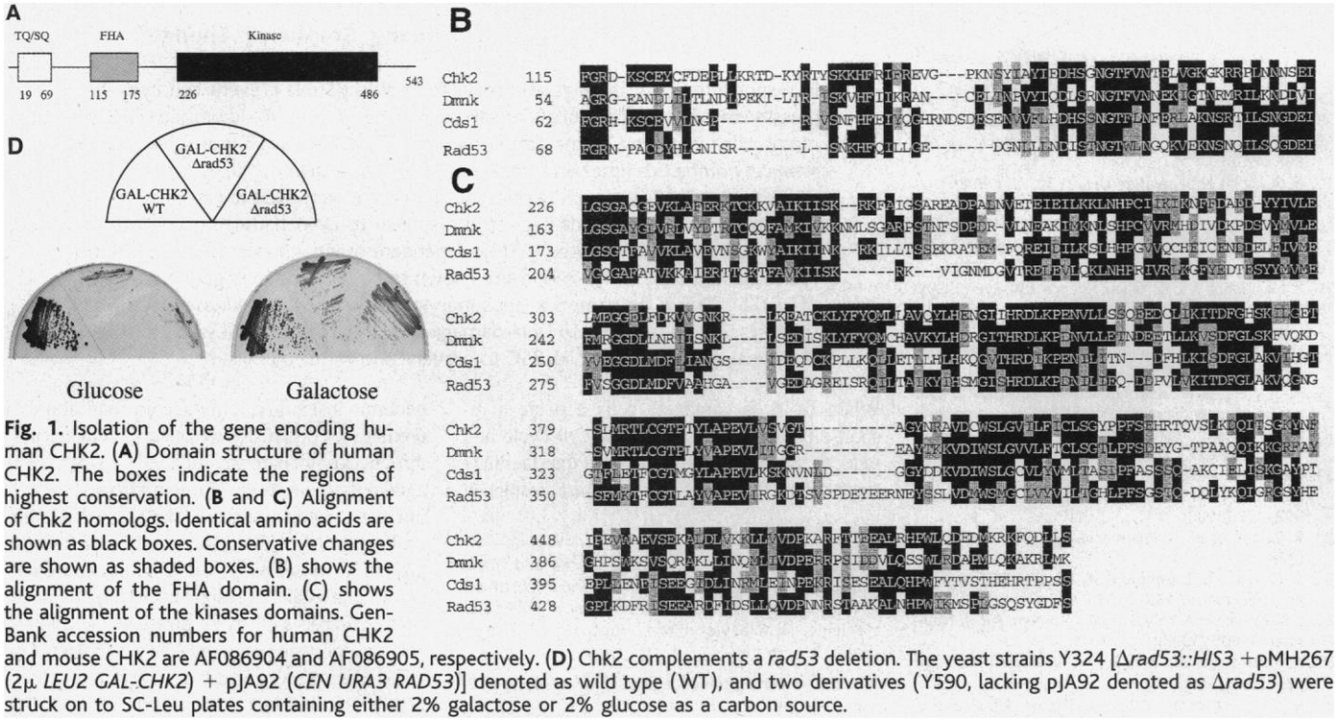


Fig. 1. Isolation of the gene encoding human CHK2. (**A**) Domain structure of human CHK2. The boxes indicate the regions of highest conservation. (**B** and **C**) Alignment of Chk2 homologs. Identical amino acids are shown as black boxes. Conservative changes are shown as shaded boxes. (B) shows the alignment of the FHA domain. (C) shows the alignment of the kinases domains. GenBank accession numbers for human CHK2 and mouse CHK2 are AF086904 and AF086905, respectively. (**D**) Chk2 complements a *rad53* deletion. The yeast strains Y324 [Δ*rad53::HIS3* +pMH267 (2μ *LEU2 GAL-CHK2*) + pJA92 (*CEN URA3 RAD53*)] denoted as wild type (WT), and two derivatives (Y590, lacking pJA92 denoted as Δ*rad53*) were struck on to SC-Leu plates containing either 2% galactose or 2% glucose as a carbon source.

violet (UV) light or γ irradiation showed reduction in mobility during SDS–polyacrylamide gel electrophoresis (SDS-PAGE) when compared to Chk2 from untreated cells (Fig. 2C). Inhibition of DNA replication also caused a slight reduction in mobility. Rad53 also shows more extensive mobility alterations in response to DNA damage rather than replication blocks (*12*). These results indicate that, like Rad53, Chk2 may participate in transduction of the DNA damage and replicational stress signals.

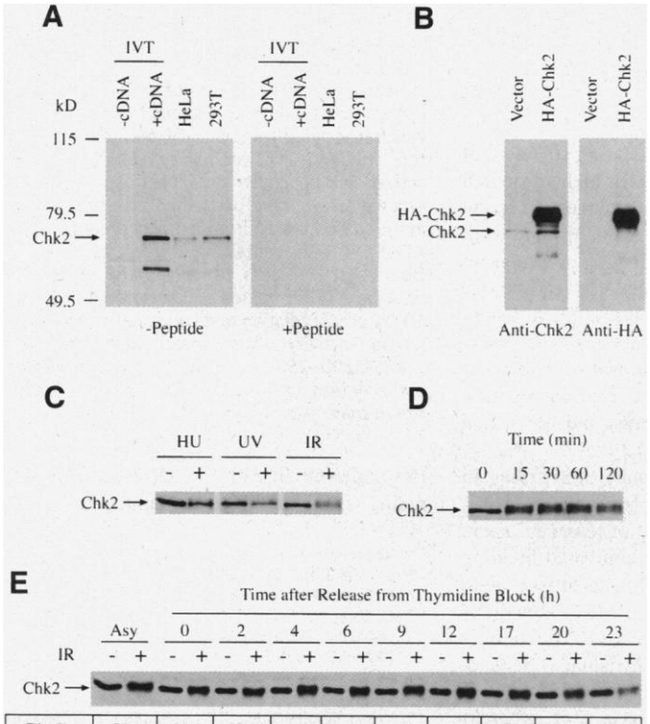
A kinetic analysis revealed rapid Chk2 modification within 15 min of γ irradiation (Fig. 2D), which suggests that Chk2 modification is part of the initial response to double-strand breaks. Chk2 does not alter its mobility during progression through the cell cycle in the absence of DNA damage, but it can be modified in response to γ irradiation at all stages of the cycle (Fig. 2E).

The redundancy between Chk1 and Cds1 during replication blocks (*10*) suggests that they might share common regulatory targets. We analyzed the ability of Chk2 to phosphorylate key regulators of Cdk tyrosine phosphorylation: Cdc25A, Cdc25B, and Cdc25C. Chk2 immunoprecipitated from 293T cells was capable of phosphorylating glutathione S-transferase (GST) fusion proteins of Cdc25A, Cdc25B, and Cdc25C (Fig. 3A) and it also autophosphorylated (*16, 20*). Immunoprecipitation of kinase activity was blocked by the presence of excess antigenic peptide. Bacterially expressed GST-Chk2 but not GST-Chk2 (D347A), a catalytically inactive mutant, also phosphorylated all three Cdc25 proteins (*16*).

We mapped the site of phosphorylation on Cdc25C. GST-Chk2, but not the catalytically inactive mutant, phosphorylated a 57–amino acid region of the Cdc25C protein (residues 200 through 256) fused to GST (Fig. 3B). This 57–amino acid motif contains four possible sites of phosphorylation. Ser^{216} is the main site of phosphorylation in vivo (*21*). A mutant Cdc25C fragment in which Ser^{216} was changed to Ala, GST-Cdc25C(200–256) (S216A), was a poor substrate for both Chk2 and Chk1, confirming Ser^{216} as a site of phosphorylation (Fig. 3C). Furthermore, we designed a peptide, GLFRAPSMPENLNR (*18*), in which Tyr^{212} was changed to Phe and Ser^{214} was changed to Ala and in which only Ser^{216} (underlined) was a possible phosphoacceptor, and we found that it was a very good substrate for Chk2 (*16*).

To examine Chk2 regulation, we immunoprecipitated Chk2 from 293T cells treated with γ irradiation and measured its kinase activity toward Cdc25 substrates. Immunoprecipitated Chk2 phosphorylated the Cdc25C fragment but not the mutant S216A derivative. Chk2 activity was increased 5.6-fold in response to γ irradiation (Fig. 3D). The 5.6-fold increase represents the minimum change in the specific activity of the kinase, because the modified form of the kinase is more difficult to detect by protein immunoblot. Chk2 is also activated in response to HU and UV treatment (Fig. 3E). The alteration in mobility and increased kinase activity of Chk2 in response to DNA damage are due to phosphorylation because treatment of Chk2 isolated from damaged cells with lambda phosphatase reversed the mobility alteration and the increased kinase activity (Fig. 3F). If the overlapping specificity of Chk1 and Chk2 kinases is conserved in *S. pombe*, it could explain the phenotype of the *chk1cds1* double mutant in response to replication blocks.

Activation of Rad53 and Cds1 is dependent on the ATM homologs *MEC1* and *rad3*, respectively. To determine whether ATM regulates Chk2, we examined Chk2 modification in a cell line lacking ATM and in the same line into which a functional ATM gene was reintroduced on an episomal vector (*22*). Cells lacking ATM showed no modification of the Chk2 protein or activation of Chk2 kinase activity in response to γ irradiation (Fig. 4, A and B). However, expression of a wild-type ATM cDNA in these cells restored both modification and activation of Chk2. This indicates that ATM is an upstream regulator of Chk2 and establishes a pathway for cell cycle arrest in response to DNA damage. Cells defective for other genes involved in the DNA damage response such as *BRCA1* (*23*) and *BRCA2* (*24*) showed normal regulation of Chk2 (Fig. 4A). We have also ob-



G1 (%)	59.1	54.9	23.2	5.5	5.5	8.3	67.4	63.3	29.4	16.6
S (%)	22.6	41.1	71.9	86.6	56.2	7.4	6.6	29.7	57.4	48.8
G2/M (%)	17.2	2.9	3.2	6.3	36.9	80.9	23.8	3.9	9.9	30.9

Fig. 2. Modification of the 60-kD Chk2 protein in response to DNA damage and DNA replication blocks. (**A**) Human Chk2 is a 60-kD protein. Protein from in vitro translation mixture without (−cDNA) or with (+cDNA) Chk2 cDNA, HeLa cell extracts, or 293T cell extracts were fractionated by SDS-PAGE and immunoblotted with affinity-purified antibodies to Chk2 in the absence (−peptide) or presence (+peptide) of competing peptide. (**B**) 293T cells were transfected with vector alone or with the vector expressing HA-Chk2 under CMV control. Proteins from these cells were fractionated by SDS-PAGE and immunoblotted with antibody to Chk2 or with antibodies to HA. (**C**) Modification of Chk2 in response to DNA damage and replication blocks. 293T cells were untreated or were treated with 20 Gy of γ irradiation or 50 J/m^2 of UV radiation and collected after 2 hours, or grown in 1 mM hydroxyurea for 24 hours before analysis. Proteins from these cells were fractionated by SDS-PAGE and immunoblotted with antibodies to Chk2. (**D**) Kinetics of Chk2 modification in response to γ irradiation. 293T cells were treated with 20 Gy of γ irradiation, collected at the indicated times, and immunoblotted with antibodies to Chk2. (**E**) Response of Chk2 to DNA damage throughout the cell cycle. HeLa cells were synchronized by double thymidine block and released. At the indicated times, cells were either untreated or irradiated with 20 Gy and collected 2 hours later. Samples were analyzed for DNA content by fluorescence-activated cell sorting or for Chk2 protein by immunoblotting. The percentages of cells in G_1, S, and G_2/M are shown below the relevant times.

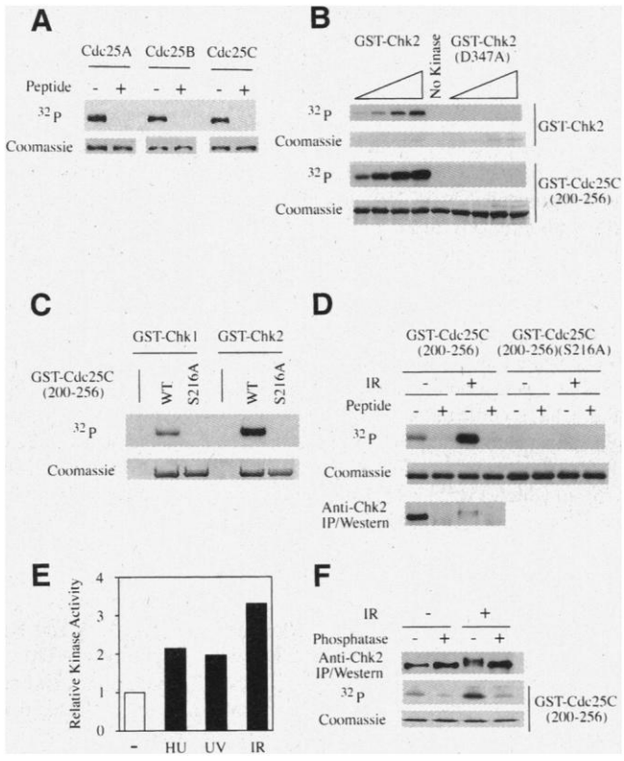


Fig. 3. Activation of Chk2 in response to DNA damage and phosphorylation of Cdc25C on an inhibitory residue. **(A)** Phosphorylation of Cdc25A, Cdc25B, and Cdc25C by Chk2. Chk2 was immunoprecipitated from 293T cells and incubated with [γ-^{32}P]ATP and GST-Cdc25A, GST-Cdc25B, or GST-Cdc25C. Proteins were resolved by SDS-PAGE, and the GST-Cdc25 proteins were visualized by autoradiography and Coomassie staining below for (A) through (E). **(B)** GST-Chk2 and GST-Chk2 (D347A) (kinase defective) were purified from *Escherichia coli* and increasing amounts were incubated with GST-Cdc25C(200–256) and [γ-^{32}P]ATP as in (A). **(C)** Phosphorylation of a Cdc25C fragment on Ser^{216} by Chk2. GST-Chk1 and GST-Chk2 were incubated with GST-Cdc25C-(200–256) or GST-Cdc25C-(200–256) (S216A) and [γ-^{32}P]ATP as in (A). **(D)** Activation of Chk2 in response to DNA damage and phosphorylation of Cdc25C on Ser^{216}. Chk2 kinase was immunoprecipitated in the absence (−peptide) or presence (+peptide) of competing peptide from extracts prepared from 293T cells treated without (−IR) or with (+IR) 20 Gy of γ irradiation. Immunoprecipitates were incubated with either GST-Cd25C(200–256) or GST-Cdc25C(200–256) (S216A) and [γ-^{32}P]ATP as in (A). Chk2 protein present in immunoprecipitates was determined by immunoblotting. **(E)** Chk2 kinase is activated in response to HU and UV. Assays were performed as in (D) on cells treated with 50 J/m^2 of UV light, 20 Gy of γ irradiation, or 1 mM HU and harvested after 2 hours. **(F)** Chk2 modification and activation are due to phosphorylation. Chk2 was immunoprecipitated from γ-irradiated cells as in **(D)**, and the immunoprecipitates were treated with or without 100 U of lambda phosphatase for 2 hours, then assayed for mobility alteration or kinase activity as in (D).

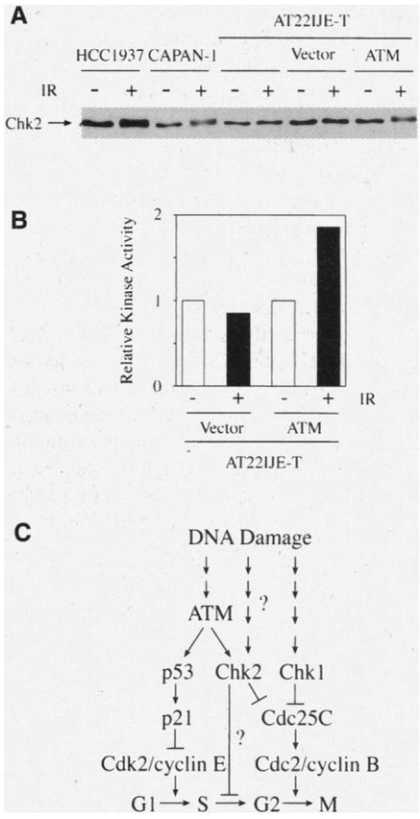


Fig. 4. Control of Chk2 activation through ATM in response to DNA damage. **(A)** HCC1937 (homozygous BRCA1 mutant cells) (*23*), CAPAN-1 (homozygous BRCA2 mutant cells) (*24*), AT22IJE-T (homozygous ATM mutant cells; left), AT22IJE-T cells containing the vector alone (middle), or AT22IJE-T cells containing the vector expressing ATM (right) (*22*) were untreated (−IR) or treated (+IR) with 10 Gy of γ irradiation and harvested after 1 hour. Protein from these cells was fractionated by SDS-PAGE and immunoblotted with anti-Chk2 antibodies. **(B)** Chk2 kinase activation is dependent on a functional ATM gene. Kinases assays were performed on Chk2 protein immunoprecipitated from extracts prepared from AT22IJE-T cells containing the vector alone or from AT22IJE-T cells containing the vector expressing ATM untreated (−) or treated (+) with 10 Gy of γ irradiation and harvested after 1 hour. Kinase activity was measured with Gst-Cdc25C(200–256) as substrate. **(C)** The genetic pathway leading from DNA damage to Cdk control in mammals.

served that treatment of ATM mutant lines with much higher levels of γ irradiation could cause some Chk2 modification, which indicates that an ATM-independent pathway might be capable of regulating Chk2 function in the presence of high levels of damage (*16*).

Our results indicate that Chk2 was functionally conserved throughout eukaryotic evolution. Chk2 kinase is activated by DNA damage and replication blocks and can directly phosphorylate Cdc25C on an inhibitory residue. The fact that two checkpoint kinases can directly phosphorylate Cdc25C on an inhibitory residue strengthens the notion that DNA damage and replicational stress regulate the S-to-mitosis and G_2-to-mitosis transitions through control of Cdc2 tyrosine phosphorylation. These results suggest a model (Fig. 4C) whereby in response to DNA damage and possibly to replication blocks ATM activates p53 to control G_1 arrest and activates Chk2, and possibly Chk1, which in turn phosphorylate Cdc25C on Ser^{216}, leading to inhibition of Cdc25C's ability to dephosphorylate and activate Cdc2/cyclin B complexes.

Because both Chk1 and Chk2 kinases may have redundant functions regarding Cdc25C regulation, why would a cell need both pathways? The requirements for inactivation of Cdc25C activity in G_2 could be higher than in S phase and require multiple activities to prevent mitosis. Alternatively, these kinases may work in different stages of the cell cycle or respond to different signals. They might also regulate different pools of Cdc25 or even different family members. It is likely that although they share some substrates, they also have different targets, such as those involved in mediating radiation-resistant DNA synthesis and prevention of late origin firing for Chk2 (*13*).

Although there are many similarities in mammalian and fungal checkpoint systems, there also are important differences. First, structurally *ATM* is most similar to budding yeast *TEL1* whereas *ATR* is most similar to *MEC1*. However, although *MEC1* functions in regulation of *RAD53*, *ATM* controls *CHK2*'s response to DNA damage. Second, the response of Cds1 to DNA damage is primarily limited to S phase (*10*), whereas Chk2 can respond throughout the cell cycle. Identification of the central signal transducers *ATM*, *ATR*, *CHK1*, and *CHK2* should facilitate understanding of how DNA damage signaling is accomplished in mammals.

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18. Single-letter abbreviations for the amino acid residues are as follows: A, Ala; C, Cys; D, Asp; E, Glu; F, Phe; G, Gly; H, His; I, Ile; K, Lys; L, Leu; M, Met; N, Asn; P, Pro; Q, Gln; R, Arg; S, Ser; T, Thr; V, Val; W, Trp; and Y, Tyr.
19. Cells were lysed in 20 mM tris-HCl buffer (pH 8.0) containing 100 mM NaCl, 0.5% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml), antipain (2 μg/ml), and 1 mM phenylmethylsulfonyl fluoride (PMSF) for 15 min at 4°C. After centrifugation at 10,000*g* for 10 min, proteins were separated by SDS-PAGE and transferred to nitrocellulose membranes. After incubation with nonfat milk (5%) in TBST [tris-HCl (pH 7.5), 100 mM NaCl, and 0.05% Tween 20], the membranes were incubated with anti-Chk2 or anti-HA (BAbCO), and then with horseradish peroxidase–conjugated secondary antibodies (Promega), and detected by enhanced chemiluminescence (Amersham).
20. Cell extracts were incubated with anti-Chk2 and protein A beads (Pharmacia) for 1 hour at 4°C. The precipitated beads were washed four times with 20 mM tris-HCl (pH 8.0), 0.1% Triton X-100, 10 mM NaF, 1 mM Na_3VO_4, aprotinin (2 μg/ml), leupeptin (2 μg/ml), antipain (2 μg/ml), and 1 mM PMSF and then three times with 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, and 10 mM $MnCl_2$. Kinase reactions contained immunoprecipitated endogenous Chk2 bound to protein A beads and GST-Cdc25 substrates in 20 mM Hepes (pH 7.4), 10 mM $MgCl_2$, 10 mM $MnCl_2$, 40 μM adenosine triphosphate (ATP), and 15 μCi [γ-^{32}P]ATP for 30 min at 30°C. Proteins were separated by SDS-PAGE and visualized by Coomassie blue staining and autoradiography. ^{32}P incorporation into GST-Cdc25 substrates was quantitated with a PhosphorImager (Molecular Dynamics).
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dMi-2, a Hunchback-Interacting Protein That Functions in *Polycomb* Repression

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Early in *Drosophila* embryogenesis, gap gene products directly repress transcription of homeotic (HOX) genes and thereby delimit HOX expression domains. Subsequently, Polycomb-group proteins maintain this repression. Currently, there is no known molecular link between gap and Polycomb-group proteins. Here, dMi-2 is identified as a protein that binds to a domain in the gap protein Hunchback that is specifically required for the repression of HOX genes. Genetic analyses show that dMi-2 participates in both Hunchback and Polycomb repression in vivo. Hence, recruitment of dMi-2 may serve as a link between repression of HOX genes by Hunchback and Polycomb proteins.

The design of animals depends on spatially restricted expression of HOX genes (*1*). In the early *Drosophila* embryo, segmentation gene products that are locally expressed delimit the domains of HOX gene expression (*2*, *3*). Gap proteins, such as Hunchback (Hb), bind directly to regulatory sequences of HOX genes and repress their transcription in cells outside of HOX expression domains (*4*, *5*). Although HOX genes need to be continuously repressed in these cells and in their descendants, gap proteins are only transiently available. The role of the Polycomb-group (PcG) gene products is to maintain repression of HOX genes throughout development (*1*, *6*–*8*). To identify proteins that may act as a molecular link between the Hb repressor and PcG proteins, we used Hb protein as a bait in a yeast two-hybrid screen.

Using LexA-Hb as bait, we isolated cDNAs representing six different genes (*9*). In interaction tests with various unrelated LexA baits, proteins encoded by three of the six cDNAs interacted exclusively with Hb (Fig. 1A). Among these proteins, the hip76 clone product exhibited the strongest interaction with Hb. We isolated multiple cDNA clones (*10*) that span a complete open reading frame (ORF) encoding a 1982–amino acid protein with high sequence similarity to the human autoantigen Mi-2 (*11*). We refer to the *Drosophila* protein as dMi-2. dMi-2 contains five conserved sequence motifs (*11*) that are also present in the two human Mi-2 proteins and in two *Caenorhabditis elegans* ORFs (*12*): two chromodomains (*13*), a DNA-stimulated adenosine triphosphatase (ATPase) domain (*14*), two PHD finger motifs (*15*), a truncated helix-turn-helix motif resembling the DNA-binding domain of c-myb (*16*), and a motif with similarity to the first two helices of an HMG domain (*17*).

To map the dMi-2–interacting domain in Hb, we generated Hb fragments and tested them for dMi-2 interaction in yeast two-hybrid assays (Fig. 1B). dMi-2 interacted very strongly with sequences overlapping the D domain, a stretch of amino acids that is conserved between Hb proteins of different insect species (*18*). Mutations in the D box cause extensive derepression of HOX genes of the Bithorax complex (BXC) (*2*) (see below). Both D box alleles are premature termination codons, suggesting that the D domain and its COOH-terminal flanking sequences are critical for repression of BXC genes (*19*). Our interaction tests (Fig. 1B) show that this protein portion of Hb interacts with dMi-2. In vitro binding assays with bacterially expressed dMi-2 and Hb proteins confirmed that these proteins bind directly to each other (*20*). Thus, dMi-2 binds to a portion of Hb that appears to be critical for repression of BXC genes.

In situ hybridization to polytene chromosomes revealed that *dMi-2* maps to subdivision 76D (*21*). In a screen for zygotic lethal mutations in this region, we identified five complementation groups (*21*).

To test whether any of these five complementation groups encode dMi-2, we sequenced the *dMi-2* coding regions of several

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