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CDK2-Dependent Phosphorylation of FOXO1 as an Apoptotic Response to DNA Damage

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Materials and Methods

Plasmids. Plasmids for wild type and Akt phosphorylation-resistant mutant FLAG-FOXO1 (FOXO1-WT and AAA, originally named FKHR-WT and FKHR-AAA, where the three Akt phosphorylation sites Thr24, Ser256, and Ser319 were converted to alanine) and the luciferase reporter construct, 3xIRS, which contains three copies of the FOXO response element in the promoter of the IGFBP1 gene, were kindly provided by K.L. Guan (S1). Plasmids for amino acid substitution mutants of FOXO1 (S249A, S298A, S249A/S298A, S249D) were generated by polymerase chain reaction (PCR)-based mutagenesis (Stratagene). Expression vectors for various truncated forms of FOXO1, PTEN, and p27KIP1, and the FasL promoter reporter construct have been described previously (S2-S4). Expression vectors for FOXO3a and FOXO4 were kindly provided by M.E. Greenberg and J.L. Bos, respectively (S5, S6). Full-length cDNAs for human cyclin E and CDK2 in viral vectors were kindly provided by M. Pagano (New York University) and P.A. Khavari (Stanford University), respectively (S7, S8), and tagged-(HA or V5) or untagged- expression vectors of these genes were generated in the backbone plasmid pcDNA3.1 by PCR. Kinase-dead CDK2 (CDK2-KD) was generated by converting Thr160 to alanine (T160A) in human CDK2 via PCR-based mutagenesis.
An active mutant of CDK2, CDK2-AF, was generated by converting Thr14 and Tyr15 to alanine and phenylalanine, respectively via PCR-based mutagenesis. A luciferase reporter construct of p21WAF1, p21-Luc (-1895 ~ +16bp), which contains a functional p53-binding site, was kindly provided by W.S. El-Deiry (University of Pennsylvania). GST-cyclin E, GST-CDK2, various GST-FOXO1 fusion proteins (see Fig. 1A), GST-FOXO3a (amino acids 148-280), and GST-FOXO4 (amino acids 92-288) were generated with the pGEX-4T-1 vector (Amersham Pharmacia Biosciences). GST-FOXO3a-S173A and GST-FOXO4-S268A were generated by PCR-based mutagenesis. Purified GST-cyclin A and RB C-terminal fusion proteins were purchased from Cell Signaling Technology.

Small interference RNA (siRNAs) and siRNA-resistant plasmids. Smart pools of siRNAs for FOXO3a, FOXO4, Foxo1 (mouse FOXO1), Foxo6 (mouse FOXO6), CDK2, Chk1, p53, p27KIP1 and GFP were purchased from Dharmacon. The following gene-specific and control siRNAs were also obtained from Dharmacon: siCONTROL Non-Targeting siRNA#1 (5'-UAGCGACUAAACACACAUCAA-3'), siCONTROL Non-Targeting siRNA#2 (5'-UAAGGCUAUGAAGAGAUAC-3'), Chk2 siRNA-a (5'-GAAAUUGCACUGUCACUAAUU-3'), Chk2 siRNA-b (5'-AAACGCGUCCUUUGAAUAAU-3'), and a FOXO1-specific siRNA (5'-CCAGAUGCCUAUAACACAUU-3'). Three silent mutations, which were introduced into the wild-type and phosphorylation-resistant mutant (S249A) of FOXO1 to generate the silencing-resistant proteins (FOXO1-WTsr and FOXO1-S249Asr), are underscored in the sequence of the FOXO1-specific siRNA.
**Antibodies and chemicals.** A rabbit polyclonal antibody against phosphorylated S249 of FOXO1 (S249-p) was raised by using the phosphorylated peptide NPEGKSGKpSPRRRAAS, and purified over a peptide-affinity column. Other antibodies were: anti-FOXO1, anti-phospho-FOXO1 at S256 (S256-p), and anti-PARP polyclonal (Cell Signaling Technology); anti-FOXO3a (Upstate); anti-FLAG (M2) (Sigma); anti-Bim polyclonal (Chemicon); anti-CDK2 polyclonal (M2), anti-Chk1, anti-14-3-3ζ (C-16); anti-CBP (A-22); anti-p53 (DO-1); anti-p27KIP1 (F8) and anti-Erk2 monoclonal (Santa Cruz Biotechnology); anti-V5 (Invitrogen); anti-manganese superoxide dismutase (MnSOD) (Stressgen Bioreagents); anti-hemagglutinin (HA) (Roche Applied Science). The anti-Chk2 antibody was described previously (S9). LY294002 and roscovitine were purchased from Calbiochem. UCN-01 was generously provided by E. Sausville (National Cancer Institute, NIH).

**Real-Time PCR.** Two-step real-time polymerase chain reaction was performed using cDNA prepared from RNA isolated from different cell lines. First strand cDNAs were synthesized using a kit from Invitrogen. Real-time PCR was performed using SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI PRISM 7900 HT Fast Real Time System following the manufacturer’s instructions. Both forward and reverse primers were used at a final concentration of 900 nM. Relative quantitation was used to determine relative expression levels by the comparative $C_T$ method using the formula $2^{-\Delta\Delta C_T}$ where $C_T$ is the threshold cycle of amplification. The followings are the primer sequences used for PCR: FOXO1 forward, 5’-AACCTGGCATTACAGTTGGCC-3’; FOXO1 reverse, 5’-
AAATGCAGGAGGCATGACTACGT-3'; FOXO3a forward, 5’-
TCAATCAGAACTTGCTCCACCA-3'; FOXO3A reverse, 5’-
GGACTCACTCAAGCCCATGTTG-3'; FOXO4 forward, 5’-
TTTTCTCCTGTGCCAATTagggg-3'; FOXO4 reverse, 5’-
TCCAACAGCATGCTCATCTTTG-3'; FOXO6 forward, 5’-
AACAATTTTATTCATGAAATATGCTG-3', FOXO6 reverse, 5’-
CCCTTCTCCCATATTATAAGTGTc-3'; Foxo6 forward, 3’-
CCTCTCTCCCATATTATAAGTGTC-3'; Foxo6 reverse, 5’-
GTCGTATAAAAGTACTTGTTTAACAA-3'; GAPDH forward, 5’-
GAAGGTGAAGGTCCGGAGTC-3'; GAPDH reverse, 5’-
GAAGATGGTGATGGGATTTTCC-3’.

Cell culture and transfection. The prostate cancer cell lines DU145, LNCaP and PC-3, breast cancer cell line MCF7, and ‘normal’ (non-tumorigenic) breast epithelial cell line MCF10A, were purchased from American Type Culture Collection (ATCC). The wild-type MEF cells were obtained from Dr. Junjie Chen’s laboratory. Prostate cancer cell lines were grown in RPMI 1640 supplemented with 10% FBS except when stated otherwise. MCF7 cells were cultured in DMEM supplemented with 10% FBS. MCF10A cells were cultured in Mammary Epithelial Growth Medium (MEGM) (Clonetics). MEF cells were grown in DMEM supplemented with 15% FBS. NIH 3T3 and HEK 293 cells were maintained in MEM and DMEM, respectively, supplemented with 10% FBS. Transfections by electroporation and measurement of luciferase activity were performed as described (S2). Approximately 75-90% transfection efficiencies were routinely
achieved. LNCaP-FOC4, a stable cell line of LNCaP expressing wild-type FLAG-FOXO1, was established and maintained in RPMI 1640 supplemented with 10% FBS plus 400 µg/ml G418.

*Luciferase assay.* Luciferase assays were performed as described (S3). Briefly, cells were transfected with firefly luciferase reporter constructs of FOXO transcription factors (3xIRS or FasL promoter), renilla luciferase reporter plasmids and indicated plasmids. Cells were harvested and cell lysates were prepared by adding lysis buffer directly to the cells on ice. Firefly luciferase and renilla luciferase activities were determined using a dual luciferase kit (Promega), and renilla luciferase activities of cells were used as internal controls.

*Immunoprecipitation, immunoblotting, and in vitro kinase assay.* Immunoprecipitations were carried out using an immunoprecipitation kit (Roche Applied Science). Immunoblotting was performed as described (S2). Kinase assays were carried out in the presence of [γ-32P]ATP by using an in vitro kinase buffer purchased from Cell Signaling Technology.

*Cell sorting and nuclear morphometry cell death assay.* In order to analyze apoptotic death of transfected cells, cells were co-transfected with an expression vector for the enhanced green fluorescence protein (EGFP). Twenty four hours after transfection, GFP-positive (transfected) cells were sorted and re-plated. At the indicated time points, apoptotic cell death was analyzed as described (S10-S12). Briefly, cells were collected
and fixed with a solution of 1.5% formaldehyde, 40% methanol, and 10% acetic acid. Hoechst (Sigma-Aldrich Corp.) was added to a final concentration of 1 μg/ml, and cells were incubated for 10 min at room temperature. Cells were placed on slides and viewed under UV and phase contrast illumination (Cal Zeiss Axiophot). Cells scored as “dead” exhibited signs of hyperfluorescent chromatin (condensation) and/or nuclear fragment. This method was used routinely for apoptosis measurement except stated otherwise.

**Propidium iodide exclusion apoptosis assays.** Apoptosis analyses using this method were performed as described (S3). Briefly, cells were treated with permeabilizing solution (0.1% sodium citrate, 0.1% Triton X-100, 50 μg/ml propidium iodide), and hypodiploid cells were counted by flow cytometry. Percentage of cells in sub-G1 was analyzed using the WINMDI 2.8 software.

**In vitro protein synthesis and GST-pull down assay.** 35S-labelled CDK2 proteins were synthesized by using TnT Quick Coupled Transcription/Translation Systems (Promega). GST protein and GST-fusion proteins for CDK2, cyclin E, FOXO1, FOXO3a, and FOXO4 were purified from the BL21 Star (DE3) *Escherichia coli* strain (Invitrogen), and GST pull-down assays were performed as described (S2).

**Immunofluorescence chemistry and confocal microscopy.** Cells grown on coverslips (Eppendorf Scientific, Inc., Hamburg, Germany) were washed briefly in 1× PBS and fixed for 20 min in 2% paraformaldehyde (Toussins) in 1× PBS. Cells were permeabilized by incubating with 0.2% Triton X-100 for 15 min. Cells were washed three times in 1×
PBS and incubated in blocking buffer (5% goat serum in 1x PBS) for 1 h at room temperature. Cells were incubated with a mouse anti-FLAG monoclonal antibody (1:1000) for 1 h at room temperature. After washing with 1x PBS three times for 5 min each, cells were incubated for 1 h at room temperature with the secondary Alexa Fluor 488 goat anti-mouse IgG conjugate (Molecular Probes) (1:1000) prepared in blocking buffer for 1 h at room temperature. Coverslips were washed with three changes of 1x PBS for 5 min each and mounted in Vectashield Mounting Medium with 4',6-diamidino-2-phenylindole (DAPI) (Vector Laboratories). Cells were analyzed with a laser-scanning microscope LSM510. An argon ion laser was used to excite fluorescein isothiocyanate and UV laser was used to excite DAPI.

Statistics. Experiments with three or four replicates were carried out and statistical analyses were performed by Student's t test. Values of P < 0.05 are considered significant.

SOM Text

CDK2 primarily phosphorylates FOXO1 at S249 in vitro

To test whether CDK2 is able to phosphorylate FOXO1 at S249 in vitro, this residue was converted to alanine (S249A) in FO1-2, and kinase assays were performed using this mutated form of FO1-2 as substrate. This single mutation completely abolished the CDK2-mediated phosphorylation of FO1-2 (Fig. 1D), suggesting that S249 is a CDK2 phosphorylation site in vitro. Apart from FO1-2, however, mutation of S249A in another CDK2-phosphorylated segment FO1-3, which shares the S249 residue with FO1-2, failed to completely abrogate the CDK2-mediated phosphorylation (fig. S1C), indicating
additional potential phosphorylation site(s) in FO1-3. There are eight S/T-P sites in FO1-3 (fig. S1D); therefore, three truncated GST fusion proteins were generated from FO1-3. Kinase assays eliminated the last four S/T-P motifs as possible phosphorylation sites (fig. S1E). Amino acid substitution studies of the other four S/T-P motifs in FO1-3 demonstrated that a mutation at serine 298 (S298A) also reduces phosphorylation of FO1-3, although it is not as efficient as the S249A mutation (fig. S1C). This was supported by the finding that the phosphorylation level of the double mutation (S249A/S298A) is very comparable to the single mutant S249A, but not S298A (fig. S1C). Thus, results from the in vitro kinase assays suggest that CDK2 preferentially phosphorylates FOXO1 at S249 within the consensus CDK-phosphorylation motif (fig. S1B).

**FOXO1 interacts with CDK2 in vitro and in vivo**

To better understand the functional interaction between FOXO1 and CDK2, we also examined whether FOXO1 interacts with CDK2. Expression vectors of FLAG-FOXO1 and V5-CDK2 were transfected into LNCaP prostate cancer cells, and co-immunoprecipitation assays were performed. As demonstrated in fig. S3A, ectopically expressed FOXO1 formed a complex with tagged CDK2 in these cells. A similar result was obtained with NIH 3T3 and HEK 293 cells. Next we examined the interaction between endogenous FOXO1 and CDK2 proteins. Both proteins exist as a complex in LNCaP cells (fig. S3, B and C). These data suggest that both ectopically expressed and endogenous FOXO1 associate with CDK2 in different cell types.
In order to determine whether these proteins interact \textit{in vitro} and which domains of FOXO1 are required for this interaction, GST pull-down assays were performed. Bacterially expressed GST-CDK2 fusion protein was purified and added to a lysate of LNCaP cells. Endogenous FOXO1 protein was pulled down by GST-CDK2, but not the GST protein (fig. S3D). A similar result was obtained with NIH 3T3 cells. To determine whether activation of CDK2 enhances its interaction with FOXO1, purified cyclin E was combined with GST-CDK2 prior to the pull-down assay. As shown in fig. S3D, the binding of CDK2 to FOXO1 was similar regardless of the presence or absence of cyclin E. This suggests that CDK2 interacts with FOXO1 independently of its activation status. To determine which region(s) of FOXO1 are required to interact with CDK2, a series of truncated GST-FOXO1 fusion proteins (see middle panel in Fig. 1B and Fig. 1A) were used for in vitro protein binding assays. CDK2 interacted strongly with two fragments, FO1-3 and FO1-5, weakly with FO1-2, but not with FO1-1, FO1-4 or GST itself (fig. S3E). These data indicate that FOXO1 contains two major CDK2-interaction sites, which are present in the regions of 267-419 and 503-655, respectively. Next, a series of C-terminal truncated forms of FLAG-FOXO1 (fig. S3F) were transfected into LNCaP cells, and co-immunoprecipitation assays were performed to confirm the CDK2 interaction regions \textit{in vivo}. In agreement with our previous report (S2), these truncated proteins were expressed at similar levels. However, only deletion of amino acids 262-655 (FOXO1:1-261) dramatically reduced the \textit{in vivo} association of FOXO1 with CDK2 (fig. S3G). This finding suggests that \textit{in vivo} interaction between CDK2 and FOXO1 can be mediated by FO1-3 and FO1-5 in concert or individually. Together, these results indicate that CDK2 interacts with FOXO1 both \textit{in vitro} and \textit{in vivo}. 
CDK2 inhibits the transcriptional activity of FOXO1

In order to assess the effect of CDK2 on the transcriptional activity of FOXO1, we first examined the effect of CDK2 on the endogenous FOXO proteins in LNCaP cells. As expected, very low transcriptional activity of FOXO proteins was observed in this PTEN-mutated cell line, presumably because of the inhibitory effect imparted by the constitutive activation of Akt (fig. S4A). Indeed, restoring PTEN expression resulted in a dramatic increase in transcriptional activity of FOXOs. This effect was diminished by ectopic expression of CDK2 and cyclin E (fig. S4A). Transcriptional activities of FOXO3a and FOXO4 are not affected by forced expression of CDK2 and cyclin E (fig. S9, D and E). Given that expression level of FOXO6 in LNCaP cells is extremely low (fig. S9A), the effect of CDK2 on endogenous FOXOs is likely mediated by its inhibition of FOXO1.

We were also interested to determine whether expression of CDK2 affects the activation of FOXO1 by inhibition of the endogenous Akt in LNCaP cells. As expected, treatment of LNCaP cells with the PI3-kinase inhibitor LY294002 resulted in a significant increase in transcriptional activity of FOXO1 (fig. S4B). However, this effect was blocked by co-transfection of CDK2 and cyclin E (fig. S4B). In contrast, inhibition of endogenous CDK2 by roscovitine in LY294002-treated cells resulted in a further increase in the activity of FOXO1 (fig. S4C). Furthermore, forced expression of the CDK2 inhibitor p27^KIP1 in DU145 cells resulted in an increase in the transcriptional activity of FOXO1, while silencing of p27^KIP1 decreased FOXO1 activity (fig. S5A). Together, these findings suggest that increased activity of FOXO1 caused by PTEN expression or inhibition of
Akt can be antagonized by overexpression of cyclin E and CDK2, and that inhibition of CDK2 and Akt results in a super activation of FOXO1. Moreover, converting S249 to alanine largely increased the transcriptional activity of FOXO1 (fig. S4D). Little or no difference in transcriptional activity was detected between the S249A single mutant and the S249A/S298A double mutant (fig. S4E), suggesting that phosphorylation of FOXO1 at S249 is critical for CDK2-mediated inhibition of its transcriptional activity.

Effects of CDK2-mediated phosphorylation of FOXO1 on expression of endogenous FOXO-target genes and cell survival

Next, we examined the effect of inhibition of CDK2-mediated phosphorylation of FOXO1 on cell survival by focusing on the expression of endogenous FOXO-target genes including the pro-apoptotic gene Bim, the stress resistance genes MnSOD and catalase, and cell cycle regulator p27\(^{\text{KIP1}}\) \((S13-S16)\). It is known that FOXO1 expression induces apoptosis. To determine whether CDK2-mediated phosphorylation affects the pro-apoptotic function of FOXO1, LNCaP cells were transfected with or without FOXO1, and the activities of endogenous Akt or CDK2 were inhibited by treating cells with LY294002 or roscovitine, respectively. Activation of FOXO1 by either LY294002 or roscovitine was evident with a decrease or loss of phosphorylation of FOXO1 at Ser256 and Ser249. Expression of MnSOD, but not catalase, was increased in cells treated with roscovitine in the presence or absence of the inhibitor of Akt (fig. S6A), suggesting that MnSOD-catalase axis-mediated detoxification \((S17)\) due to activation of FOXO1 is not completely functional in prostate cancer cells. Because of the low level of endogenous FOXO1, LY294002 treatment resulted in only a very limited increase in the
expression of endogenous Bim (fig. S6B). However, an increase in Bim expression was readily apparent in cells treated with LY294002 plus roscovitine (fig. S6B). In agreement with the induction of Bim expression, the greatest level of apoptosis was observed in cells transfected with FOXO1 in the presence of the CDK2 inhibitor roscovitine (fig. S6C). Cleavage of the nuclear poly(ADP-ribose) polymerase (PARP), a marker of apoptosis, followed a similar pattern (fig. S6B). These data indicate that inhibition of CDK2 activity enhances the apoptosis-inducing function of FOXO1.

Moreover, treatment of DU145 cells with camptothecin increased expression of p27KIP1 (fig. S5B). This effect was abolished by knocking down of the endogenous FOXO1 gene (fig. S5B), suggesting that FOXO1 is the key factor mediating the camptothecin-induced expression of p27KIP1. Furthermore, abrogation of camptothecin-mediated induction of p27KIP1 by siRNA did not affect DNA damage-induced cell death (fig. S5C). Thus, expression of p27KIP1 is affected by DNA damage via a FOXO1-dependent mechanism, but increased expression of p27KIP1 has no effect on DNA damage-induced apoptosis.

Effect of CDK2 on the interaction of FOXO1 with CBP and 14-3-3

In order to further look into the underlying mechanism of CDK2-mediated inhibition of FOXO1, we also assessed the interaction of FOXO1 with nuclear co-activator CBP and its binding partner 14-3-3. Consistent with the finding that FOXO1 protein localizes in the cytoplasm, interaction of FOXO1 with the nuclear protein CBP was decreased in cells transfected with CDK2 and cyclin E, and this effect was abolished by treatment of roscovitine (fig. S6E). Moreover, CDK2-promoted cytoplasmic localization of FOXO1
appears to be independent of the binding of FOXO1 to 14-3-3, since no changes were observed in the binding of FOXO1 to 14-3-3, at least the zeta isoform, which has been shown to interact with FOXO proteins (S5) (fig. S6F).

Cellular localization of the FOXO1 protein and apoptosis in cells with low or high degree of DNA damage

Little or no reduction in the phosphorylation of FOXO1 at S249 or apoptosis was detected in cells exposed to a low dose of camptothecin, even though CDK2 activity decreased transiently (fig. S8, A and B). However, inhibition of CDK2-mediated phosphorylation of FOXO1 and apoptosis were observed in cells treated with a high concentration of camptothecin (fig. S8, A and B). FOXO1 proteins were observed primarily in the nuclei under this condition (fig. S8C). These data suggest that activation of the checkpoint pathways by low doses of DNA damaging agents arrests the cell cycle and provides time for DNA repair, but sustained inhibition of FOXO1 phosphorylation at S249 by massive DNA damage allows FOXO1 to be localized in the nucleus, thereby reaching a critical threshold of activity to contribute to apoptosis.
Supporting Figures

**Figure S1A**

**Figure S1B**

| Consensus CDK-phosphorylation motif | FOXO1 (h) 245 | KSGKPGRRAA | FOXO1 (m) 242 | KSGKPGRRAA | Foxx6 (m) 173 | KIGTPFRRAV | RB - T5 1 | MPPTRPMRFAA | RB - S612 608 | SVVSSPPKGRS | RB - S788 784 | RIFSPFRLPS | RB - S811 807 | SPLKSPKISSS |
|-----------------------------------|----------------|-------------|----------------|-------------|----------------|-------------|-------------|----------------|----------------|----------------|-------------|----------------|-------------|----------------|-------------|
| Histone H1B 14 | PVKSPAAAKA | Histone H1B 169 | KVSKSPKAKA | Histone H1B 185 | KVAKSFKAPKA |
| p27Kip1 103 | SVEGPFPRPFL | p107 971 | QQPSPFPLSQ | p107 1305 | KGWGSPRLKK |
| p107 1317 | SADSPARKVC | MAPM/B23 195 | SIRDTPAKWQ | MAPM/B23 230 | RQEKTPTKPG |
| p53 311 | MISSSPQKXX | Smad3 4 | ILRTPPIPYKR | Smad3 175 | MIFETRFPGGL |
| Smad3 269 | PINFGPRHHL | SRCR2 3287 | CTFFSEPAGQA |
**fig. S1.** CDK2 phosphorylates FOXO1 primarily at S249 _in vitro_. (A) Inhibition of CDK2-mediated phosphorylation of FOXO1 by p27KIP1. Reconstituted cyclin E/CDK2 and cyclin A/CDK2 kinase assays, using the GST-FOXO1 fusion proteins FO1-2 and FO1-3 as substrates, were performed in the presence or absence of the CDK inhibitor p27KIP1. After the reaction, 32P-labelled proteins were detected by SDS-PAGE, followed by autoradiography. (B) The putative consensus CDK phosphorylation motif (K/R)(S/T)PX(K/R) (S, serine or T, threonine, which is the CDK phosphorylation residue; P, proline; K, lysine; R, arginine; X, any amino acid) found in human and mouse FOXO1, Foxo6 and other known CDK target proteins. Known CDK phosphorylation proteins (p53, Smad3 and BRCA2), lacking the consensus motif, are also included for comparison. (C) Reconstituted cyclin E/CDK2 kinase assays using the wild-type (WT) and amino acid substitution mutants of FO1-3 as substrates. Phosphorylation levels of each substrate were quantified, and the relative level of the mutants was compared to that of wild-type substrate. (D) Schematic diagram of the three overlapping GST-FOXO1 fusion proteins in the FO1-3 region of FOXO1. The eight putative CDK phosphorylation sites (S/T-P motifs) are indicated. (E) Reconstituted cyclin E/CDK2 kinase assays using the GST-fusion proteins shown in (D) as substrates.
**fig. S2.** Specificity of the antibody (S249-p) generated against the peptide containing the S249 phosphorylation site. (A) LNCaP cells were transfected with FLAG-FOXO1. At 48 h after transfection, cells were harvested and lysed with IP buffer. Whole cell lysates were subjected to immunoprecipitation with anti-FLAG antibody M2. Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting with anti-S249-p antibody (lane1), pre-immune sera (lane 2), anti-S249-p antibody pre-blocked with the peptide containing the phosphorylated (NPEGGKSGKpSPRRRAAS, lane 3) or non-phosphorylated (NPEGGKSGKSPRRRAAS) S249 (lane 4). (B) Wild-type FLAG tagged FOXO1 was transfected into LNCaP cells. FLAG-FOXO1 proteins were immunoprecipitated with M2, and treated with or without protein phosphatase (PPase) from bacteriophage λ in the presence or absence of protein phosphatase inhibitors sodium fluoride and sodium orthovanadate. Treated proteins were analyzed by Western blot with antibodies for phosphorylated (upper panel) and total FOXO1 protein (lower panel). (C) LNCaP cells were transfected with plasmids for wild-type and S249A mutant of FOXO1 and expression vector for CDK2-AF, as indicated. At 48 h after transfection, cells were lysed and IP was performed as in (A). Immunoprecipitated proteins were analyzed by SDS-PAGE and Western blotting with antibodies for S249-p, FOXO1, and CDK2.
**fig. S3.** CDK2 interacts with FOXO1 *in vitro* and *in vivo*. (A) Co-immunoprecipitation of FLAG-FOXO1 and V5-CDK2 from LNCaP cells with an anti-FLAG antibody. IP, immunoprecipitation; IB, immunoblotting. (B) Co-immunoprecipitation of endogenous FOXO1 and CDK2 in LNCaP cells with an anti-CDK2 antibody. (C) Co-immunoprecipitation of endogenous FOXO1 and CDK2 in LNCaP cells with an anti-FOXO1 antibody. (D) Whole cell lysates of LNCaP were subjected to GST pull-down by bacterially expressed GST-CDK2 in the presence or absence of cyclin E. (E) *In vitro* binding of wild-type CDK2 to GST-FOXO1 fusion proteins. (F) Diagram of a series of FLAG-tagged C-terminal truncated FOXO1 mammalian expression vectors. The forkhead DNA binding domain (FKD), nuclear localization signal domain (NLS), nuclear export signal domain (NES), and transactivation domain (TAD) of the protein are indicated. (G) Co-immunoprecipitation of endogenous CDK2 and C-terminal truncation forms of FLAG-FOXO1 from LNCaP cells with an anti-FLAG antibody.
**fig. S4.** CDK2 inhibits the transcriptional activity of FOXO1. (A) The effect of CDK2 expression on transcriptional activity of endogenous FOXO proteins in LNCaP cells. LNCaP cells were transfected with a luciferase reporter construct containing the promoter of IGFBP-1 gene (3xIRS), and plasmids as indicated. At 36 h after transfection, luciferase activities were measured and analyzed as described in Materials and Methods. * P< 0.05; **P < 0.05. (B) Effect of CDK2/cyclin E on the activation of FOXO1 by PI3K inhibitor LY294002. LNCaP cells were transfected with plasmids as indicated. At 24 h after transfection, cells were treated with LY294002 (20 µM) for 24 h. Luciferase measurements and statistical analysis were performed as in (A). * P< 0.05; **P < 0.05. (C) Effect of inhibition of the endogenous CDK2 on the activation of FOXO1. Wild-type FOXO1 expression vector and the FasL promoter reporter construct were transfected into LNCaP cells. At 24 h after transfection, cells were treated with LY294002 (20 µM) in combination with or without the CDK2 inhibitor roscovitine (30 µM) for 24 h. Luciferase measurement and statistical analysis were performed as in (A). (D) Effect of mimic S249 phosphorylation on FOXO1 transactivation. Expression vectors for the wild-type and phosphorylation-mimicking mutant (S249D) of FOXO1, PTEN, and an empty control vector, as well as the FasL promoter reporter construct were transfected into LNCaP cells. At 36 h after transfection, cells were collected for luciferase analysis. Luciferase measurement and statistical analysis were performed as in (A). (E) LNCaP cells were transfected with plasmids as indicated. At 36 h after transfection, luciferase activities were measured and analyzed as in (A).
**Fig. S5.** Expression and function of p27KIP1 in cells with DNA damage. (A) Effect of p27KIP1 on transcriptional activity of FOXO1. DU145 cells were transfected with plasmids and/or siRNAs as indicated. At 48 h after transfection, cells were harvested and lysed, either for measurement of luciferase activity or immunoblotting with anti-p27KIP1 antibody. Immunoblotting of Erk2 was used as a loading control. *P < 0.05; **P < 0.05. (B) Effect of DNA damage on expression of p27KIP1. DU145 cells were mock transfected, or transfected with non-specific control siRNA or FOXO1-specific siRNA. At 48 h after transfection, cells were treated with vehicle or different concentrations (100 nM or 1 µM) of camptothecin (CPT). Cells were collected and lysed for expression of p27KIP1 and FOXO1 at 24 h after CPT treatment. (C) Effect of knocking down of p27KIP1 on CDK2 activity and DNA damage-induced apoptosis. DU145 cells were mock transfected or transfected with non-specific control siRNA and p27KIP1-specific siRNA. At 24 h after transfection, cells were treated with or without CPT (1 µM). Cells were harvested at 48 h after CPT treatment. Apoptotic cell death, expression of p27KIP1, and CDK2 kinase activity were analyzed.
**fig. S6.** Effect of CDK2-mediated phosphorylation of FOXO1 on cell death, and protein interaction of FOXO1 with CBP and 14-3-3. (A) LNCaP cells were transfected with or without wild-type FOXO1 for 36 h, and treated with LY294002 (20 µM) in the presence or absence of roscovitine (30 µM) for 48h. Expression of MnSOD was analyzed by Western blot. (B) Effect of inhibition of CDK2 on expression of FOXO-regulated gene Bim. LNCaP cells were transfected with or without wild-type FOXO1 for 36 h, and treated with LY294002 (20 µM) in the presence or absence of roscovitine (30 µM) for 24h. Expression of Bim and PARP was analyzed by Western blot. (C) LNCaP cells were transfected and treated as indicated in (B). Apoptosis was analyzed at 48 h after roscovitine treatment and quantified using a morphometric cell death assay. (D) Amino acid sequences of the putative nuclear localization signal (NLS) motifs at the C-terminal end of the DNA binding domains of human FOXO1 and mouse Foxo1 and Foxo6. Basic amino acid residues in the NLS motif are marked in green. Three arginines, which were demonstrated previously to play a major role in the nuclear localization of FOXO3a, are highlighted with a rectangle. The CDK2 sites are underlined. The CDK2 phosphorylation site is in red. (E, F) Co-immunoprecipitation of FLAG-FOXO1 with endogenous CBP or 14-3-3ζ. LNCaP (E) and LNCaP or DU145 (F) cells were transfected with plasmids or siRNAs as indicated. At 42 h after transfection, cells were treated with 30 µM of roscovitine or vehicle for 6 h. Cells were harvested and lysed for immunoprecipitation with an anti-FLAG antibody, and immunoblotting for CBP and FOXO1 (E) or S249-p and 14-3-3ζ (F). IP, immunoprecipitation; IB, immunoblotting.
**fig. S7.** Effect of camptothecin and γ-irradiation on CDK2-dependent phosphorylation of FOXO1. (A) LNCaP-FOC4 cells were treated with (+) or without (-) 1.25 µM of camptothecin (CPT). At 16 h after treatment, cells were collected and cell extracts were subjected to immunoprecipitation (IP) with an anti-FLAG antibody, immunoblotting (IB) with antibodies for S249-p and FOXO1, and *in vitro* CDK2 kinase assay using histone H1 as substrate. (B) PC-3 cells were transfected with an expression vector of FLAG-FOXO1 for 48 h, and cells were treated with (+) or without (-) 1.25 µM CPT. At 16 h after treatment, cells were collected and cell extracts were analyzed as in (A). (C) LNCaP-FOC4 cells were treated with (+) or without (-) 20 Gy of γ-irradiation (γ-Irr). At 2 h after γ irradiation, cells were collected and cell extracts were subjected to immunoprecipitation (IP) with anti-FLAG antibody, immunoblotting (IB) with antibodies for S249-p and FOXO1, and *in vitro* CDK2 kinase assay using histone H1 as substrate. (D) Effect of Chk1 inhibitor UCN-01 on S249 phosphorylation. LNCaP-FOC4 cells were pre-treated with (+) or without (-) 200 nM of the Chk1 inhibitor UCN-01 for 1 h, and then treated with 1.25 µM CPT or mock-treated for 16 h. IP and IB were performed as in (A). (E) PC-3 cells were transfected with an expression vector of FLAG-FOXO1, two independent Chk2-specific siRNAs, or two control siRNAs. At 48 h after transfection, cells were treated with 1.25 µM CPT or mock-treated for 16 h. IP and IB were performed as in (A).
**Fig. S8.** Effect of low or high doses of camptothecin on CDK2 inhibition, FOXO1 activation, Bim expression, and cell death. (A) DU145 cells were treated with low (10 nM) or high (1 µM) concentration of camptothecin for different periods of time. FOXO1 and CDK2 proteins were immunoprecipitated. Expression of S249-p and FOXO1 was examined by Western blotting, and CDK2 activities were analyzed by *in vitro* kinase assay using histone H1 as substrate. An additional aliquot of cells were lysed and analyzed directly by Western blot for Bim expression. (B) Apoptosis analysis in DU145 cells treated with different doses of camptothecin at different time points as indicated. Similar results were obtained from two independent experiments. (C) Cellular localization of ectopically expressed wild-type FOXO1 in DU145 cells with or without DNA damage. DU145 cells grown in culture medium containing 5% FBS were transfected with FLAG-tagged FOXO1. At 36 h after transfection, cells were mock treated (DMSO), or treated with different concentrations of CPT (10 nM or 1 µM). At 24 h after CPT treatment, cells were subjected to immunofluorescent chemistry. Quantification of a representative experiment is shown on the right. Similar results were obtained from three independent experiments.
**fig. S9.** Effect of CDK2 on transcriptional activity and function of FOXO3a and FOXO4. (A) Total RNA from DU145, LNCaP, MCF7 and MCF10A cell lines were subjected to quantitative real-time RT-PCR for the expression of FOXO1, FOXO3a, FOXO4 and FOXO6. A PCR result from human brain cDNA library (Invitrogen) was included as a positive control for FOXO6 expression. Results are presented as expression levels of these genes relative to those in DU145 cells. (B) Upper panel, alignment of amino acid sequences around potential CDK phosphorylation sites in FOXO3a and FOXO4. The putative consensus CDK phosphorylation motif (K/R)(S/T)PX(K/R) is also included for comparison (S, serine or T, threonine, which is the CDK phosphorylation residue; P, proline; K, lysine; R, arginine; X, any amino acid). Lower panel, diagrams showing the locations of the CDK2 site (S249) in FOXO1, the potential site S173 in FOXO3a, and S268 in FOXO4. FKD, forkhead DNA binding domain; NLS, nuclear localization signal.
**fig. S9 – continued.** (C) Upper panel: Reconstituted cyclin A-CDK2 and cyclin E-CDK2 kinase assays. Reconstituted cyclin A-CDK2 (0.1 µg, left) and reconstituted cyclin E-CDK2 (0.1 µg, right) *in vitro* kinase assays were performed using 0.2 µg of the following substrates: RB-C, GST, GST-FOXO1 fusion protein FO1-2, wild-type and mutated (S173A) GST-FOXO3a (148-280), or wild-type and mutated (S268A) GST-FOXO4 (92-288). Lower panel: Protein substrates indicated by Coomassie blue staining. (D) Effect of CDK2 on transcriptional activity of ectopically expressed FOXO3a. DU145 cells were transfected with FOXO reporter gene 3xIRS, HA-tagged FOXO3a expression vector, plasmids for wild-type and mutated CDK2 and cyclin E, CDK2 siRNAs or cyclin E alone. At 36 h after transfection, cells were harvested for luciferase measurement (upper panel), or Western blotting with an anti-CDK2 antibody (lower panel). (E) Effect of CDK2 on transcriptional activity of ectopically expressed FOXO4. DU145 cells were transfected with FOXO reporter gene 3xIRS, expression vectors for HA-tagged wild-type and mutated (S268A) FOXO4, plasmids for wild-type and mutated CDK2 and cyclin E, a pool of CDK2 siRNAs. At 36 h after transfection, cells were harvested for luciferase measurement (upper panel), or Western blotting with an anti-CDK2 antibody (lower panel). (F) Effect of silencing of FOXO1, FOXO3a and FOXO4 on DNA damage-induced cell death. DU145 cells were transfected with expression plasmids for enhanced green fluorescent protein (EGFP) and siRNAs for FOXO1, FOXO3a or FOXO4. Twenty-four hours after transfection, cells were sorted and re-plated. At 24 hours after re-plating, cells were collected and Western blotted for expression of FOXO1 and FOXO3a (lower panel), analyzed by RT-PCR for FOXO4 expression, or treated with or without 1 µM of CPT. At 48 h after CPT treatment, three sets of cells were analyzed for apoptosis (upper panel). * P < 0.05.
**fig. S10.** Role of FOXO transcriptional factors in DNA damage-induced apoptosis. (A) DU145 cells were transfected with FOXO1 specific siRNA or a control siRNA. At 48 h after transfection, cells were treated with 1.25 µM camptothecin (CPT). Apoptosis was counted by flow cytometry and percentage of cells in sub-G1 was analyzed by the WINMDI software. The result of a representative experiment is shown in the left panel, and quantification of results from three independent experiments is shown in the right panel. (B) DU145 cells were transfected with expression plasmids for enhanced green fluorescent protein (EGFP), plasmids and siRNAs as indicated. Twenty-four hours after transfection, cells were sorted and re-plated in medium containing 2.5% FBS. At 24 hours after re-plating, cells were treated with 1.25 µM CPT. At 48 h after CPT treatment, three sets of cells were analyzed for apoptosis. (C) DU145 cells were transfected and cultured as in Fig. 4C. At 48 h after CPT treatment, three sets of cells were analyzed for luciferase activity. *P < 0.05; **P < 0.05.
**fig. S11.** Role of the FOXO1 pathway in DNA damage-induced apoptosis in p53-proficient cancer and normal cells. (A) MCF7 breast cancer cells were transfected with plasmids and siRNAs as indicated, and expression plasmids for enhanced green fluorescent protein (EGFP). Cells were sorted and re-plated 24 h after transfection. At 24 hours after re-plating, cells were collected and Western blotted for expression of FOXO1 and p53 (middle panel) or treated with 1 µM CPT. At 48 h after CPT treatment, three sets of cells were analyzed for apoptosis (top panel). Luciferase activity of the reporter constructs was measured in viable cells in triplicate (bottom panel). * P < 0.05; **P < 0.05; ***P < 0.05.
**fig. S11 - continued.** (B) MCF7 cells were transfected with non-specific siRNA, FOXO1-specific siRNA, silencing resistant wild-type and S249A mutant of FOXO1 (FOXO1-WTsr and FOXO1-S249Asr), an active mutant CDK2-AF, and expression plasmid for enhanced green fluorescent protein (EGFP) as indicated. Twenty-four hours after transfection, cells were sorted and re-plated. At 24 hours after re-plating, cells were collected and Western blotted for expression of FOXO1 and CDK2 (lower panel) or treated with 1 µM CPT. At 48 h after CPT treatment, three sets of cells were analyzed for apoptosis (upper panel). * P < 0.05; **P < 0.05. (C) MCF10A ‘normal’ (non-tumorigenic) breast epithelial cells were transfected with expression plasmids for enhanced green fluorescent protein (EGFP) and siRNAs for FOXO1 and p53. Twenty-four hours after transfection, cells were sorted and re-plated. At 24 hours after re-plating, cells were collected and Western blotted for expression of FOXO1 and p53 (lower panel) or treated with 1 µM CPT. At 36 h after CPT treatment, three sets of cells were analyzed for apoptosis (upper panel). * P < 0.05; **P < 0.05. (D) Wild-type MEF cells cultured in DMEM containing 15% FBS were transfected with expression plasmids for enhanced green fluorescent protein (EGFP), and siRNAs for mouse Foxo1 and Foxo6. Twenty-four hours after transfection, cells were sorted and re-plated. At 24 hours after re-plating, cells were collected and Western blotted for expression of Foxo1 (lower panel), analyzed by RT-PCR for Foxo6 expression, or treated with or without 1 µM of CPT. At 48 h after CPT treatment, three sets of cells were analyzed for apoptosis (upper panel). *P < 0.05.
References