福井大学リポジトリ
UNIVERSITY OF FUKUI
REPOSITORY

Activation of FOXO1 by Cdk1 in cycling cells and postmitotic neurons

メタデータ	言語: eng 出版者: 公開日: 2012-04-25 キーワード (Ja): キーワード (En): 作成者: YUAN, Zengqiang, BECKER, Esther B.E., MERLO, Paola, YAMAD, Tomoki, DIBACCO, Sara, KONISHI, Yoshiyuki, SCHAEFER, Erik M., BONNI, Azad メールアドレス: 所属:
URL	http://hdl.handle.net/10098/5229

Activation of FOXO1 by Cdk1 in Cycling Cells and Postmitotic Neurons

Zengqiang Yuan,[1]*† Esther B. E. Becker,[1]* Paola Merlo,[1] Tomoko Yamada,[1] Sara DiBacco,[1] Yoshiyuki Konishi,[1] Erik M. Schaefer,[2] Azad Bonni[1]‡

Activation of cyclin-dependent kinase 1 (Cdk1) has been linked to cell death of postmitotic neurons in brain development and disease. We found that Cdk1 phosphorylated the transcription factor FOXO1 at Ser^{249} in vitro and in vivo. The phosphorylation of FOXO1 at Ser^{249} disrupted FOXO1 binding with 14-3-3 proteins and thereby promoted the nuclear accumulation of FOXO1 and stimulated FOXO1-dependent transcription, leading to cell death in neurons. In proliferating cells, Cdk1 induced FOXO1 Ser^{249} phosphorylation at the G_2/M phase of the cell cycle, resulting in FOXO1-dependent expression of the mitotic regulator Polo-like kinase (Plk). These findings define a conserved signaling link between Cdk1 and FOXO1 that may have a key role in diverse biological processes, including the degeneration of postmitotic neurons.

The protein kinase Cdk1 is a key mediator of neuronal cell death that is relevant to brain development and degeneration (*1*–*6*). As a major apoptotic kinase, Cdk1 might be expected to orchestrate a program of gene expression that activates the cell death machinery. Because Cdk1 resides in the cytoplasm in neurons (*1*, *5*, *6*), we reasoned that Cdk1 might regulate gene expression through proteins that shuttle between the cytoplasm and nucleus. The FOXO transcription factors undergo nucleocytoplasmic shuttling and control cell death (*7*, *8*). We therefore investigated the role of FOXO proteins in propagating the Cdk1 cell death signal to the nucleus in postmitotic neurons.

FOXO1 contains a conserved putative Cdk1 phosphorylation site within the forkhead domain at Ser^{249} (fig. S1A). Cdk1 catalyzed the phosphorylation of FOXO1 in vitro (Fig. 1A) (*9*). Cdk1 also phosphorylated the FOXO1 forkhead domain (FOXO1FD) in vitro, but failed to phosphorylate a FOXO1FD mutant in which Ser^{249} was replaced with alanine (FOXO1FD S249A) (fig. S1B). We generated an antibody that specifically recognizes FOXO1 that is phosphorylated at Ser^{249} (*9*). The phosphoS249-FOXO1 antibody recognized recombinant FOXO1 or FOXO1FD that was phosphorylated by Cdk1 in vitro but did not recognize unphosphorylated FOXO1 or the FOXO1FD S249A mutant that was incubated with Cdk1 in vitro (Fig. 1B and fig. S1C). We expressed cyclin B and Cdk1 in 293T cells together with FOXO1 or a S249A FOXO1 mutant. Immunoblotting of total lysates or FOXO1 immunoprecipitates of transfected cells revealed that Cdk1 increased the amount of phosphorylated FOXO1 at Ser^{249} in cells (Fig. 1C and fig. S1D). In other experiments, depletion of endogenous Cdk1 by RNA interference (RNAi) reduced the FOXO1 phosphorylation in cells (Fig. 1D and fig. S1E), which suggested a requirement for endogenous Cdk1 in the FOXO1 phosphorylation at Ser^{249} in cells.

We tested whether the activation of endogenous Cdk1 induced the phosphorylation of endogenous FOXO1 at Ser^{249} in neurons. Endogenous Cdk1 is activated in cerebellar granule neurons upon inhibition of membrane depolarization (*1*, *3*). We found that the amount of FOXO1 Ser^{249} phosphorylation was higher in neurons deprived of membrane-depolarizing concentrations of KCl (5 mM KCl) than in neurons maintained in depolarizing medium (30 mM KCl) (Fig. 1E). The Cdk1 inhibitor roscovitine reduced the FOXO1 Ser^{249} phosphorylation in neurons deprived of depolarization (Fig. 1E). Thus, endogenous Cdk1 appears to mediate activity deprivation–induced phosphorylation of endogenous FOXO1 at Ser^{249} in neurons.

The identification of Cdk1-induced phosphorylation of FOXO1 at Ser^{249} led us to test whether the FOXO1 phosphorylation mediated the ability of Cdk1 to trigger cell death in neurons. Because endogenous Cdk1 is required for apoptosis of activity-deprived neurons (*1*, *3*), we determined the role of FOXO1 in apoptosis of neurons deprived of activity. We transfected neurons with the U6/foxo RNAi or control U6 plasmid. FOXO RNAi reduced the expression of FOXO1 in primary granule neurons and

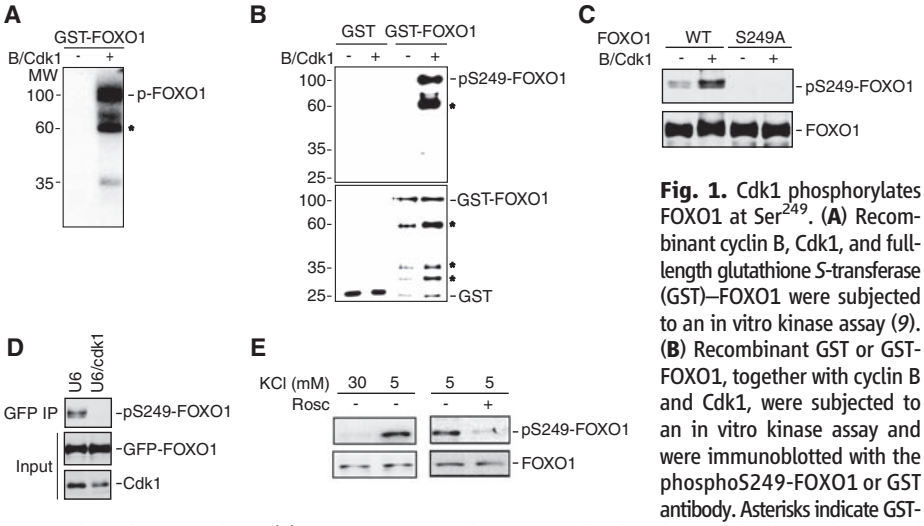

Fig. 1. Cdk1 phosphorylates FOXO1 at Ser^{249}. (**A**) Recombinant cyclin B, Cdk1, and full-length glutathione *S*-transferase (GST)–FOXO1 were subjected to an in vitro kinase assay (*9*). (**B**) Recombinant GST or GST-FOXO1, together with cyclin B and Cdk1, were subjected to an in vitro kinase assay and were immunoblotted with the phosphoS249-FOXO1 or GST antibody. Asterisks indicate GST-FOXO1 degradation products. (**C**) Lysates of 293T cells transfected with cyclin B and Cdk1 or the control vector, together with the green fluorescent protein (GFP) fusion protein GFP-FOXO1 or the GFP-FOXOS249A mutant, were immunoblotted with the phosphoS249-FOXO1 antibody or a mouse monoclonal FOXO1 antibody. (**D**) Lysates of Neuro2A cells transfected with the control U6 or U6/cdk1 RNAi plasmid and GFP-FOXO1 were immunoprecipitated with the GFP antibody and immunoblotted with the phosphoS249-FOXO1 antibody. Lysates were also immunoblotted with the GFP or Cdk1 antibody. (**E**) Lysates of granule neurons maintained in membrane-depolarizing medium (30 mM KCl) or in which depolarization was inhibited (5 mM KCl) in the presence of the Cdk1 inhibitor roscovitine (10 μM) or its vehicle [dimethyl sulfoxide (DMSO)] were immunoblotted with the phosphoS249-FOXO1 or FOXO1 antibody.

[1]Department of Pathology, Harvard Medical School, 77 Avenue Louis Pasteur, Boston, MA 02115, USA. [2]Center for Signal Transduction, Cellular Analysis Business Unit/BioDiscovery Division, Invitrogen Corporation, 94 South Street, Hopkinton, MA 01748, USA.

*These authors contributed equally to this work.
†Present address: Institute of Biophysics, Chinese Academy of Sciences, 15 Datun Road, Beijing 100101, China.
‡To whom correspondence should be addressed. E-mail: azad_bonni@hms.harvard.edu

Downloaded from www.sciencemag.org on March 20, 2008

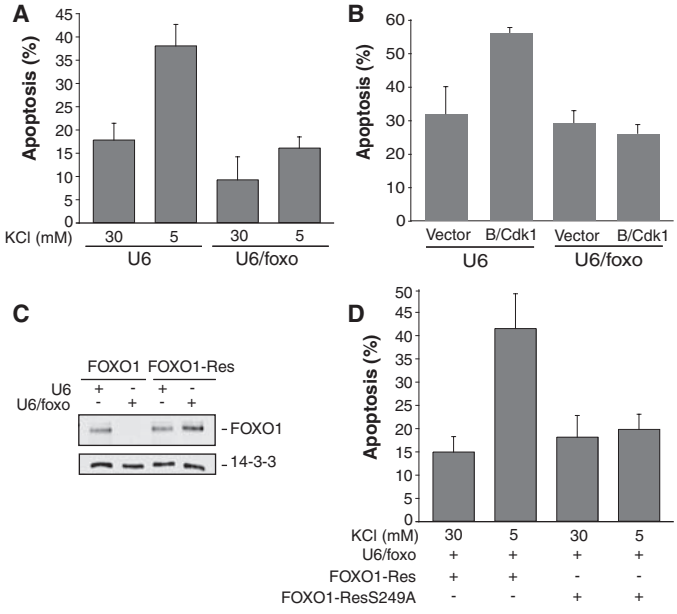

Fig. 2. Cdk1-FOXO1 signaling mediates activity deprivation–induced neuronal death. (**A**) Granule neurons transfected with the U6/foxo RNAi or control U6 plasmid were placed in membrane-depolarizing medium (30 mM KCl) or deprived of membrane depolarization (5 mM KCl) for 30 hours and subjected to analysis of cell death (*9*). FOXO RNAi attenuated activity deprivation–induced neuronal cell death [mean ± SEM, $n = 4$; $P < 0.005$, analysis of variance (ANOVA) followed by Fisher's PLSD post hoc test] but not cell death in depolarized neurons (see also fig. S2C). (**B**) Granule neurons transfected with the U6/foxo RNAi or control U6 plasmid, together with cyclin B and Cdk1 or the control vector, were analyzed as in (A). FOXO RNAi inhibited cyclin B and Cdk1–induced cell death (mean ± SEM, $n = 3$; $P < 0.005$, ANOVA followed by Fisher's PLSD post hoc test). (**C**) Lysates of COS cells transfected with the U6/foxo RNAi or control U6 plasmid together with FOXO1 or an RNAi-resistant rescue form of FOXO1 (FOXO1-Res) were immunoblotted with a FOXO1 or 14-3-3 antibody (*9*). (**D**) Granule neurons transfected with the U6/foxo RNAi plasmid together with FOXO1-Res or the FOXO1-ResS249A mutant were analyzed as in (A). FOXO1-Res, but not FOXO1-ResS249A, induced cell death in activity-deprived neurons in the background of FOXO RNAi (mean ± SEM, $n = 3$; $P < 0.05$, ANOVA followed by Fisher's PLSD post hoc test).

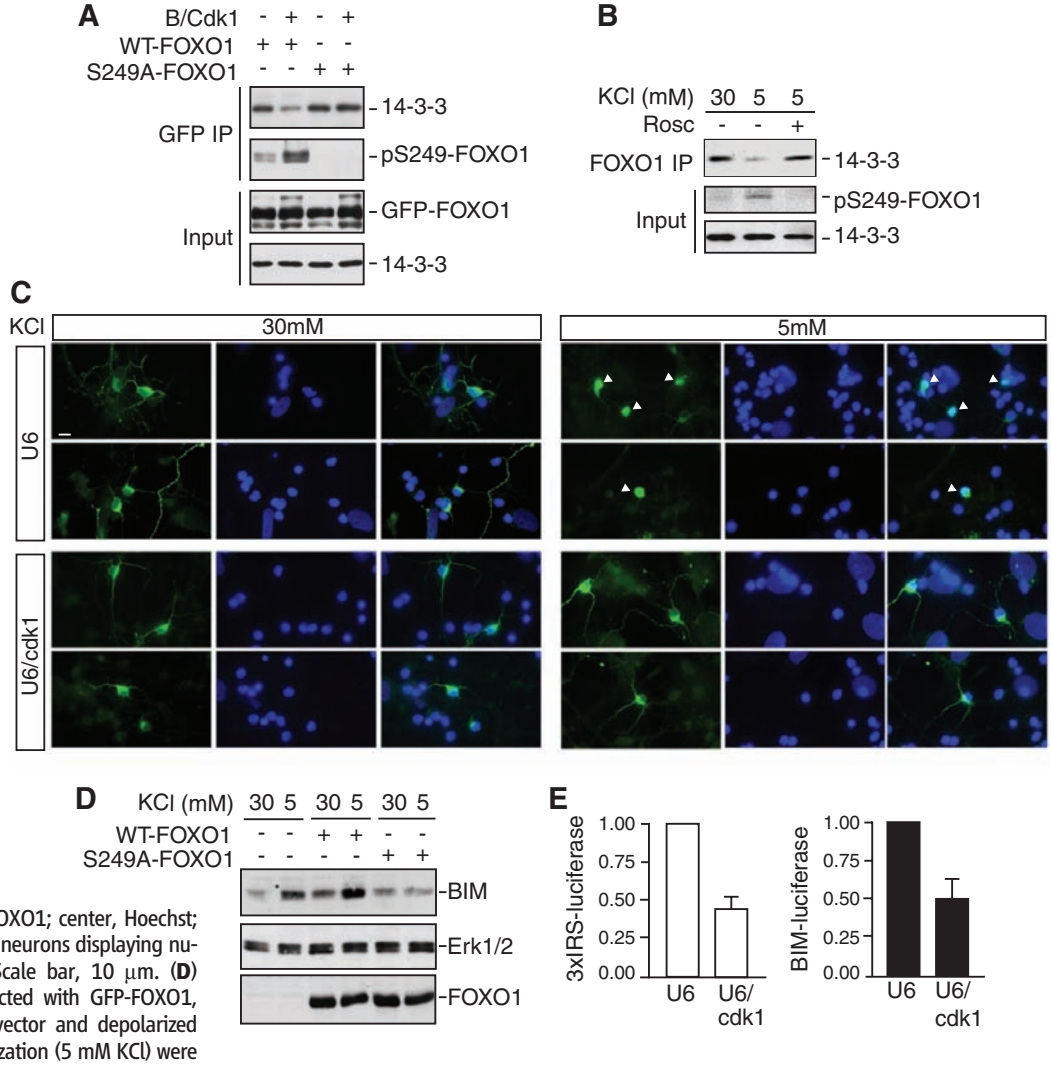

Fig. 3. FOXO1 Ser^{249} phosphorylation disrupts the FOXO1–14-3-3 interaction and promotes nuclear accumulation of FOXO1, leading to activation of transcription. (**A**) Lysates of 293T cells transfected with GFP-FOXO1 or GFP-FOXO1S249A, together with cyclin B and Cdk1 or the control vector, were immunoprecipitated with the GFP antibody and immunoblotted with the 14-3-3 or phosphoS249-FOXO1 antibody. Lysates were also immunoblotted with the GFP or 14-3-3 antibody. (**B**) Lysates of granule neurons maintained in depolarizing medium (30 mM KCl) or deprived of depolarization (5 mM KCl), in the presence of the Cdk1 inhibitor roscovitine (10 μM) or its vehicle (DMSO), were immunoprecipitated with the FOXO1 antibody and immunoblotted with the 14-3-3 antibody. Lysates were immunoblotted with the phosphoS249-FOXO1 or 14-3-3 antibody. (**C**) Granule neurons transfected with GFP-FOXO1 together with the U6/cdk1 RNAi or control U6 plasmid maintained in depolarizing medium (30 mM KCl) or deprived of depolarization (5 mM KCl) were analyzed by fluorescence microscopy. Representative images are shown (left, GFP-FOXO1; center, Hoechst; right, merged). Arrowheads indicate neurons displaying nuclear localization of GFP-FOXO1. Scale bar, 10 μm. (**D**) Lysates of granule neurons transfected with GFP-FOXO1, GFP-FOXO1S249A, or the control vector and depolarized (30 mM KCl) or deprived of depolarization (5 mM KCl) were immunoblotted with the BIM, Erk1/2, or FOXO1 antibody. (**E**) Granule neurons transfected with the U6/cdk1 RNAi or control U6 plasmid together with the 3xIRS-luciferase (left) or BIM-luciferase (right) reporter gene and tk-renilla were deprived of depolarization and subjected to luciferase assays. Cdk1 knockdown reduced the expression of the 3xIRS (mean ± SEM, $n = 3$; $P < 0.001$, *t* test) and BIM-luciferase (mean ± SEM, $n = 3$; $P < 0.005$, *t* test) reporter gene.

Downloaded from www.sciencemag.org on March 20, 2008

protected neurons against cell death induced by suppression of depolarization (Fig. 2A and fig. S2A) (*9*). FOXO RNAi also suppressed the ability of expression of cyclin B and Cdk1 to induce cell death in neurons (Fig. 2B). To determine the importance of the phosphorylation of FOXO1 at Ser^{249} in activity deprivation–induced neuronal apoptosis, we constructed an expression plasmid encoding an RNAi-resistant form of FOXO1 (FOXO1-Res) (*9*). Whereas FOXO RNAi induced depletion of FOXO1 encoded by wild-type cDNA, FOXO RNAi failed to induce efficient depletion of FOXO1-Res (Fig. 2C). Expression of FOXO1-Res triggered apoptosis in FOXO-depleted neurons deprived of activity (Fig. 2D). By contrast, a FOXO1-Res mutant in which Ser^{249} was replaced with alanine (FOXO1-ResS249A) failed to effectively induce neuronal cell death in the background of FOXO RNAi (Fig. 2D). Thus, the Ser^{249} phosphorylation appears to be required for the ability of FOXO1 to mediate activity deprivation–induced neuronal apoptosis.

We characterized the mechanism by which the Cdk1-induced phosphorylation of FOXO1 at Ser^{249} stimulates FOXO1-dependent neuronal cell death. Ser^{249} lies within a conserved short FOXO1 peptide motif that includes the Akt site of phosphorylation, Ser^{256} (fig. S1A). Because Ser^{256}-phosphorylated FOXO1 interacts with 14-3-3 proteins, leading to FOXO1's cytoplasmic sequestration and inhibition (*7*, *8*), we tested whether phosphorylation of FOXO1 at Ser^{249} might regulate FOXO1's interaction with 14-3-3 proteins. Although FOXO1 or its forkhead domain interacted efficiently with 14-3-3 proteins, expression of Cdk1 reduced these interactions (Fig. 3A and fig. S3A). However, Cdk1 failed to inhibit the interaction of FOXO1S249A with 14-3-3 proteins (Fig. 3A). Cdk1 did not affect Ser^{256} phosphorylation in FOXO1 or its forkhead domain (fig. S3, A and B), which suggests that the Ser^{249} phosphorylation inhibits the interaction of Ser^{256}-phoshorylated FOXO1 with 14-3-3 proteins.

To ascertain whether endogenous Cdk1 might regulate the interaction of endogenous FOXO1 with 14-3-3 proteins in neurons, we assayed the association of these proteins in granule neurons in the presence or absence of membrane depolarization. In depolarized neurons, endogenous FOXO1 interacted with endogenous 14-3-3 proteins (Fig. 3B). However, withdrawal of depolarization, which stimulates the activity of endogenous Cdk1 in neurons (*1*, *3*), reduced the interaction (Fig. 3B). The Cdk1 inhibitor roscovitine blocked the ability of activity deprivation to disrupt FOXO1's association with 14-3-3 proteins (Fig. 3B). These findings suggest that endogenous Cdk1-induced phosphorylation of FOXO1 at Ser^{249} releases FOXO1 from sequestration by 14-3-3 proteins in neurons.

Because 14-3-3 proteins sequester FOXO proteins in the cytoplasm (*7*, *8*), we characterized the consequences of the Ser^{249} phosphorylation on the subcellular localization of FOXO1. Whereas FOXO1 was localized predominantly in the cytoplasm in depolarized neurons, FOXO1 accumulated in the nucleus in neurons deprived of activity (Fig. 3C and fig. S4, A to C). Depletion of endogenous Cdk1 by RNAi reduced the nuclear accumulation of FOXO1 in activity-deprived neurons (Fig. 3C and fig. S4, A to C). In other experiments, expression of cyclin B and Cdk1 triggered the nuclear accumulation of wild-type FOXO1 but not FOXO1S249A in neurons (fig. S5). These results suggest that Cdk1-induced phosphorylation of FOXO1 Ser^{249} stimulates the nuclear accumulation of FOXO1 in neurons.

The finding that Ser^{249}-phosphorylated FOXO1 accumulates in the nucleus led us to test whether Cdk1-FOXO1 signaling might activate FOXO-dependent transcription. Overexpression of Cdk1 augmented the ability of FOXO1, but not of FOXO1S249A, to stimulate the expression of a luciferase reporter gene controlled by a FOXO-responsive promoter (3xIRS-luciferase) in granule neurons (fig. S6) (*9*). Withdrawal of membrane depolarization induced the endogenous expression of the FOXO-responsive apoptotic gene *BIM* in granule neurons (Fig. 3D). Expression of FOXO1S249A, but not of FOXO1, reduced the induction of endogenous BIM expression upon activity deprivation (Fig. 3D). Depletion of endogenous Cdk1 by RNAi in neurons reduced the expression of both the 3xIRS-luciferase reporter gene and a reporter gene controlled by the BIM promoter (Fig. 3E). Together, these results suggest that endogenous Cdk1-induced phosphorylation of FOXO1 at Ser^{249} stimulates FOXO1-dependent transcription, leading to BIM expression and consequent neuronal cell death.

Overexpression of the Cdk1-related protein kinase Cdk2 was reported to phosphorylate FOXO1 at Ser^{249}, thereby inducing the nuclear exclusion and inhibition of FOXO1 in prostate cancer cells (*10*). We found that overexpression of Cdk2 or Cdk1 stimulated FOXO1-dependent transcription and failed to promote the nuclear export of

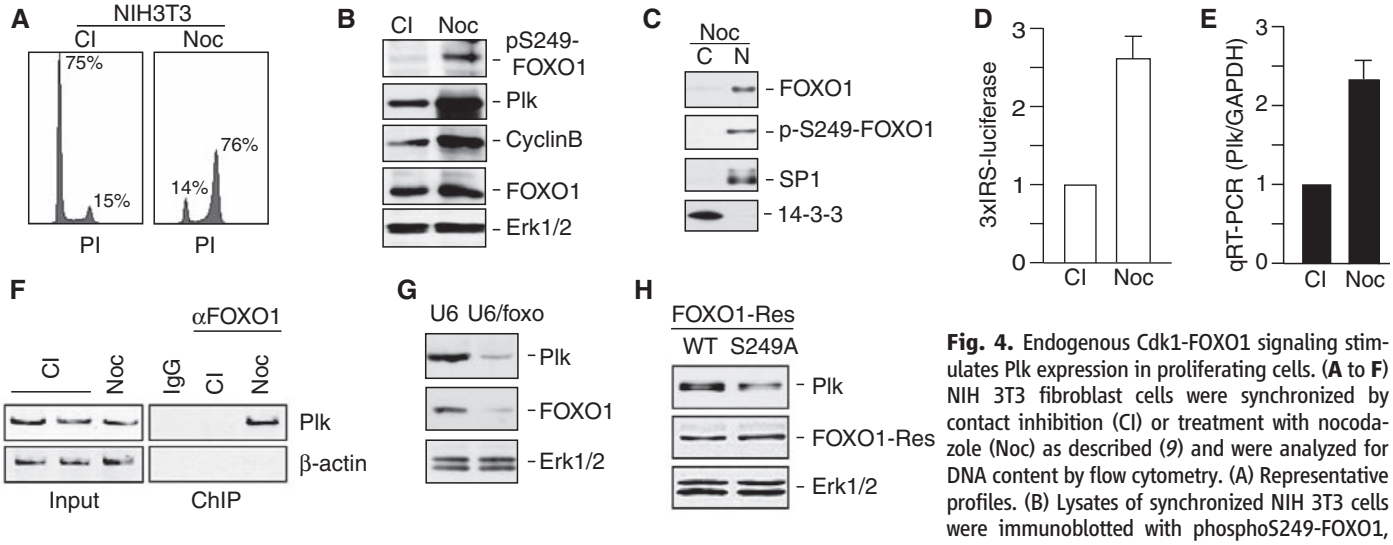

Fig. 4. Endogenous Cdk1-FOXO1 signaling stimulates Plk expression in proliferating cells. (**A** to **F**) NIH 3T3 fibroblast cells were synchronized by contact inhibition (CI) or treatment with nocodazole (Noc) as described (*9*) and were analyzed for DNA content by flow cytometry. (A) Representative profiles. (B) Lysates of synchronized NIH 3T3 cells were immunoblotted with phosphoS249-FOXO1, Plk1, cyclin B, FOXO1, or Erk1/2 antibody. (C) Lysates of NIH 3T3 cells synchronized in G_2/M were fractionated into cytoplasmic and nuclear components and immunoblotted with the FOXO1, phosphoS249-FOXO1, SP1, or 14-3-3 antibody. The 14-3-3 and SP1 proteins indicate the cytoplasmic and nuclear compartments, respectively. (D) NIH 3T3 cells transfected with 3xIRS-luciferase reporter gene and synchronized as in (A) were subjected to luciferase assay. FOXO-dependent reporter expression was increased in G_2/M-enriched relative to G_0/G_1-enriched NIH 3T3 cells (mean ± SEM, $n = 5$; $P < 0.01$, *t* test). (E) The amount of Plk mRNA was induced in G_2/M-enriched relative to G_0/G_1-enriched NIH 3T3 cells (mean ± SEM, $n = 3$; $P < 0.001$, *t* test). (F) Chromatin immunoprecipitation analysis of synchronized NIH 3T3 cells, using a rabbit immunoglobulin G control or a FOXO1 antibody. (**G**) NIH 3T3 cells transfected with U6-GFP/foxo RNAi or U6-GFP control RNAi plasmid were sorted on the basis of GFP expression. Lysates of sorted cells were immunoblotted with the Plk, FOXO1, or ERK1/2 antibody. (**H**) NIH 3T3 cells transfected with U6-GFP/foxo RNAi plasmid and FOXO1-Res or FOXO1-ResS249A were sorted on the basis of GFP expression. Lysates of sorted cells were analyzed as in (G).

Downloaded from www.sciencemag.org on March 20, 2008

FOXO1 in diverse cell types, including prostate cancer cells (figs. S7 and S8) (*9*). We confirmed that overexpression of Cdk2 induced phosphorylation of FOXO1 at Ser^{249} (fig. S9A). However, the FOXO1 Ser^{249} phosphorylation was not reduced in Cdk2-deficient fibroblasts (fig. S9, B and C) (*9*), which suggests that endogenous Cdk2 may not be uniquely required for the FOXO1 Ser^{249} phosphorylation in proliferating cells.

To determine the consequences of endogenous FOXO1 Ser^{249} phosphorylation in proliferating cells, we characterized the FOXO1 phosphorylation in distinct phases of the cell cycle in which Cdk1 activity is low (G_0 or G_1) or high (G_2/M) in synchronized DU145 prostate cancer cells (fig. S10), NIH 3T3 fibroblasts (Fig. 4), and murine embryonic fibroblasts (MEFs) (fig. S11). Endogenous FOXO1 phosphorylation was low in cells enriched for the G_0 or G_1 phase and high in cells enriched for the G_2/M transition of the cell cycle (Fig. 4B and figs. S10B, S11B, and S12). These findings suggest that phosphorylation of endogenous FOXO1 at Ser^{249} coincides with endogenous Cdk1 activity in the cell cycle.

We assessed the effect of phosphorylation of endogenous FOXO1 at Ser^{249} on the subcellular localization of FOXO1 in proliferating cells. In subcellular fractionation assays, endogenous FOXO1 and Ser^{249}-phosphorylated FOXO1 in particular were associated with the nuclear fraction in G_2/M-enriched cells (Fig. 4C and figs. S10C and S11C). We also assessed the effect of phosphorylation of endogenous FOXO1 at Ser^{249} on FOXO-dependent transcription in proliferating cells. Expression of the FOXO-responsive reporter gene was higher in G_2/M-enriched cells than in G_0/G_1-enriched cells (Fig. 4D and figs. S10D and S11D).

The gene promoter of the mitotic regulator Polo-like kinase (Plk) harbors conserved FOXO binding sites, and Plk transcription is induced by FOXO3 at the G_2/M transition (*11*). We therefore tested whether the gene encoding Plk might represent a direct target of FOXO1 in proliferating cells. Plk mRNA and protein levels were higher at the G_2/M transition than in the G_0/G_1 phase of the cell cycle (Fig. 4, B and E, and figs. S10, B and E, and S11, B and E). In addition, FOXO1 occupied the Plk gene promoter at G_2/M but not at G_0/G_1 (Fig. 4F and fig. S10F). FOXO RNAi reduced endogenous Plk levels in cells (Fig. 4G). Expression of the RNAi-resistant FOXO1-Res, but not FOXO1-ResS249A, restored Plk expression in cells in the background of FOXO RNAi (Fig. 4H and fig. S13). These results suggest that the gene encoding Plk represents a G_2/M-specific target gene of Ser^{249}-phosphorylated FOXO1 in proliferating cells.

Our study reveals an intimate and conserved signaling link between the protein kinase Cdk1 and the transcription factor FOXO1. Cdk1 phosphorylates FOXO1 at Ser^{249} and thereby disrupts FOXO1's interaction with 14-3-3 proteins, driving FOXO1 into the nucleus to activate a cell death program of gene expression in neurons. Cdk1-FOXO1 signaling also operates at the G_2/M transition of the cell cycle in proliferating cells and thereby stimulates the expression of the mitotic regulator Plk. The Cdk1-FOXO1 signaling pathway may thus have diverse functions in cellular homeostasis, including regulation of neuronal death and degeneration in brain development and disease.

References and Notes

1. Y. Konishi, M. Lehtinen, N. Donovan, A. Bonni, *Mol. Cell* **9**, 1005 (2002).
2. E. B. Becker, A. Bonni, *Prog. Neurobiol.* **72**, 1 (2004).
3. M. Kurita, T. Kuwajima, I. Nishimura, K. Yoshikawa, *J. Neurosci.* **26**, 12003 (2006).
4. V. Khurana *et al.*, *Curr. Biol.* **16**, 230 (2006).
5. I. Vincent, G. Jicha, M. Rosado, D. W. Dickson, *J. Neurosci.* **17**, 3588 (1997).
6. J. Busser, D. S. Geldmacher, K. Herrup, *J. Neurosci.* **18**, 2801 (1998).
7. L. P. Van Der Heide, M. F. Hoekman, M. P. Smidt, *Biochem. J.* **380**, 297 (2004).
8. B. M. Burgering, G. J. Kops, *Trends Biochem. Sci.* **27**, 352 (2002).
9. See supporting material on *Science* Online.
10. H. Huang, K. M. Regan, Z. Lou, J. Chen, D. J. Tindall, *Science* **314**, 294 (2006).
11. B. Alvarez, A. C. Martinez, B. M. Burgering, A. C. Carrera, *Nature* **413**, 744 (2001).

12. We thank J. W. Harper, S. Puram, and other members of the Bonni laboratory for helpful discussions and critical reading of the manuscript. Supported by a pilot grant from the Paul Glenn Laboratories (A.B.), NIH grant NS047188 (A.B.), and a Uehara Memorial Foundation fellowship (T.Y.).

Supporting Online Material

www.sciencemag.org/cgi/content/full/319/5870/1665/DC1
Materials and Methods
SOM Text
Figs. S1 to S13
References

30 October 2007; accepted 21 February 2008
10.1126/science.1152337

www.sciencemag.org on March 20, 2008