Table 1. Crystallographic statistics. GlpF was overexpressed, purified, and crystallized as described (6), replacing glycerol by xylose (15% w/w) for (GlpF-G)A and (GlpF-G)B, a nontransported substrate (6). Single crystals were subject to x-ray diffraction at ALS beam line 5.0.2 using a CCD detector (Quantum IV). The crystals were in space group I422 and were isomorphous to crystals previously grown in 15% (w/w) glycerol. The structures were determined by direct isomorphous replacement using the protein component of 1FX8 and refined with CNS (27). The RMSD between 1FX8 (at 2.2 Å resolution) and (GlpF-G)A is 0.26 Å, and (GlpF-G)B is 0.51 Å and GlpF F200T is 0.20 Å, the RMSD between (GlpF-G)A and (GlpF-G)B is 0.18 Å.

<table>
<thead>
<tr>
<th>Crystal</th>
<th>(GlpF-G)A</th>
<th>(GlpF-G)B</th>
<th>GlpFW48F/F200T</th>
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<tr>
<td>Wavelength (Å)</td>
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<td>1.1</td>
<td>1.0</td>
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<td>Resolution (Å)</td>
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<td>30−2.8</td>
<td>35−2.1</td>
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<tr>
<td>Observations</td>
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<td>51474</td>
<td>128187</td>
</tr>
<tr>
<td>Unique</td>
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<td>8696</td>
<td>25710</td>
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<tr>
<td>Unit cell size (Åx, Åy in Å)</td>
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<td>96.23, 184.37</td>
<td>96.93, 185.43</td>
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<td>87.6</td>
<td>97.9</td>
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<tr>
<td>Completeness (%)</td>
<td>14.0</td>
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</tr>
<tr>
<td></td>
<td>(I/σ(I))</td>
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</tbody>
</table>

Refinement statistics

- Number of reflections: 9774/1144
- Number of nonhydrogen atoms: 997
- Unobserved residues: 1-5, 260-281
- Resolution (Å): 30−2.7
- Rmerge (%): 22.9/26.1
- Average B factor (Å²): 54.4
- Bond length deviation (Å): 0.007
- Bond angle deviation (°): 1.26

DNA Repair Pathway Stimulated by the Forkhead Transcription Factor FOXO3a Through the Gadd45 Protein

Hien Tran, Anne Brunet, Jill M. Grenier, Sandeep R. Datta, Albert J. Fornace Jr., Peter S. DiStefano, Lillian W. Chiang, Michael E. Greenberg

The signaling pathway from phosphoinositide 3-kinase to the protein kinase Akt controls organismal life-span in invertebrates and cell survival and proliferation in mammals by inhibiting the activity of members of the FOXO family of transcription factors. We show that mammalian FOXO3a also functions at the G1 to M checkpoint in the cell cycle and triggers the repair of damaged DNA. By gene array analysis, FOXO3a was found to modulate the expression of several genes that regulate the cellular response to stress at the G2-M checkpoint. The growth arrest and DNA damage response gene Gadd45a appeared to be a direct target of FOXO3a that mediates part of FOXO3a's effects on DNA repair. These findings indicate that in mammals FOXO3a regulates the resistance of cells to stress by inducing DNA repair and thereby may also affect organismal life-span.

The binding of growth factors to specific receptor tyrosine kinases activates the phosphoinositide 3-kinase (PI3K) and the serine-threonine kinase Akt (also called protein kinase B or PKB). Akt promotes cell survival and proliferation in part by directly phosphorylating and inhibiting members of the FOXO subfamily of forkhead transcription factors (1–3). In the nematode Caenorhabditis elegans, null mutants of the PI3K-Akt pathway lead to the activation of the worm FOXO transcription factor DAF-16, resulting in either an extension of adult life-span or, during development, an entrance into the long-lived larval stage termed dauer (4, 5). In both cases, the PI3K-Akt pathway mutants develop a resistance to stress that may account for the longevity phenotype observed (4, 6). One possibility is that in the mutant background, the activation of FOXO transcription factors may mediate the resistance to stress because DAF-16’s activity is required for the transcriptional up-regulation of cytosolic catalase and superoxide dismutase, scavenger proteins that protect against oxidative damage (7, 8). In mammals, the role of the forkhead transcription...
Report

Factors in the response to stress is, as yet, unclear.

One mechanism by which cells protect themselves against oxidative stress is by repairing the damage to their DNA and proteins that occurs upon exposure to environmental stress (9). Furthermore, this capacity to repair DNA damage is closely correlated with an increased longevity in mammals (9). Therefore, we considered the possibility that the transcription factor FOXO3a, a mammalian forkhead family member that is similar in sequence to DAF-16, induces a program of gene expression that allows for the repair of DNA damage caused by oxidative stress. Under conditions of DNA damage, cycling cells arrest at one of two critical cell cycle checkpoints (G1-S or G2-M phase of the cell cycle, before and after DNA replication) allowing the cells time to repair their DNA (10).

Overexpression of FOXO3a induces a potent G1 arrest (3), but FOXO3a’s role in G2-M progression has not been explored. Consistent with a possible role for FOXO3a in the G2-M phase of the cell cycle, FOXO3a was localized in the nucleus in cells passing through the G2 phase of the cell cycle [Web fig. 1 (11)]. To assess the role of FOXO3a in G2-M progression, we generated a line of Rat-1 fibroblasts that express a fusion protein consisting of FOXO3a fused at its COOH terminus to the ligand-binding domain of the estrogen receptor (TM-ER) (11). In TM-ER, the FOXO3a moiety was mutated such that the key regulatory sites of phosphorylation, Thr32, Ser253, and Ser315, were converted to key regulatory sites of phosphorylation, FOXO3a moiety was mutated such that the receptor (TM-ER) (11) fused at its COOH terminus to the ligand-binding domain of the estrogen receptor (TM-ER) (11). In TM-ER, the FOXO3a moiety was mutated such that the key regulatory sites of phosphorylation, Thr32, Ser253, and Ser315, were converted to alanine so that Akt could no longer phosphorylate these sites. The ER domain functions as a molecular switch that rapidly turns on the activity of the FOXO3a protein when TM-ER expressing cells are exposed to 4OHT. Using antibodies that recognize the TM-ER protein, we found that in the absence of 4OHT, the TM-ER fusion protein was sequestered in the cytoplasm (Fig. 1A)(13). However, 1 hour after treatment of cells with 4OHT (1 μM), the TM-ER protein was found almost exclusively in the nucleus (Fig. 1A). We tested the ability of TM-ER to drive FOXO3a-dependent transcription in response to 4OHT. Activated TM-ER enhanced transcription of a luciferase reporter gene containing three canonical FOXO binding sites within its promoter [forkhead response element (FHRE) luciferase] (Fig. 1B). 4OHT did not induce the reporter gene in cells that did not express TM-ER (Fig. 1B), indicating that the transactivation of the reporter was not a nonspecific effect of 4OHT treatment. 4OHT also did not induce FHRE luciferase expression in cells that expressed a mutant form of TM-ER that has a deletion in the DNA binding domain (TMΔDB-ER) (11) (Fig. 1B).

To examine the role of FOXO3a during progression from G2 to M phase, we expressed Rat-1 cells stably expressing TM-ER to aphpidicolin to arrest the cells in early S phase (11). The aphpidicolin was then washed out to release the cells from the S phase block while FOXO3a activity was simultaneously induced by the addition of 4OHT. Fluorescence-activated cell sorting (FACS) analyses of the Rat-1 cells stably expressing TM-ER revealed a G2-M delay (Fig. 2A). 4OHT alone did not affect the progression of control Rat-1 fibroblasts through the G2 and M phases of the cell cycle (Fig. 2B). Importantly, FACS analyses of Rat-1 clones stably expressing TMΔDB-ER showed that treatment of cells with 4OHT no longer elicited a G2-M delay, indicating that FOXO3a induces G2-M delay by binding to DNA (Fig. 2B). The results obtained by FACS analyses were corroborated by immunocytochemistry experiments with the use of an antibody that specifically stains cells that are in the G2, and M phases of the cell cycle [Web fig. 2, A and B (11)].

To verify that the G2 delay observed in TM-ER-expressing clones also occurs in cells expressing endogenous FOXO3a, we treated Rat1 cells with the PI3K inhibitor LY294002 (LY), which promotes the transactivation of the reporter was not a nonspecific effect of 4OHT treatment. 4OHT also did not induce FHRE luciferase expression in cells that expressed a mutant form of TM-ER that has a deletion in the DNA binding domain (TMΔDB-ER) (11) (Fig. 1B).

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To confirm that FOXO3a induces the repair of damaged DNA, we used a quantitative polymerase chain reaction (PCR)-

**Fig. 1.** Inducible activation of FOXO3a. (A) Rat1 cells expressing the inducible form of FOXO3a (TM-ER) were incubated in the presence (+) or absence (−) of 4OHT (1 μM) for 1 hour. The subcellular localization of TM-ER was monitored by immunofluorescence using the antibody to HA. (B) Control Rat 1 cells (CTL) and Rat 1 cells expressing TM-ER or TMΔDB-ER were cotransfected with the FHRE-luciferase reporter gene. One day after transfection, cells were incubated in the presence (+) or absence (−) of 1 μM 4OHT for 6 hours, and luciferase assays were performed. The graph represents the mean and SEM of three independent experiments conducted in duplicates.
FOXO3a (the cell cycle (Table 1), consistent with the involvement in apoptosis and the regulation of analysis, we identified several genes involved in FOXO3a activation. From this clustering applied to identify genes regulated after comparing, and a clustering algorithm was NAs and expressed sequence tags (ESTs) to DNA microarrays containing 12,000 cDNA.

4OHT addition and hybridized the mRNA ing Rat 1 cells at 0, 1, 3, and 8 hours after ger RNA (mRNA) from TM-ER– expressing Rat 1 cells at various times in the presence or absence of 4OHT (500 nM). FACS analysis was performed as described in (A). The data presented corresponds to the mean of two independent experiments. (C) Rat1 cells were released from an S phase block at various times in the presence or absence of LY (25μM). FACS analysis was performed as described in (A). The experiment presented is representative of two independent experiments.

Based assay to detect the presence of damaged DNA templates that had been repaired (11). We cotransfected a DNA template damaged by 5000J/m 2 of UV irradiation together with various FOXO3a constructs and an undamaged control DNA template. Repair of the template was assessed by measuring its amplification by PCR and was quantified by real-time fluorescence detection. As with the promoter reactivation experiments, TM but not TMΔDB induced repair of the damaged template (Fig. 3C).

We used high-density cDNA microarrays to identify FOXO3a-regulated genes that might mediate the G2-M delay and DNA repair response. We isolated messenger RNA (mRNA) from TM-ER–expressing Rat 1 cells at 0, 1, 3, and 8 hours after 4OHT addition and hybridized the mRNA to DNA microarrays containing 12,000 cDNAs and expressed sequence tags (ESTs) (16, 17). Transcriptional profiles were compared, and a clustering algorithm was applied to identify genes regulated after FOXO3a activation. From this clustering analysis, we identified several genes involved in apoptosis and the regulation of the cell cycle (Table 1), consistent with the previously known functions ascribed to FOXO3a (1, 3). FOXO3a also increased expression of a group of genes that takes part in the cellular response to stress. Expression of these genes was not increased in control Rat 1 cells treated with 4OHT.

Several of the genes expressed in response to activation of TM-ER also showed increased expression when cells were treated with the PI3K inhibitor LY (Table 1). Of these genes, Gadd45a appeared to be a good candidate for mediating the effects of FOXO3a on G2-M progression and DNA repair. All three members of the Gadd45 family, Gadd45a, Gadd45β, and Gadd45γ, are expressed in response to stress stimuli that induce DNA damage (18-20). The GADD45A promoter contains three FOXO transcription factor binding sites (21). Of these sites, one is in a region that is conserved across species, suggesting that its function is critical. Lastly, Gadd45a is important for DNA repair and cell cycle arrest at the G2-M checkpoint (22-24).

To verify that FOXO3a regulates Gadd45a expression, we isolated mRNA at
hour 0, 1 hour, and 3 hours after exposure of TM-ER–expressing cells to 4OHT. By real-time quantitative reverse transcriptase–PCR (RT-PCR), we found that expression of the Gadd45A mRNA was induced upon exposure of TM-ER–expressing cells to 4OHT (Fig. 4A) (11). Western analysis with a Gadd45 antibody revealed that the abundance of Gadd45A protein increased in three independent TM-ER–expressing clones in response to 4OHT treatment (Fig. 4B) (13). In contrast, 4OHT did not increase abundance of Gadd45A mRNA or protein in cells that did not express the TM-ER protein (Fig. 4, A and B), nor did 4OHT cause accumulation of the Gadd45A protein in cells that expressed TMΔDB-ER (Fig. 4B).

To determine if the induction of Gadd45A transcription is mediated by the direct binding of FOXO3a to the promoter of the Gadd45A gene, we examined the effects of FOXO3a on the activity of the human GADD45A promoter. An analysis of the human GADD45A promoter revealed the presence of one conserved consensus FHRE, ATAAACAA, at 505 nucleotides 5′ of the transcriptional start site, and two suboptimal FHREs, TAAACAAA and TTGTATGG, located 377 and 803 nucleotides 5′ of the transcriptional start site, respectively. To assess the ability of FOXO3a to regulate GADD45A transcription, we cotransfected CCL39 fibroblasts with various FOXO3a constructs together with a reporter gene in which the GADD45A promoter drives the expression of luciferase activity (Fig. 5A). A mutant of FOXO3a that does not bind DNA (TMΔDB) did not activate GADD45A promoter expression (Fig. 6A).

GADD45A promoters in which we replaced the core consensus sequence AACA of FHRE-2 within the GADD45A promoter with GGGG and also replaced the AC within the core consensus sequence of FHRE-3 with GG (P-2/3m) showed diminished activity in response to FOXO3a (P < 0.05, t test) (Fig. 5B). Thus, direct DNA binding of FOXO3a to FHRE-2 and FHRE-3 of the GADD45A promoter appears to be important for the transactivation of the GADD45A promoter.

To determine if FOXO3a promotes UV expression of Gadd45a in cells exposed to UV irradiation, we transfected CCL39 fibroblasts with a luciferase reporter containing either the wild-type GADD45A promoter or the double mutant GADD45A promoter construct (P-2/3m) and exposed the cells to 30 J/m² of UV irradiation for 6 hours, and luciferase assays were performed (P < 0.005, t test).

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Gadd45a

or

Asterisk indicates statistical difference from bars) were transfected as described in Fig. 5A. Twenty-four hours after transfection, luciferase assays were performed. The data presented represent the mean and SEM of six independent experiments. (b) Gadd45a−/− MEFs (solid bars) and WT MEFs (open bars) were transfected as described in Fig. 5A. Twenty-four hours after transfection, luciferase assays were performed.

Table 1. Identification of FOXO3a target genes by transcriptional profiling.

<table>
<thead>
<tr>
<th>Genes</th>
<th>Function</th>
<th>Microarray</th>
<th>Northern</th>
<th>FHRE</th>
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<tr>
<td>DNA repair</td>
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<tr>
<td>GADD45 and PA26</td>
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<td></td>
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<td>Tumor suppressor</td>
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<tr>
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<td>Cysteine protease</td>
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<tr>
<td></td>
<td>Bcl-2 family member</td>
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</table>

Our findings suggest that an important role for the forkhead transcription factors in stress-resistance and the aging process may be conserved in mammals. An organism’s ability to respond to stress, in particular the capacity of cells to repair damage to DNA, correlates with an increased longevity (13). We propose that under low stress conditions, FOXO3a may promote DNA repair, whereas under higher levels of stress, FOXO3a may induce a program of cell death. This graded response to stress stimuli would protect cells from damage but also facilitate the removal of heavily damaged cells, resulting in an overall increase in the life-span of the organism.

References and Notes

1. A. Brunet et al., Cell 96, 857 (1999).
11. Supplementary Web material is available on Science Online at www.sciencemag.org/cgi/content/full/296/5567/530/DC1.
13. Western blot, immunofluorescence experiments, and luciferase assays were performed as described (1). The GADD45a (H-165) antibody was purchased from Santa Cruz.
14. A. Brunet, H. Tran, M. E. Greenberg, unpublished data.
17. Isolation of mRNA and transcriptional profiling experiments were performed as described previously in (16) and as described in (11).
21. The plasmid encoding the GADD45α promoter driving the luciferase reporter gene and HA-GADD45α in a mammalian expression vector were kind gifts of D. Haber and were described previously (27).
27. D. P. Harkin et al., Cell 97, 575 (1999).
28. We thank members of the Greenberg lab for their support. In particular, we thank A. Nigh and W. Chen for help with the quantitative real-time PCR; A. West, J. Zieg, and R. Dolmetsch for their comments on the manuscript; B. Demple for helpful discussions and critical reading of manuscript; and A. Flint for his help in analyzing the FACS samples at the Howard Hughes FACS Facility. Supported by a Senior Scholars Award from the Ellison Foundation, NIH grant PO1-HD24926, and Mental Retardation Research Center grant, NHIP30-HD18655 (M.E.G.). A.B. was supported by a Goldenson Ben- reng Fellowship. M.E.G. also acknowledges the generous contribution of the F. M. Kirby Foundation to the Division of Neuroscience.