Cell, Vol. 96, 857–868, March 19, 1999, Copyright ©1999 by Cell Press

# Akt Promotes Cell Survival by Phosphorylating and Inhibiting a Forkhead Transcription Factor

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## Summary

**Survival factors can suppress apoptosis in a transcription-independent manner by activating the serine/threonine kinase Akt, which then phosphorylates and inactivates components of the apoptotic machinery, including BAD and Caspase 9. In this study, we demonstrate that Akt also regulates the activity of FKHRL1, a member of the Forkhead family of transcription factors. In the presence of survival factors, Akt phosphorylates FKHRL1, leading to FKHRL1's association with 14-3-3 proteins and FKHRL1's retention in the cytoplasm. Survival factor withdrawal leads to FKHRL1 dephosphorylation, nuclear translocation, and target gene activation. Within the nucleus, FKHRL1 triggers apoptosis most likely by inducing the expression of genes that are critical for cell death, such as the Fas ligand gene.**

## Introduction

Survival factors suppress the intrinsic cell death machinery and thereby prevent apoptosis, a naturally occuring cell death process that is critical for the development of multicellular organisms (Jacobson et al., 1997). Execution of apoptosis is ensured by a series of transcription-independent events that include the activation of cascades of cysteine proteases, termed caspases, and result in cell disintegration followed by engulfment by surrounding cells (for review, Steller, 1995). Under some circumstances, apoptosis is a transcription-dependent process that requires the upregulation of death genes, including the tumor suppressor p53 (Khanna and Lavin, 1993), the proapoptotic member of the Bcl2 family Bax (Miyashita and Reed, 1995), and certain cytokines, such as the Fas ligand (Friesen et al., 1996; Kasibhatla et al., 1998; Le-Niculescu et al., 1999) or TNF$\alpha$ (Sung et al., 1991). These cytokines, by binding to their cognate cell surface receptors, may in turn induce caspase cascades and thereby propagate the process of apoptosis (Nagata, 1997; Ashkenazi and Dixit, 1998).

Recently, a signaling pathway by which extracellular stimuli suppress apoptosis has been characterized. Survival factors, such as insulin-like growth factor 1 (IGF1) and neurotrophins, bind to their cell surface receptors and trigger the activation of several kinases, including the phosphatidylinositol 3-kinase (PI3K) (Fruman et al., 1998) and the $Ca^{2+}$/calmodulin-dependent kinase kinase (Yano et al., 1998). These kinases in turn lead to the activation of a serine/threonine kinase termed Akt or PKB (for reviews, Datta and Greenberg, 1998; Downward, 1998). Akt plays a central role in promoting the survival of a wide range of cell types (Dudek et al., 1997; Kauffmann-Zeh et al., 1997; Kennedy et al., 1997; Songyang et al., 1997). Recently, two Akt substrates that are components of the intrinsic cell death machinery have been identified: the Bcl2 family member BAD (Datta et al., 1997; del Peso et al., 1997) and the protease Caspase 9 (Cardone et al., 1998; Y. Gotoh et al., unpublished data). In both cases, phosphorylation of these proteins by Akt suppresses their proapoptotic function, thereby accounting at least in part for the potent survival effects of Akt.

If Akt is a general mediator of cell survival, it is possible that this kinase catalyzes the phosphorylation of other substrates in addition to BAD and Caspase 9. One possibility that remained to be examined was that Akt might phosphorylate and regulate the activity of transcription factors that control cell death genes. This hypothesis is supported by the observation that Akt translocates into the nucleus upon exposure of cells to survival factors (Andjelkovic et al., 1997). Although Akt targets within the nucleus remained to be identified, genetic studies of the PI3K/Akt signaling pathway in the nematode *Caenorhabditis elegans* have established that this signaling cascade suppresses the function of the transcription factor DAF16, which belongs to the Forkhead family (FH) (Lin et al., 1997; Ogg et al., 1997; Paradis and Ruvkun, 1998). Analysis of the DAF16 sequence reveals three sites that conform to the consensus site phosphorylated by Akt (**R**X**R**XX*S/T*) (Alessi et al., 1996), suggesting that DAF16 and perhaps other FH transcription factors might be direct targets of Akt.

Three members of the human FH family, termed FKHR (Galili et al., 1993), FKHRL1 (Hillion et al., 1997; Anderson et al., 1998), and AFX (Borkhardt et al., 1997), are similar in sequence to DAF16 and represent the mammalian counterparts of DAF16 (Ogg et al., 1997). The genes for these human DAF16 homologs were first identified at chromosomal breakpoints in human tumors (Galili et al., 1993; Borkhardt et al., 1997; Hillion et al., 1997; Anderson et al., 1998). Notably, the three putative Akt phosphorylation sites present in the DAF16 sequence are also found in the human homologs, raising the possibility that these human DAF16 counterparts might also be Akt targets.

In the present study, we show that human FKHRL1 is a direct target of Akt phosphorylation and that Akt

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phosphorylation of FKHRL1 results in FKHRL1 retention in the cytoplasm and the inhibition of FKHRL1-dependent transcription. Survival factor withdrawal induces FKHRL1 dephosphorylation and translocation to the nucleus. Within the nucleus, the dephosphorylated FKHRL1 induces target genes such as Fas ligand and triggers apoptosis. We propose that growth factors, by triggering the Akt-dependent phosphorylation and inactivation of FKHRL1 function, suppress the transcription of death genes, such as the Fas ligand gene, and thereby promote cell survival.

## Results

### Akt Phosphorylates FKHRL1 In Vitro

We initially tested the FKHRL1 isoform as an Akt substrate, since Northern blot experiments had shown that the FKHRL1 mRNA is the most ubiquitously expressed of the three mammalian DAF16 homologs (Anderson et al., 1998). To determine whether FKHRL1 is a substrate of Akt in vitro, we produced in bacteria a GST-FKHRL1 fusion protein and a series of mutants in which one or three of the putative Akt phosphorylation sites (T32, S253, and S315) were replaced by a nonphosphorylatable alanine residue. A constitutively active form of Akt immunoprecipitated from 293T cells effectively phosphorylated wild-type GST-FKHRL1 in an in vitro kinase assay (Figure 1A). FKHRL1 phosphorylation by Akt required the presence of active Akt in the immune complex, since an inactive form of Akt was unable to phosphorylate the FKHRL1 protein (Figure 1A).

To determine whether Akt phosphorylates FKHRL1 at T32, S253, and/or S315, we tested as Akt substrates mutants of FKHRL1 in which just one of these sites was converted to an alanine (Figure 1A). Whereas the S315A mutant was phosphorylated to the same extent as the wild-type FKHRL1 protein, the T32A and the S253A mutants were phosphorylated at a significantly lower level, indicating that T32 and S253 of FKHRL1 are likely to be Akt targets. However, both the T32A and S253A mutants displayed residual phosphorylation by Akt (Figure 1A), suggesting that when one of these sites is mutated, the other site is still phosphorylated. By contrast, a FKHRL1 protein in which all three of the putative Akt phosphorylation sites were converted to alanines was no longer detectably phosphorylated by Akt (Figure 1A), indicating that there are no other sites within FKHRL1 that are significantly phosphorylated by Akt in vitro.

To confirm that T32 and S253 are the sites of Akt-catalyzed phosphorylation in vitro, we raised antibodies to phosphopeptides that correspond to these two phosphorylation sites and used the antibodies in immunoblotting experiments (Figure 1B). The anti-phospho T32 peptide antibody recognized wt FKHRL1 that was incubated with a constitutively active form of Akt in an in vitro kinase assay but failed to recognize unphosphorylated FKHRL1 (Figure 1B, top panel). Moreover, the anti-phospho T32 antibody did not recognize FKHRL1 in which T32 had been converted to an alanine, but it was able to effectively recognize FKHRL1 in which S253 or S315 was converted to an alanine (Figure 1B, top panel). These findings indicate that the anti-phospho T32 antibody specifically recognizes FKHRL1 when it is phosphorylated at T32 and demonstrate that Akt phosphorylates FKHRL1 at T32 in vitro. By a similar strategy, we demonstrated that the anti-phospho S253 antibody is specific to FKHRL1 that is phosphorylated at S253 and that Akt phosphorylates FKHRL1 at S253 in vitro (Figure 1B, middle panel).

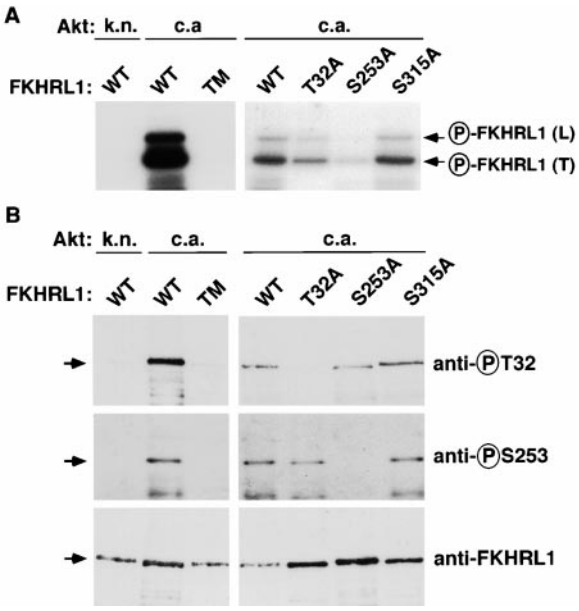


Figure 1. Akt Phosphorylates FKHRL1 In Vitro

(A) A constitutively active form of Akt (c.a.) or kinase inactive mutant (k.n.) expressed in 293T cells was immunoprecipitated with the anti-HA antibody and incubated in the presence of $^{32}$P-ATP and 5 μg of GST-FKHRL1 fusion proteins, wild type (WT), T32A/S253A/S315A triple mutant (TM), or each of the single mutants. L, full-length GST-FKHRL1 protein; T, smaller fragment of the GST-FKHRL1 protein that is possibly a degradation product.

(B) Samples obtained in (A) were analyzed by immunoblot with the antibodies directed against phospho-T32 FKHRL1 (top panel) or phospho-S253 FKHRL1 (middle panel) or with the antibody directed against total FKHRL1 (bottom panel).

### Akt Phosphorylates FKHRL1 In Vivo

To determine whether Akt phosphorylates FKHRL1 at T32, S253, and/or S315 within cells, we generated HA epitope–tagged versions of wild-type and phosphorylation mutants of FKHRL1. These constructs were transfected into 293T cells and the expression of FKHRL1 monitored by immunoblotting with an anti-FKHRL1 antibody (Figure 2A, bottom panel). The phosphorylation of FKHRL1 at T32 or S253 was monitored by immunoblotting with anti-phospho FKHRL1-specific antibodies (Figure 2A, top and middle panels). When 293T cells were grown in the presence of growth factors, the anti-phospho T32 and anti-phospho S253 antibodies recognized wild-type FKHRL1 but not the corresponding single mutants (T32A and S235A, respectively), the corresponding double mutants, or the triple mutant of FKHRL1 (Figure 2A). These findings confirm the specificity of the anti-phospho T32 and anti-phospho S253 antibodies and indicate that in the presence of growth factors, conditions under which the PI3K/Akt pathway is active, FKHRL1 is phosphorylated within cells at both T32 and S253.

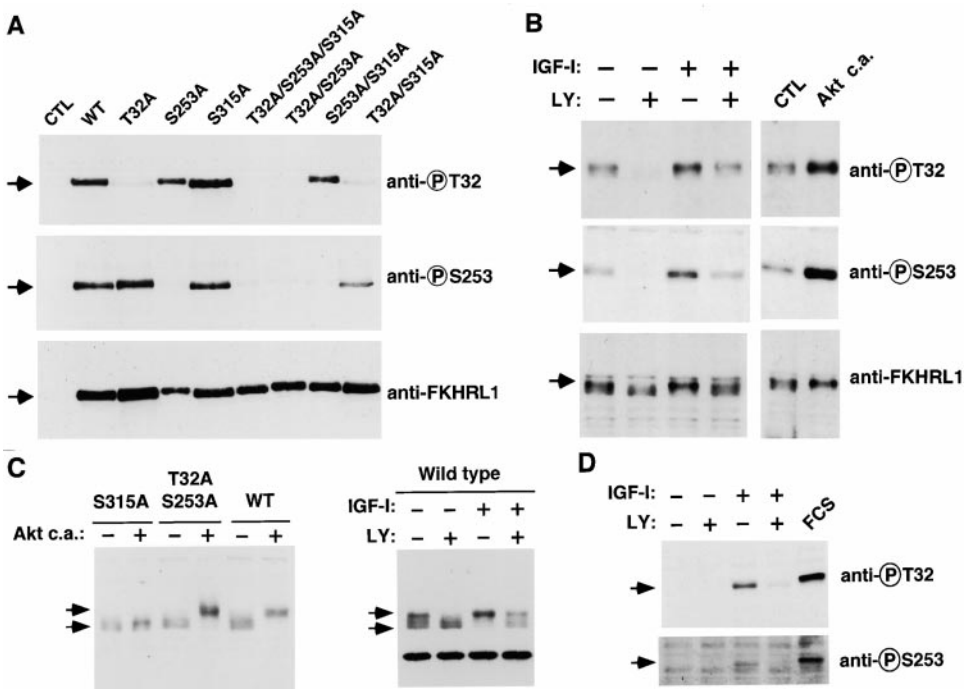


Figure 2. Akt Induces Phosphorylation of FKHRL1 In Vivo

(A) 293T cells were transiently transfected with constructs encoding FKHRL1 tagged with the HA epitope (WT or mutants) or with an empty vector (CTL). Whole-cell extracts were resolved by SDS–PAGE and immunoblotted with the antibodies directed against phospho-T32 (top panel) or phospho-S253 (middle panel). The total level of protein was assayed by immunoblot using the anti-FKHRL1 antibody (bottom panel).

(B) CCL39 fibroblasts were transiently cotransfected with wt FKHRL1 plasmid and an empty vector (CTL) or a constitutively active Akt construct (c.a.). They were serum starved for 20 hr, then incubated with 10 μM LY for 1 hr and stimulated with 50 ng/ml IGF1 for 20 min. Cell lysates were analyzed by immunoblot with the antibodies directed against phospho-T32 (top panel) or phospho-S253 (middle panel). The total level of protein was assayed by immunoblot using the anti-FKHRL1 antibody (bottom panel).

(C) CCL39 fibroblasts were transiently cotransfected with FKHRL1 plasmids (WT or mutants) and an empty vector (−) or a constitutively active Akt construct (+) and were treated as in (B) for the right panel. Cell lysates were resolved by SDS–PAGE (6%) and immunoblotted with the anti-HA antibody.

(D) CCL39 fibroblasts were serum starved for 20 hr, then incubated with 10 μM LY for 1 hr and stimulated with 50 ng/ml IGF1 or 20% FCS for 20 min. Immunoblotting experiments were performed as in (B).

To determine whether FKHRL1 phosphorylation is induced by the addition of survival factors, we expressed HA-tagged FKHRL1 in the IGF1-responsive fibroblast cell line CCL39 and examined the phosphorylation status of FKHRL1 using the phosphoantibodies in immunoblot experiments. We found that a 20 min exposure of fibroblasts to IGF1 induced the phosphorylation of FKHRL1 at T32 and S253 (Figure 2B). To determine whether IGF1 induction of FKHRL1 phosphorylation was mediated by the PI3K pathway, we examined the effect of an inhibitor of PI3K, LY 294002 (LY), on FKHRL1 phosphorylation. The phosphorylation of both T32 and S253 was inhibited by LY treatment (Figure 2B), suggesting that the endogenous PI3K pathway is necessary for FKHRL1 phosphorylation at T32 and S253 in vivo.

To determine whether Akt activity was sufficient to mediate the effect of IGF1 on FKHRL1 phosphorylation, we cotransfected CCL39 cells with a constitutively active mutant of Akt and the HA-tagged form of FKHRL1. We found that the expression of this constitutively active version of Akt induced the phosphorylation of FKHRL1 at both T32 and S253 in vivo (Figure 2B, right panels), indicating that active Akt alone is sufficient to induce the phosphorylation of FKHRL1 at T32 and S253 in vivo.

The anti-phosphoantibodies that we generated to the phosphopeptide that spans S315 were found not to be site specific (data not shown). To assay Akt's ability to catalyze the phosphorylation of FKHRL1 at S315, we took advantage of the finding that the presence of IGF1 or the expression of a constitutively active Akt induced a shift up in the mobility of wt FKHRL1 on an SDS gel (Figure 2C). When FKHRL1 S315 was mutated to an alanine, Akt no longer induced a shift in FKHRL1 mobility (Figure 2C). By contrast, mutating FKHRL1 T32 and S253 to alanines had no effect on Akt's ability to induce a shift in FKHRL1 mobility (Figure 2C). These findings indicate that the Akt-dependent shift in FKHRL1 mobility is primarily due to the Akt-dependent phosphorylation of FKHRL1 at S315. Although, as shown above, Akt phosphorylates FKHRL1 at T32 and S253, the phosphorylation of these two sites appears not to have a significant effect on the mobility of FKHRL1 on an SDS gel. The expression of constitutively active Akt induces the phosphorylation of FKHRL1 at S315 in cells, but Akt does not effectively catalyze the phosphorylation of FKHRL1 at S315 in vitro. Thus, it is possible that within cells Akt activates one or more other kinases that then trigger FKHRL1 phosphorylation at S315.

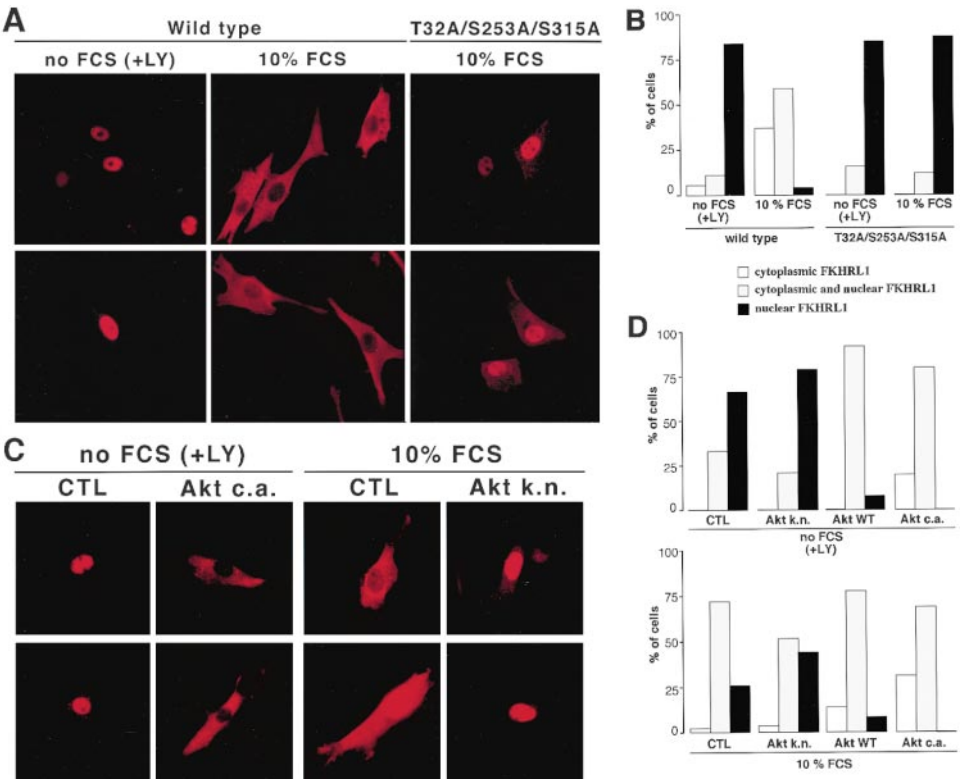


Figure 3. Akt, by Phosphorylating FKHRL1, Promotes Its Retention in the Cytoplasm

(A and B) CCL39 fibroblasts were transiently transfected with the HA-tagged FKHRL1 construct (WT or triple mutant) and were either incubated in the presence of 10% FCS, or serum starved for 20 hr and then incubated with 10 μM of LY for 1 hr. FKHRL1 was detected by immunolocalization with the anti-HA antibody. Representative pictures are shown in (A), and quantitation of the experiment is shown in (B).

(C and D) CCL39 fibroblasts were cotransfected with a wt M2-tagged FKHRL1 construct and different constructs encoding either a dominant interfering mutant form of Akt (Akt k.n.), a wild-type form of Akt (Akt WT), or a constitutively active version of Akt (Akt c.a.). Cells were either incubated in the presence of serum or were serum starved for 20 hr and then incubated with 10 μM of LY for 1 hr. FKHRL1 was detected by immunolocalization with the anti-M2 antibody. Representative pictures are shown in (C), and quantitation of the experiment is shown in (D).

We next asked whether the anti-FKHRL1 phosphoantibodies could detect the endogenous form of FKHRL1 and whether endogenous FKHRL1 becomes newly phosphorylated in response to external stimuli (Figure 2D). By immunoblot experiments with the anti-phosphoantibodies, we found that exposure of CCL39 fibroblasts to IGF1 induces the phosphorylation of a protein that corresponds in molecular weight to FKHRL1 (100 kDa). In addition, phosphorylation of the 100 kDa protein was blocked when cells were treated with the PI3K inhibitor LY (Figure 2D). Given the fact that the protein recognized by the phosphoantibodies has the correct molecular weight, it is likely that this protein is the endogenous FKHRL1 or a closely related protein. We conclude that phosphorylation of an endogenous FKHRL1-related protein is induced by IGF1 by a PI3K-dependent mechanism.

Taken together, these experiments indicate that growth factor activation of the PI3K/Akt pathway is both necessary and sufficient to induce the phosphorylation of FKHRL1 at T32, S253, and S315 within cells.

### Akt Phosphorylated FKHRL1 Is Retained in the Cytoplasm

We next investigated the effect of Akt phosphorylation on the function of FKHRL1 as a transcription factor. Genetic evidence obtained in *C. elegans* suggests that the PI3K/Akt pathway acts to antagonize the function of the FKHRL1 homolog DAF16 in the nematode (Ogg et al., 1997). There are several possible explanations for this genetic finding. For example, Akt might decrease the total level of DAF16/FKHRL1 expression. Alternatively, Akt might inhibit DAF16/FKHRL1 function as a transcriptional activator or repressor by affecting either DAF16/FKHRL1's nuclear localization, its binding to DNA, its interaction with putative partners, or its intrinsic transcriptional activation or repression function.

Since the expression of Akt did not significantly alter the expression of FKHRL1 (see Figure 2B), we asked whether Akt-induced phosphorylation of FKHRL1 might have an effect on the subcellular distribution of this transcription factor. Toward this end, we transfected the HA-FKHRL1 into fibroblasts and examined FKHRL1 subcellular localization under different conditions of PI3K/Akt activation (Figure 3). When the endogenous PI3K/Akt pathway was inhibited (serum starvation and treatment with LY), wild-type FKHRL1 was localized almost entirely within the nucleus (Figures 3A and 3B). By contrast, when the PI3K/Akt pathway was activated (treatment with 10% serum), wild-type FKHRL1 was largely excluded from the nucleus and was detected in the cytoplasm (Figures 3A and 3B).

To determine whether the subcellular localization of FKHRL1 was dependent upon Akt activation, we cotransfected into fibroblasts different versions of HA-tagged Akt together with an M2-tagged form of wild-type FKHRL1 and assessed the effect of these proteins on FKHRL1's subcellular localization (Figures 3C and 3D). When coexpressed with a constitutively active version of Akt, FKHRL1 was excluded from the nucleus and localized within the cytoplasm, even in the absence of survival factors (Figure 3C, left panel). Conversely, when coexpressed with a kinase-inactive version of Akt that is known to inhibit the function of the endogenous Akt, FKHRL1 was primarily localized within the nucleus, even in the presence of survival factors (Figure 3C, right panel). These experiments demonstrate that under these conditions, Akt activity is both necessary and sufficient to retain FKHRL1 in the cytoplasm.

To determine whether the effect of Akt on the subcellular localization of FKHRL1 was due to the phosphorylation of FKHRL1, we examined the subcellular distribution of FKHRL1 mutants in which T32, S253, and/or S315 was converted to an alanine (Figures 3A and 3B). Whereas the wild-type form of FKHRL1 was found to be predominantly cytoplasmic in cells exposed to serum, FKHRL1 in which T32, S253, and S315 were all converted to alanines was exclusively nuclear (Figures 3A and 3B). This indicates that phosphorylation at these three sites alters the subcellular distribution of FKHRL1. When not phosphorylated, FKHRL1 is localized to the nucleus. By contrast, when these three sites are phosphorylated, FKHRL1 is retained in the cytoplasm.

Detailed comparison of each of the FKHRL1 phosphorylation mutants revealed that when either T32, S253, or S315 was mutated to an alanine, the number of cells expressing FKHRL1 in the nucleus was substantially increased (data not shown). An analysis of the subcellular localization of the double mutants revealed that the mutation of two of the phosphorylation sites resulted in an even greater percentage of cells showing FKHRL1 within their nucleus (data not shown), suggesting that phosphorylation of FKHRL1 at all three sites is necessary to retain FKHRL1 completely within the cytoplasm.

Taken together, these findings indicate that growth factor stimulation via the endogenous PI3K/Akt pathway induces the phosphorylation of FKHRL1 at three sites that play an important role in sequestering FKHRL1 in the cytoplasm, away from FKHRL1's target genes in the nucleus.

### Phosphorylated FKHRL1 Binds to 14-3-3

We next considered the possibility that the phosphorylation of FKHRL1 might cause FKHRL1 sequestration in the cytoplasm by promoting FKHRL1's interaction with a protein that functions as a scaffold within the cytoplasm. One group of scaffolding proteins that specifically bind certain phospho-serine-containing proteins is the 14-3-3 family of proteins. Given that the amino acid sequence surrounding T32 is a perfect consensus site for 14-3-3 binding, except for the substitution of a Thr for a Ser (Yaffe et al., 1997), we investigated whether the T32 phosphorylated form of FKHRL1 binds to 14-3-3.

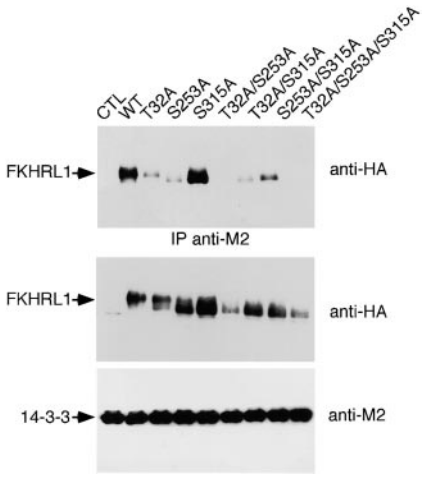


Figure 4. The Phosphorylated Form of FKHRL1 Binds to 14-3-3

293T cells were transiently cotransfected with an empty vector (CTL) or HA-tagged FKHRL1 constructs and a construct encoding the 14-3-3 zeta isoform tagged with the M2 epitope. 14-3-3 was immunoprecipitated with the anti-M2 antibody, and the immune complex was resolved by SDS–PAGE and immunoblotted with the anti-HA antibody (top panel). Total cell extracts were also analyzed by direct immunoblotting with the anti-HA antibody (middle panel) or the anti-M2 antibody (bottom panel).

To this end, we coexpressed FKHRL1 and the zeta isoform of 14-3-3 in 293T cells in the presence of growth factors. Immunoprecipitation experiments followed by immunoblotting showed that wild-type FKHRL1 interacts with 14-3-3 zeta (Figure 4). To determine whether the phosphorylation of FKHRL1 was critical for this interaction, we tested the mutants of the FKHRL1 phosphorylation sites in the coimmunoprecipitation assay (Figure 4). The S315A mutant of FKHRL1 interacted with 14-3-3 to the same extent as wild-type FKHRL1, indicating that the phosphorylation of S315 is not required for 14-3-3 binding. By contrast, both T32A and S253A single mutants of FKHRL1 interacted less efficiently than wild-type FKHRL1 with 14-3-3. Moreover, the interaction of FKHRL1 with 14-3-3 was abolished when T32 and S253 of FKHRL1 were both converted to alanines, indicating that phosphorylation of both sites contributes to 14-3-3 binding. These findings suggest that Akt may promote FKHRL1's interaction with 14-3-3 by phosphorylating FKHRL1 at T32 and S253. Thus, in growth factor–stimulated cells, a function of 14-3-3 may be to bind the phosphorylated form of FKHRL1 and thereby sequester FKHRL1 in the cytoplasm so that it is unable to regulate its target genes in the nucleus.

### Identification of DNA-Binding Sites for FKHRL1

To address FKHRL1's mechanism of action within the nucleus, we first examined the ability of FKHRL1 to interact with specific DNA sequences using a DNA mobility shift assay. Although FKHRL1 contains a "Forkhead domain" that has been shown to be a DNA-binding domain for all FH family members, it had not been determined whether FKHRL1 or DAF16 interacts directly with DNA. Moreover, the sequence of DNA recognized by FKHRL1 remained to be identified.

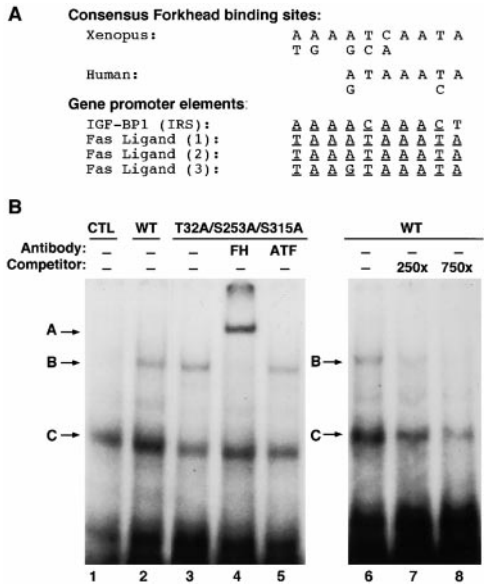


Figure 5. FKHRL1 Is Able to Bind Two Elements on DNA

(A) Comparison between the consensus Forkhead-binding sites and sites found in the IGFBP1 or in the Fas ligand promoter. The underlined nucleotides match the Forkhead consensus.

(B) 293T cells were transiently transfected with FKHRL1 wt or triple mutant. Whole-cell extracts were assayed in band shift experiments using the IRS of IGFBP1 as the $^{32}$P-labeled oligonucleotide. For supershift experiments, immunopurified antibodies to either FKHRL1 or the nonrelated factor ATF1 were added to the extracts 10 min before adding the probe. "A" indicates the supershifted FKHRL1/IRS complex; "B," the specific FKHRL1/IRS complex; and "C," a nonspecific protein/DNA complex.

We searched for DNA sequences that are similar to the previously characterized Forkhead consensus binding sites (Pierrou et al., 1994; Kaufmann et al., 1995) within promoters of growth factor–responsive genes (Figure 5A). One element that matched the FH consensus element was the insulin-responsive sequence (IRS) that had been identified in the promoter of several genes repressed by insulin in a PI3K/Akt-dependent manner, such as insulin-like growth factor–binding protein 1 (IGFBP1) (Cichy et al., 1998) (Figure 5A). We also identified a cluster of three perfect consensus sites for FH factor binding within the promoter of the Fas ligand gene, termed the FH-responsive element (FHRE) (Holtz-Heppelmann et al., 1998) (Figure 5A). Transcription of the Fas ligand gene has recently been found to be induced when neurons are deprived of survival factors (Le-Niculescu et al., 1999).

To determine whether FKHRL1 is able to bind to the IRS or the FHRE, we performed DNA mobility shift experiments using oligonucleotides corresponding to an IRS (Figure 5B) or the FHRE (data not shown) and whole cells extracts prepared from 293T cells transfected with FKHRL1 (wild type or triple mutant). We found that both the wild-type and the triple mutant form of FKHRL1 bound to the IRS of the IGFBP1 gene, leading to a shift in the mobility of the IRS DNA element on a nondenaturing polyacrylamide gel (Figure 5B, lanes 1, 2, and 3). Formation of the FKHRL1/IRS complex was inhibited by an excess of unlabeled IRS DNA but not by the inclusion of an excess of a mutant DNA sequence (Figure 5B, lanes 6, 7, 8 and data not shown). In the presence of the anti-FKHRL1 antibody, but not a control antibody, the shifted band was supershifted (Figure 5B, lanes 4 and 5), demonstrating that the shifted band was due to the binding of FKHRL1 to the IRS element. Similar experiments performed with the FHRE showed that FKHRL1 was also able to bind this site (data not shown). Taken together, these DNA mobility shift experiments indicate that FKHRL1 is capable of binding to a specific DNA sequence element that conforms to the consensus regulatory element recognized by Forkhead transcription factors.

### FKHRL1, When Not Phosphorylated by Akt, Is an Activator of Transcription

We next asked whether FKHRL1 regulates transcription when bound to an FH recognition site within the promoter of a reporter gene and, if so, what effect Akt-induced phosphorylation of FKHRL1 has on this process. Two models of transcriptional regulation are possible to account for the genetic findings obtained in *C. elegans*. First, DAF16 and its human counterparts might be activators of transcription, and Akt, by inhibiting their function, might turn off gene expression. Alternatively, DAF16 and related proteins might be repressors of transcription, and Akt, by suppressing the activity of a transcriptional repressor, might turn on gene expression. To distinguish between these two possibilities, we generated a reporter construct in which a small region of the Fas ligand promoter containing the three canonical FHREs was inserted 5′ of a basal promoter controlling the expression of the luciferase gene (FHRE-Luc reporter). The effect of FKHRL1 on reporter gene transcription was assessed after cotransfection of FKHRL1 and the luciferase reporter gene into cells (Figure 6).

We found that in the absence of survival factors, wild-type FKHRL1, which is nuclear under these conditions, effectively induced luciferase reporter gene expression (Figure 6A), indicating that FKHRL1 is an activator rather than a repressor of transcription. In the presence of IGF1, the expression of the FHRE-Luc reporter gene by FKHRL1 was significantly reduced (Figure 6A), suggesting that the PI3K/Akt pathway plays a role in inhibiting FKHRL1-dependent transcription. This was confirmed by the finding that FKHRL1-dependent transcription was dramatically suppressed when FKHRL1 was coexpressed with a constitutively active form of Akt and the FHRE-Luc reporter gene (Figure 6B).

To determine whether the effect of Akt on FKHRL1-dependent transactivation was due to Akt's ability to phosphorylate FKHRL1, we cotransfected the FHRE-Luc reporter with a mutant form of FKHRL1 in which all three of the Akt phosphorylation sites were converted to alanines. The triple mutant of FKHRL1, which localized to the nucleus, potently induced transcription of the reporter gene even in the presence of IGF1, a condition under which the endogenous PI3K/Akt pathway is active (Figure 6C). The ability of the triple mutant of FKHRL1 to induce the expression of the FHRE-Luc reporter gene was not affected by the status of Akt activity. First, the transactivating effect of the triple mutant of FKHRL1

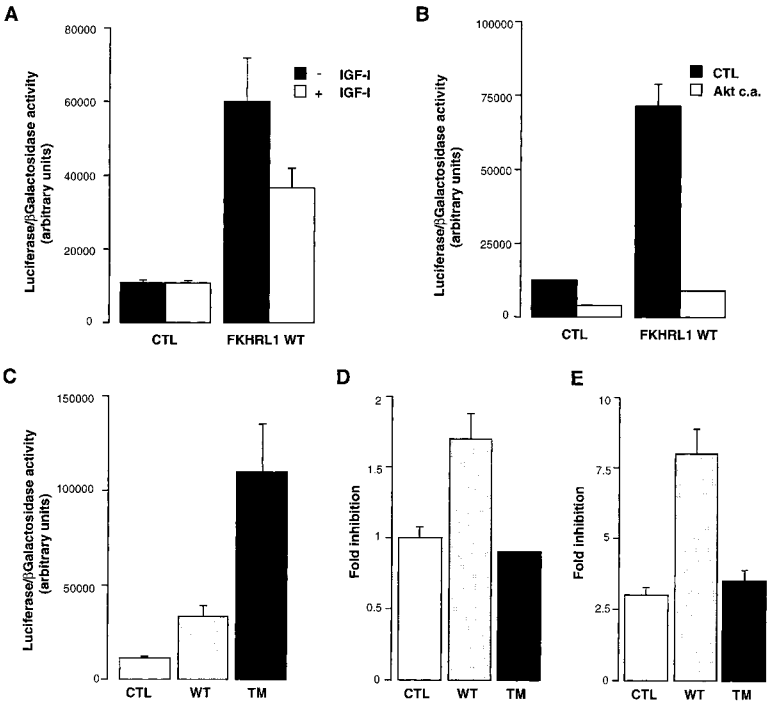


Figure 6. FKHRL1 Activates Reporter Gene Transcription, and This Activity Is Suppressed by Akt in a Phosphorylation-Dependent Manner

(A) CCL39 fibroblasts were transiently cotransfected with an empty vector (CTL) or a vector encoding wild-type FKHRL1 together with the luciferase reporter construct under the control of the portion of the Fas ligand promoter that contains three FH-binding sites (FHRE-Luc reporter). One day after transfection, cells were incubated in the presence or absence of IGF1 for 24 hr, and the luciferase activity was assayed.

(B) CCL39 fibroblasts were transiently cotransfected with an empty vector (CTL) or a vector encoding wild-type FKHRL1 together with a constitutively active form of Akt and the FHRE-Luc reporter. The cells were incubated in serum-free medium for 24 hr, and luciferase assays were performed.

(C) CCL39 fibroblasts were transiently cotransfected with an empty vector (CTL), or a vector encoding FKHRL1 wt or the triple mutant (TM) and the FHRE-Luc reporter. Cells were incubated in the presence of IGF1 for 24 hr, and luciferase assays were performed.

(D) The same experiment as in (A) was performed with the empty vector (CTL) or the wt and the triple mutant (TM) of FKHRL1. The results are presented as fold inhibition by IGF1.

(E) The same experiment as in (B) was performed with the empty vector (CTL) or the wt and the triple mutant (TM) of FKHRL1. The results are presented as fold inhibition by Akt.

Data presented represent the mean and SEM of three (B and D) or four (A and C) independent experiments conducted in duplicate. Each bar is statistically significant according to ANOVA with $p < 0.01$.

was independent of the presence or absence of IGF1 (Figure 6D). Second, the expression of a constitutively active form of Akt failed to efficiently suppress the induction of the FHRE-Luc reporter gene by the triple mutant of FKHRL1 (Figure 6E).

Taken together, these experiments indicate that in the absence of phosphorylation by Akt, FKHRL1 acts in the nucleus as an activator of transcription. By catalyzing the phosphorylation of FKHRL1, Akt prevents FKHRL1-dependent transcription mainly by promoting the exclusion of FKHRL1 from the nucleus so that this transcription factor is unable to activate its target genes.

### The Nonphosphorylated Form of FKHRL1 Induces Apoptosis by a Fas Ligand–Dependent Mechanism

We observed that FKHRL1 bound to DNA and activated transcription under conditions of growth factor withdrawal that can lead to apoptosis. This finding prompted us to consider whether FKHRL1 might regulate the expression of critical death genes and thereby induce apoptosis. Akt might then promote survival by phosphorylating FKHRL1 and inhibiting its function as a transcriptional activator.

One possible FKHRL1 target whose protein product is a mediator of cell death is the Fas ligand gene. We and others have found that the withdrawal of growth factors from several different cell types, including PC12 cells and cerebellar granule neurons, leads to the upregulation of Fas ligand mRNA and protein (Le-Niculescu et al., 1999; G. Perini, et al., unpublished data). The production of Fas ligand is important for apoptosis induced by the withdrawal of survival factors, since blocking the action of Fas ligand leads to a reduction in apoptosis (Le-Niculescu et al., 1999). We found that the Fas ligand promoter contains within its regulatory region three FHREs that bind FKHRL1 (Figure 5) and mediate FKHRL1-dependent transcription (Figure 6). Moreover, using a construct in which 2.5 kb of the Fas ligand promoter was fused to the luciferase reporter gene, we found that the triple mutant of FKHRL1 induced the activity of the Fas ligand native promoter (data not shown).

To examine the effect of FKHRL1 on apoptosis, we first used cerebellar granule neurons as a cellular model for apoptosis, since these cells have been shown both to be dependent on Akt activity for their survival and on Fas ligand for apoptosis. We transfected these neurons with the wild-type or the triple mutant form of FKHRL1 and performed a blind analysis of cell survival and apoptosis in the transfected cells by microscopic visualization of the integrity of the nucleus and the processes as previously described (Dudek et al., 1997) (Figure 7A). These experiments showed that in the presence of the survival factor IGF1, the triple mutant of FKHRL1, which is no longer phosphorylated by Akt and is localized to the nucleus, induced apoptosis when compared to a control empty vector or wt FKHRL1 (Figure 7A, left panel). The effect of mutant FKHRL1 on apoptosis was also observed in other cell types such as Jurkat T lymphocytes (Figure 7C), which are also extremely sensitive

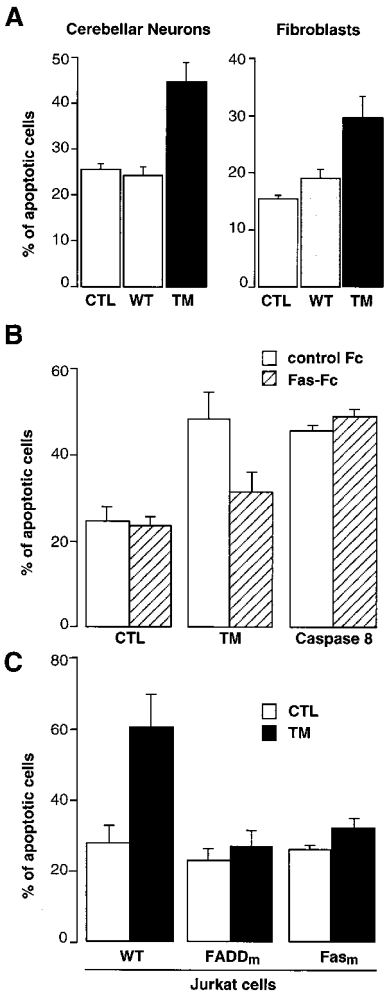


Figure 7. The Nonphosphorylatable Form of FKHRL1 Triggers Apoptosis by a Fas Ligand–Dependent Mechanism

(A) (Left) Cerebellar granule cells were transiently cotransfected with an empty vector (CTL), or a vector encoding FKHRL1 wt or the triple mutant (TM) and a vector encoding β-galactosidase. After 24 hr, cells were incubated for 20 hr with IGF1. Transfected cells were stained with the anti-β-galactosidase antibody, and DNA was stained with Hoechst. The experiments were performed in duplicate, and 100 to 200 cells per coverslips were counted in a blinded fashion. Data presented correspond to the mean and SEM of three independent experiments. The results comparing the triple mutant to the empty vector and the wt were statistically significant according to ANOVA with $p < 0.01$.

(Right) CCL39 fibroblasts were transfected and scored for apoptosis as described for neurons in left panel. Data presented represent the mean and variation of two independent experiments performed in duplicate.

(B) Cerebellar granule cells were transiently cotransfected with a vector encoding the β-galactosidase and with an empty vector (CTL), a vector encoding the triple mutant of FKHRL1 (TM) or the vector encoding Caspase 8. Cells were incubated with Fas-Fc or a control Fc (1 μg/ml) for 20 hr and apoptotic cells scored in a blinded fashion as described in (A). Data presented correspond to the mean and SEM of three independent experiments performed in duplicate. The results comparing the effect of control Fc and Fas-Fc for the triple mutant are statistically significant according to ANOVA with $p < 0.05$.

(C) Jurkat T cells (WT, FADD mutant [$FADD_m$], or Fas mutant [$Fas_m$]) were cotransfected with an empty vector (CTL) or a vector encoding the triple mutant of FKHRL1 (TM) and a vector encoding GFP. Forty-eight hours after transfection, cells were stained with Hoechst and apoptotic cells were counted. Data presented represent the mean and variation of two independent experiments.

to Fas signaling (Nagata, 1997), and CCL39 fibroblasts (Figure 7A, right panel).

To determine whether the induction of cell death by the triple mutant was mediated at least in part by its ability to induce the expression of the Fas ligand gene, we used two different approaches to block the Fas ligand signaling. The first approach employed a soluble Fas-Fc fusion protein that functions as a decoy for the newly synthesized Fas ligand and thus inhibits Fas ligand binding to its cell surface receptor Fas (Nagata, 1997). When cerebellar granule neurons were treated with Fas-Fc, but not with the control Fc, the FKHRL1-induced apoptosis was significantly reduced (Figure 7B). The inhibition of FKHRL1-induced apoptosis by Fas-Fc was specific, since Fas-Fc treatment did not affect the basal level of apoptosis nor the apoptosis induced by the expression of Caspase 8, a downstream component of the Fas signaling cascade (Figure 7B). The blocking effect of Fas-Fc on the apoptosis caused by the triple mutant of FKHRL1 was also observed in CCL39 fibroblasts (data not shown).

The second approach we employed to assess the importance of Fas ligand for FKHRL1-induced apoptosis took advantage of mutant cell lines derived from Jurkat cells that are functionally deficient in the receptor Fas or in a crucial component of the Fas signaling cascade, the adaptor protein FADD (P. J. and J. B., unpublished data). In wt Jurkat cells, as in neurons and fibroblasts, the triple mutant of FKHRL1 induced a high level of apoptosis relative to the control vector (Figure 7C). The apoptotic effect of the triple mutant of FKHRL1 was almost completely abolished when mutant FKHRL1 was expressed in Fas or FADD mutant Jurkat cells (Figure 7C), indicating that the Fas signaling cascade is required for FKHRL1 induction of apoptosis.

## Discussion

Growth factors promote cell survival at least in part by activating the PI3K and its downstream target Akt. One function of Akt is to phosphorylate and inhibit proapoptotic components of the intrinsic cell death machinery present within the cytoplasm. There is evidence that the PI3K/Akt pathway may also have an important function within the nucleus (Lin et al., 1997; Ogg et al., 1997; Cichy et al., 1998). However, the nuclear targets of the PI3K/Akt pathway and their role in cell survival are not yet well clarified. In this study, we demonstrate that the PI3K/Akt pathway regulates transcription by phosphorylating FKHRL1, a member of the Forkhead transcription factor family. This leads to FKHRL1's interaction with 14-3-3 proteins and FKHRL1's sequestration in the cytoplasm, away from its transcriptional targets. Under conditions of growth factor deprivation, the PI3K/Akt pathway is inactivated, FKHRL1 is unphosphorylated at its Akt sites, and FKHRL1 accumulates in the nucleus where it may activate death genes, including the Fas

ligand gene, and thereby participate actively in the process of apoptosis.

When fibroblasts are exposed to IGF1 or serum, Akt is activated and catalyzes the phosphorylation of FKHRL1. It is likely that additional protein kinases whose consensus sites of phosphorylation are similar to that of Akt, such as PKA or p90RSK, may regulate FKHRL1 function by phosphorylating FKHRL1 at T32, S253, and/or S315. Moreover, an analysis of the amino acid sequence of FKHRL1 reveals the presence of potential phosphorylation sites in addition to T32, S253, and S315. For example, FKHRL1 contains several serines and threonines that are flanked by amino acids that conform to the consensus site (PX*S*/*T*P) recognized by MAP kinases. Further studies will be required to determine whether protein kinases in addition to Akt play an important role in regulating FKHRL1 function.

The phosphorylation of FKHRL1 at T32 and S253 promotes FKHRL1 binding to the zeta isoform of 14-3-3. The interaction of FKHRL1 with 14-3-3 may be important for retaining the phosphorylated form of FKHRL1 in the cytoplasm and thereby for inhibiting FKHRL1's ability to activate transcription. In addition, the binding of FKHRL1 to 14-3-3 in growth factor–stimulated cells may prevent the dephosphorylation of FKHRL1 by endogenous phosphatases and in this way may prolong the action of Akt and promote cell survival.

It is not clear how the withdrawal of survival factors triggers the dephosphorylation and relocalization of FKHRL1 to the nucleus. The dephosphorylation of FKHRL1 may reflect the inactivation of Akt permitting the action of a constitutively active protein phosphatase on the transcription factor.

There are several mechanisms that may explain how the unphosphorylated form of FKHRL1 translocates to the nucleus where it activates gene expression. An analysis of other FH family members has revealed the presence of a domain enriched in basic amino acids at the end of the FH DNA–binding domain that may form part of a nuclear localization signal (NLS). Since in FKHRL1 this basic domain overlaps with the second site of Akt phosphorylation (S253), it is possible that phosphorylation of this site adds a negative charge to the positively charged basic region, thereby disrupting the function of the NLS. In growth factor–deprived cells, when Akt is inactive and FKHRL1 is dephosphorylated, the NLS may function effectively and thus promote the translocation of FKHRL1 to the nucleus. An alternative possibility is that the phosphorylation of FKHRL1 changes FKHRL1's interaction with other proteins that act either to sequester FKHRL1 in the cytoplasm when FKHRL1 is phosphorylated (e.g., 14-3-3) or to localize the unphosphorylated form of FKHRL1 within the nucleus.

Once FKHRL1 is in the nucleus, it binds to specific DNA sequence elements and functions as an activator rather than as a repressor of transcription. Our experiments demonstrate that nuclear FKHRL1 binds to a DNA sequence, the Forkhead response element (FHRE), which is found in three copies within the regulatory region of the Fas ligand gene. Under conditions of cell growth where the PI3K/Akt pathway is inactive and FKHRL1 is unphosphorylated, FKHRL1 effectively promotes the transcription of an FHRE-driven reporter gene. This suggests that under certain circumstances, FKHRL1 may control the expression of the endogenous Fas ligand gene. Consistent with this possibility, we find that a mutant form of FKHRL1 that is not phosphorylated by Akt induces apoptosis by a Fas ligand–dependent mechanism. Additional experiments will be required to determine whether the endogenous FKHRL1 also regulates Fas ligand transcription and cell death. Since the Fas ligand gene has previously been shown to be regulated by a number of transcription factors also involved in cell death regulation, including NFAT (Latinis et al., 1997; Faris et al., 1998; Holtz-Heppelmann et al., 1998), AP1 and NFκB (Kasibhatla et al., 1998), and Egr3 (Mittelstadt and Ashwell, 1998), it is possible that FKHRL1 will be found to cooperate with one or several of these other factors to induce Fas ligand transcription and apoptosis in growth factor–deprived cells. One way Akt might suppress apoptosis would be to inhibit one or several transcription factors that control the expression of death genes such as the gene encoding Fas ligand.

Although we present evidence that in postmitotic neurons, fibroblasts, or Jurkat T cells, the nonphosphorylated form of FKHRL1 plays a role in mediating cell death, it is likely that FKHRL1 in mammals and DAF16 in *C. elegans* have other biological functions as well. In fibroblasts that have been deprived of serum and have withdrawn from the cell cycle into the G0 resting state, the PI3K/Akt pathway is inactive and FKHRL1 is localized to the nucleus. It is possible that under these circumstances, FKHRL1 actively induces the expression of genes that inhibit cell cycle progression, such as the CDK inhibitors. A role for FKHRL1 in apoptosis and cell cycle control is consistent with the observation that mutations of FKHRL1 can lead to oncogenesis (Hillion et al., 1997; Anderson et al., 1998).

It is also likely that in certain cell types, FKHRL1 regulates the expression of genes that modulate metabolic functions of the cell. For example, in mammals as well as in *C. elegans*, the PI3K/Akt pathway has been found to mediate aspects of the cellular response to insulin. One of the sites to which FKHRL1 binds is present within the regulatory region of a number of genes whose transcription is repressed by insulin in a PI3K/Akt-dependent manner, such as IGF-BP1 or phosphoenolpyruvate carboxykinase (PEPCK) (O'Brien et al., 1995; Cichy et al., 1998; Liao et al., 1998). It is possible that the downregulation of genes by insulin also involves the phosphorylation and cytoplasmic sequestration of FKHRL1 or closely related transcription factors. Therefore, the PI3K/Akt signaling pathway, by phosphorylating FKHRL1, may coordinately regulate the expression of proapoptotic genes, cell cycle–regulated genes, and genes that control cellular homeostasis. It will be of interest in the future to determine the complete array of genes that are regulated by FKHRL1.

It is suggested by the experiments reported here that Akt regulates a variety of biological processes in mammals by phosphorylating FKHRL1. It is likely that in *C. elegans*, Akt phosphorylation of DAF16 leads to the relocalization of DAF16 within the cell from the nucleus to the cytoplasm, thereby providing a biochemical mechanism for the genetic finding that Akt antagonizes DAF16 function. That many aspects of the PI3K/Akt/FKHRL1

signaling pathway are conserved in organisms as diverse as *C. elegans* and mammals indicates that this signal transduction pathway will prove to be critical for the proper development and function of most, if not all, animal species.

## Experimental Procedures

### Materials

IGF1 and LY 294002 (LY) were purchased from Boehringer Mannheim and Calbiochem, respectively. Unless otherwise stated, IGF1 and LY were used at the final concentrations of 50 ng/ml and 20 μM, respectively. The anti-HA and the anti-M2 monoclonal antibodies were obtained from Boehringer Mannheim and Kodak, respectively. The anti-ATF1 antibody was purchased from UBI. The Fas-Fc fusion protein was obtained from R&D Systems and used at the final concentration of 1 μg/ml.

### Constructs

The vector encoding the HA-tagged forms of Akt, either constitutively active (myristylated-ΔPH) or inactive (KM mutant), and the CMV-LacZ, EF-LacZ, and CMV-GFP constructs used in this study have been described previously (Datta et al., 1997; Dudek et al., 1997). The vector encoding mouse Caspase 8 (Sakamaki et al., 1998) was kindly provided by Dr. S. Yonehara. The vector encoding the M2-tagged form of the zeta isoform of 14-3-3 was kindly provided by Dr. H. Fu. The FKHRL1 human cDNA (Anderson et al., 1998) was tagged at the N terminus with the HA epitope and subcloned into the pECE expression vector (Ellis et al., 1986). The mutants of each FKHRL1 phosphorylation site were generated by PCR. The wild-type FKHRL1 cDNA was tagged at the C terminus with the M2 epitope and subcloned into a modified version of the pcDNAneo3 expression vector. The cDNA encoding the first 525 amino acids or full-length FKHRL1 were subcloned into the pGex 4T3 vector (Pharmacia). Details of the constructs are available upon request. Wild-type and mutant GST-FKHRL1 protein were prepared according to the Pharmacia protocol.

A 2.5 kb fragment of the human Fas ligand promoter was kindly provided to us by Dr. C. V. Paya (Holtz-Heppelmann et al., 1998). The fragment of the Fas ligand promoter containing the three FKHRL1-binding sites located between nucleotides −743 to −648 relative to the start site of Fas ligand mRNA synthesis initiation was amplified by PCR and subcloned between the NheI and BglII site of the pGL3 vector containing a minimal SV40 promoter (Promega).

### Antibodies

Phosphopeptides of the following sequence were synthesized (Tufts Synthesis Facility, Boston, MA) (P-T32: CPQSRPRSCpTWPLQ; P-S253: CKAPRRRAVpSMDNS; P-S315: CTDFRSRTNpSNAST) and coupled to KLH (Pierce). Antibodies to the KLH-coupled peptides and purified full-length GST-FKHRL1 were obtained and purified as previously described (Datta et al., 1997).

### Cell Culture

The 293T human epithelial kidney cell line and the Chinese hamster lung fibroblast cell line CCL39 (ATCC) were cultured in DMEM supplemented with 10% FCS (GIBCO) and antibiotics (50 U/ml penicillin and 50 μg/ml streptomycin). Jurkat T lymphocytes were cultured in RPMI supplemented with 10% FCS and antibiotics. Cerebellar granule cells were obtained from P6 Long Evans rats and cultured as previously described (Dudek et al., 1997). Unless otherwise stated, cells were transfected by the calcium phosphate technique.

### Kinase Assays

293T cells were seeded in 35 mm dishes at the density of 750,000 cells per dish. They were transfected with 5 μg of the HA-Akt expression vectors. Twenty-four hours after transfection, cells were starved in serum-free medium for 20 hr. Extracts were obtained and kinase assays were performed as described (Dudek et al., 1997) using 5 μg of purified GST protein as a substrate and 5 μCi, 50 μM of $\gamma^{32}$P ATP for 1 hr at 30°C. The reaction products were resolved by SDS–PAGE (12%) and $^{32}$P-labeled proteins visualized by autoradiography.

### Immunoblotting and Immunoprecipitation

Cells were seeded in 6-well dishes at the density of $5.10^5$ cells/well. They were transfected with 5 μg of the FKHRL1 construct. Twenty-four hours after transfection, cells were incubated in serum-free DMEM for 20 hr and stimulated with IGF1. Extracts were prepared for immunoblotting or immunoprecipitation using Triton lysis buffer as described previously (Pagès et al., 1994).

### Immunolocalization

CCL39 cells were plated onto glass coverslips at a density of $10^5$ cells/well in 12-well dishes. The cells were transfected with 4 μg of the indicated plasmid. The day after transfection, cells were incubated in serum-free medium for 16 hr, and then treated with 10 μM LY for 1 hr. The cells were then fixed in 4% formaldehyde/2% sucrose and permeabilized with 0.1% Triton X100. Coverslips were incubated with the primary antibodies (anti-HA or anti-M2) for 2 hr, then incubated for 1 hr with a goat anti-mouse cy3-conjugated antibody. Coverslips were mounted in Vectastain (Vector Laboratories) and examined under epifluorescent illumination. For quantitation, 50–100 cells per coverslip were counted.

### Electrophoretic Mobility Shift Assay

293T cells were seeded in 6-well plates at a density of 750,000 cells/well. 293T cells were transfected with 2.5 μg of the various FKHRL1 plasmids and 2.5 μg of the plasmid encoding the constitutively active form of Akt. Whole-cell extracts were prepared by lysing the cells in a Triton lysis buffer as described (Pagès et al., 1994). Complementary oligonucleotides corresponding to the IGFBP1 IRS (5′-ATTGCTAGCAAGCAAAACAAACCGCTAGCTTA-3′) or the Fas ligand site (5′-AATAGATCTTAAATAAATAGATCTTTA-3′) were annealed and end-labeled with $\gamma^{32}$P ATP. The binding reactions were performed and analyzed on a nondenaturing gel as described (Pierrou et al., 1994). For supershift studies, 1 μl of immunopurified anti-FKHRL1 antibody or the anti-ATF1 antibody was preincubated for 10 min on ice with the cell extract. DNA–protein complexes were visualized by autoradiography.

### Luciferase Assay

CCL39 cells were seeded in 24-well plates, at a density of $10^5$ cells/well, and were cotransfected with 0.25 μg of the FKHRL1 plasmid of interest, 0.5 μg of the Luciferase reporter gene, 0.25 μg of the EF-LacZ construct, and 1 μg of carrier DNA (pBluescript). Two days after transfection, cells were lysed in 100 μl of lysis buffer, and the luciferase activity of one-fifth of the samples was assayed according to the Promega protocol. β-galactosidase activity was assayed as described (Maniatis et al., 1989).

### Survival Assays

Cerebellar granule neurons were seeded onto glass coverslips in 24-well plates at a density of $10^7$ cells/well and at DIV5-6 were cotransfected with 2 μg of the construct of interest, 1 μg of the CMV-GFP plasmid, and 0.5 μg of the CMV-LacZ plasmid as described (Dudek et al., 1997). Twenty-four hours after transfection, cells were incubated for 20 hr in the presence of IGF1 and incubated or not with Fas-Fc or Fc alone (1 μg/ml). The transfected neurons were then fixed in 4% paraformaldehyde/2% sucrose. Immunofluorescence analyses were performed as described above using the anti-β-galactosidase antibody (Pharmacia), and nuclei were stained by Hoechst 33258 (2.5 μg/ml). Apoptotic nuclei were counted in a blinded manner.

CCL39 fibroblats were transfected with 4 μg of the relevant plasmid and 1 μg of the plasmid encoding GFP. They were treated in the same way as described for neurons, and apoptotic nuclei of GFP-positive cells were counted in a blinded manner.

Jurkat cells were transfected by electroporation (250 V, 950 μF, Biorad) with 16 μg of the relevant plasmids and 4 μg of the plasmid encoding GFP. GFP-positive cells were scored for apoptosis as described above.

**Acknowledgments**

We thank Dr. H. Fu, Dr. C. V. Paya, and Dr. S. Yonehara for kindly providing reagents. We thank all members of the Greenberg laboratory for helpful discussions, particularly S. R. Datta, Y. Gotoh, and A. J. Shaywitz. We thank J. Zieg, J. M. Kornhauser, and J. Sage for the help in the preparation of the figures. This research was supported by a Mental Retardation Research Center grant (HD 18655) and by grants from National Institutes of Health (CA43855), Daiichi Pharmaceuticals, and F. M. Kirby foundation to M. E. G. and partially supported by a National Institutes of Health grant (CA46595) and a Hoechst Roussel Marion Exploratory Award to J. B.; A. Brunet was supported by an EMBO Long-Term Fellowship and is currently supported by a Human Frontier Long-Term Fellowship. A. Bonni is supported by a Postdoctoral Research Fellowship for Physicians from the Howard Hughes Medical Institute. M. Z. L. is supported by a Graduate Fellowship from National Defense Science and Engineering. P. J. is supported in part by a National Science Foundation Predoctoral Fellowship.

Received January 5, 1999; revised February 12, 1999.

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