Multiple elements regulate nuclear/cytoplasmic shuttling of FOXO1: characterization of phosphorylation- and 14-3-3-dependent and -independent mechanisms

Xiangshan ZHAO*†[1], Lixia GAN*†[1], Haiyun PAN*†, Donghui KAN*†, Michael MAJESKI*†, Stephen A. ADAM‡ and Terry G. UNTERMAN*†[2]

*Department of Medicine, University of Illinois at Chicago College of Medicine, and Medical Research Unit, Chicago, IL 60612, U.S.A., †VA Chicago Health Care System (West Side), 820 South Damen Avenue, Chicago, IL 60612, U.S.A., and ‡Department of Cell and Molecular Biology, Northwestern University Medical School, Chicago, IL 60611, U.S.A.

FOXO1, a Forkhead transcription factor, is an important target of insulin and growth factor action. Phosphorylation of Thr-24, Ser-256 and Ser-319 promotes nuclear exclusion of FOXO1, yet the mechanisms regulating nuclear/cytoplasmic shuttling of FOXO1 are poorly understood. Previous studies have identified an NLS (nuclear localization signal) in the C-terminal basic region of the DBD (DNA-binding domain), and a leucine-rich, leptomycin-B sensitive NES (nuclear export signal) located further downstream. Here, we find that other elements in the DBD also contribute to nuclear localization, and that multiple mechanisms contribute to nuclear exclusion of FOXO1. Phosphorylation of Ser-319 and a cluster of nearby residues (Ser-322, Ser-325 and Ser-329) functions co-operatively with the nearby NES to promote nuclear exclusion. The N-terminal region of FOXO1 (amino acids 1–149) also is sufficient to promote nuclear exclusion, and does so through multiple mechanisms. Amino acids 1–50 are sufficient to promote nuclear exclusion of green fluorescent protein fusion proteins, and the phosphorylation of Thr-24 is required for this effect. A leucine-rich, leptomycin B-sensitive export signal is also present nearby. Phosphorylated FOXO1 binds 14-3-3 proteins, and co-precipitation studies with tagged proteins indicate that 14-3-3 binding involves co-operative interactions with both Thr-24 and Ser-256. Ser-256 is located in the C-terminal region of the DBD, where 14-3-3 proteins may interfere both with DNA-binding and with nuclear-localization functions. Together, these studies demonstrate that multiple elements contribute to nuclear/cytoplasmic shuttling of FOXO1, and that phosphorylation and 14-3-3 binding regulate the cellular distribution and function of FOXO1 through multiple mechanisms. The presence of these redundant mechanisms supports the concept that the regulation of FOXO1 function plays a critical role in insulin and growth factor action.

Key words: Forkhead, 14-3-3 protein, gene regulation, growth factor, insulin, transcription.

INTRODUCTION

FOXO proteins form a sub-group of the Forkhead (or FOX) family of transcription factors [1] and play an important role in mediating effects of insulin and growth factors on metabolism, and in cellular proliferation and survival [2]. Three major mammalian FOXO proteins have been identified, FOXO1, FOXO3a and FOXO4 (also known as FKHR1, FKHRL1 and AFX, respectively) [3], and a recent report indicates that another FOXO protein (FOXO6) may play a role in the developing central nervous system [4]. Genetic studies demonstrate that a FOXO protein (DAF-16) is a major target for insulin-like signalling in *Caenorhabditis elegans* [5], and recent findings indicate that FOXO proteins also are important targets for insulin-like signalling in *Drosophila* [6–8]. Genetic studies also indicate that FOXO proteins are important targets for insulin signalling in the mammalian liver [9] and in the development and normal function of the islets of Langerhans [10].

Signalling by insulin and growth factors results in the phosphorylation of FOXO proteins at three highly conserved predicted PKB (protein kinase B) sites, corresponding to Thr-24, Ser-256 and Ser-319 in human FOXO1 [11–13] (Figure 1A). Phosphorylation at these sites suppresses transactivation and promotes the redistribution of FOXO proteins outside of the nucleus and localization in the cytoplasmic compartment [12–15]. Because the regulation of the nuclear/cytoplasmic distribution of FOXO proteins is thought to be important in determining their biological activity, we sought to better understand the elements involved in regulating nuclear/cytoplasmic shuttling of FOXO proteins and mediating the effects of phosphorylation on the cellular distribution and function of FOXO proteins.

Previous studies indicate that the phosphorylation of Ser-256 at the C-terminal end of the DBD (DNA-binding domain) is required for effective phosphorylation of Thr-24 and Ser-319 by PKB or other PI 3-kinase (phosphoinositide 3-kinase)-dependent kinases [15,16]. Ser-256 is located within a basic region of the DBD and we have reported that phosphorylation at this site can impair DNA-binding activity [17]. We and others also have reported that this basic region at the C-terminal end of the DBD (Figure 1A, L1) can function as an NLS (nuclear localization signal) when expressed in-frame with GFP (green fluorescent protein), and that replacing a cluster of three basic arginine residues in this region (corresponding to Arg-251, Arg-252 and Arg-253 in FOXO1)

Abbreviations used: DBD, DNA-binding domain; NES, nuclear export sequence; GFP, green fluorescent protein; PKB, protein kinase B; NLS, nuclear localization signal; CK1, casein kinase 1; BCS, bovine calf serum; DMEM, Dulbecco's modified Eagle's medium; HBSS, Hank's balanced salt solution; DAPI, 4′,6-diamido-2-phenylindole-2HCl; IGF, insulin-like growth factor; PI 3-kinase, phosphoinositide 3-kinase.

[1] These authors contributed equally to this work.

[2] To whom correspondence should be addressed at: Room 6229 Medical Research Unit, VA Chicago Health Care System (West Side), 820 South Damen Avenue, Chicago, IL 60612, U.S.A. (e-mail Unterman@uic.edu).

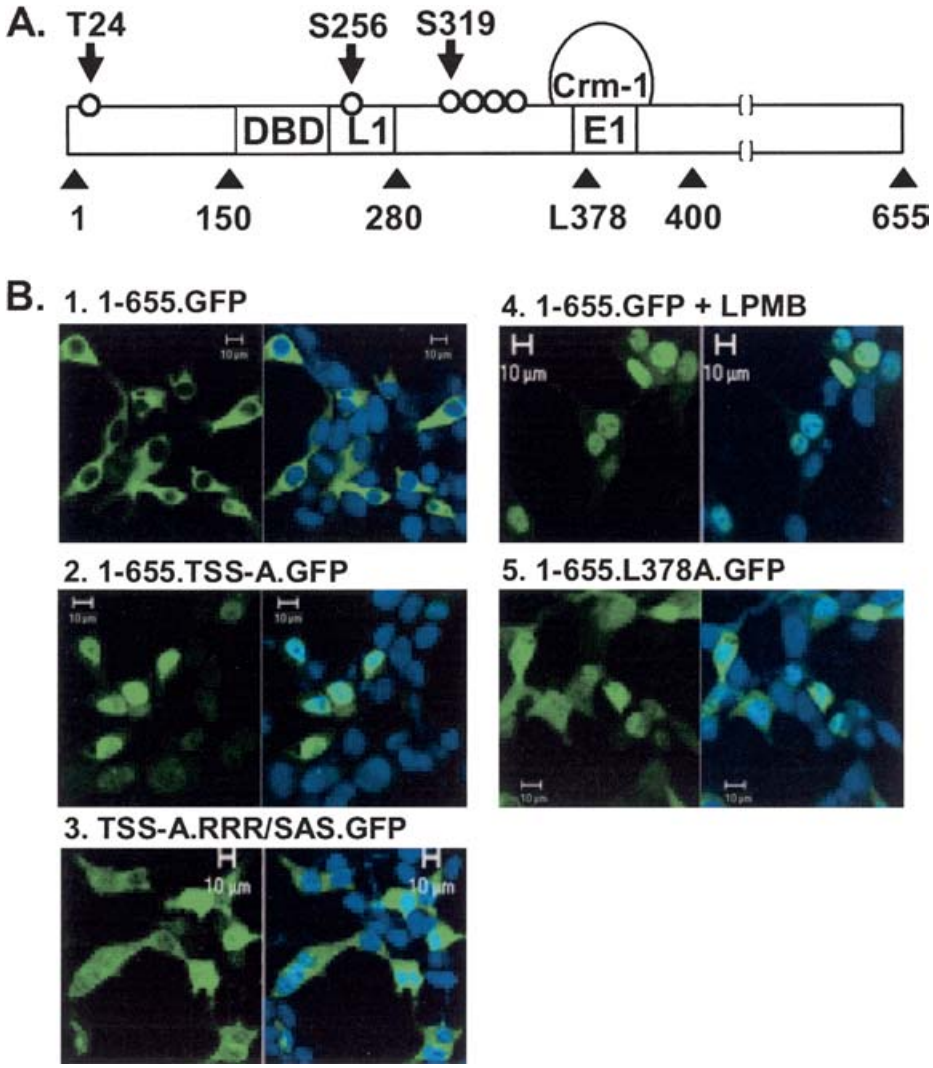

Figure 1 Cellular trafficking of FOXO1–GFP fusion proteins in HEK-293 cells

(**A**) Known phosphorylation sites and known nuclear localization and export signals in FOXO1. Previous studies have shown that the three predicted PKB phosphorylation sites (Thr-24, Ser-256 and Ser-319) are phosphorylated in response to insulin and growth factors through PI 3-kinase-dependent pathways. Following the phosphorylation of Ser-319, two nearby residues (Ser-322 and Ser-35) are phosphorylated by CK1, while Ser-329 is phosphorylated constitutively by DYRK1A. The basic region at the C-terminal end of the DBD contributes to DNA binding and can function as an NLS (L1). A leucine-rich NES (E1) is located C-terminal to the DBD and the cluster of phosphorylation sites associated with Ser-319. The function of this export signal is disrupted by treatment with leptomycin B, indicating that its function is mediated through interaction with Crm-1 or a related factor. (**B**) Function of known FOXO1 nuclear localization and export signals in HEK-293 cells. HEK-293 cells were plated on to polylysine-coated slides, then transfected with vectors expressing FOXO1 in-frame with GFP. Cells were fixed and stained with DAPI prior to visualization of GFP fusion proteins (green) and nuclei (blue) by laser confocal microscopy. Cells are shown expressing: panel 1, fusion proteins containing full-length wild-type FOXO1 (1-655.GFP); panel 2, a fusion protein where Thr-24, Ser-256 and Ser-319 have been replaced by alanine (1-655.TSS-A.GFP); panel 3, a fusion protein where Thr-24, Ser-256 and Ser-319 are replaced by alanine and where Arg-251, Arg-252 and Arg-253, which are critical for the function of the known NLS (L1), are replaced by serine, alanine and serine respectively (TSS-A.RRR/SAS.GFP); panel 4, the wild-type fusion protein after 3 h treatment with leptomycin B (1-655.GFP + LPMB); or panel 5, a fusion protein where Leu-378 is replaced by alanine (FOXO1.L378A.GFP), which disrupts the function of the known NES (E1). Scale bars, 10 μm.

with neutral amino acids effectively disrupts the function of this NLS [17,18]. However, phosphorylation of Ser-256 does not appear to be sufficient to disrupt nuclear localization of FOXO1 [17], and the phosphorylation of Thr-24 and Ser-319 appear to be critical for nuclear exclusion of FOXO1 [17].

Rena et al. [19] and Woods et al. [20] have reported that the phosphorylation of Ser-319 is required for phosphorylation of Ser-322 and Ser-325 by CK1 (casein kinase 1) [19] and studies in living cells suggest that this cluster of phosphorylation sites, together with the phosphorylation of Ser-329 by DYRK1A [20], may promote association with Ran, a nuclear transport protein, and nuclear/cytoplasmic translocation of FOXO1 [19]. Interestingly, a leucine-rich NES (nuclear export signal) is also located C-terminal to the DBD of FOXO1 [21], and the function of this NES is inhibited by leptomycin B, indicating that it interacts with Crm-1 (Figure 1A). However, it has not been determined whether the presence of this NES is required for the ability of Ser-319 and nearby phosphorylation sites to promote cytoplasmic localization of FOXO1.

Early studies revealed that the phosphorylation of FOXO proteins results in the binding of 14-3-3 proteins [12], and recent findings support the concept that 14-3-3 binding promotes the nuclear/cytoplasmic redistribution of FOXO proteins [15,22]. Thr-24 is located within a consensus 14-3-3-binding motif (Arg-Ser-Xaa-pSer/pThr-Xaa-Pro; where p indicates a phosphoryalted amino acid) [23], and phosphorylation of Thr-24 is required for 14-3-3

binding by FOXO1 [15]. 14-3-3 proteins are located primarily in the cytoplasm, and form dimers that bind to target sites co-operatively [24], which suggests that 14-3-3 proteins may tether FOXO proteins to other cytoplasmic factors [12]. Recent studies by Brunet et al. [22] indicate that 14-3-3 proteins can translocate into the nucleus where they may interact with FOXO proteins. Although early studies suggested that 14-3-3 proteins may contain an NES [25], this may not be the case [22], and the mechanisms by which the phosphorylation of Thr-24 and the binding of 14-3-3 proteins contribute to FOXO trafficking are not clear.

In the present study we provide evidence that multiple elements in the DBD contribute to nuclear localization of FOXO1, that the phosphorylation of Ser-319 and nearby residues may enhance the function of the nearby NES located C-terminal to the DBD, and that multiple elements in the N-terminal region of FOXO1 (including Thr-24 and a nearby leucine-rich export signal) promote cytoplasmic localization of fusion proteins. Co-precipitation studies indicate that 14-3-3 proteins interact co-operatively with Thr-24 and with residues in the C-terminal region of the FOXO1 DBD, where 14-3-3 binding may interfere with DNA-binding and nuclear-localization functions. Together, these studies demonstrate that multiple elements contribute to nuclear/cytoplasmic shuttling of FOXO1, and that phosphorylation and 14-3-3 binding regulates the cellular distribution and function of FOXO1 by multiple mechanisms.

MATERIALS AND METHODS

Expression vectors and cell transfection studies

Residues in the human FOXO1 cDNA were mutated and restriction sites for subcloning created by site-directed mutagenesis in the pAlter.Max (Promega) vector according to the manufacturer's directions. Mutations were confirmed by dideoxy sequencing in the University of Illinois at Chicago Sequence Center. Relevant amino acids in FOXO1 were replaced with alanine unless otherwise indicated. Fragments of FOXO1 were subcloned in-frame with the C-terminal GFP tag in the pEGFP-N1 vector (Clontech) or the N-terminal FLAG tag in pFLAG-CMV (Sigma).

For localization studies, HEK-293 cells were plated on glass slides (Nalge Nunc International) coated with poly-L-lysine (Sigma) and fed with Opti-MEM medium (Invitrogen) supplemented with 5% BCS (bovine calf serum; Invitrogen) plus antibiotics in 5% CO_2 at 37 °C. Then, 2 days later, cells were washed twice with HBSS (Hank's balanced salts solution; Sigma), re-fed with DMEM (Dulbecco's modified Eagle's medium; Invitrogen) with 1 mg/ml fatty acid-free BSA (Sigma), and then transfected with 0.8 μg of plasmid DNA with LIPOFECTAMINE 2000 (Invitrogen) for 4 h. Transfected cells were rinsed twice with HBSS, then re-fed with DMEM/BSA and stabilized for 18 h prior to a 3 h treatment with or without 20 nM leptomycin B (Sigma), 50 μM LY294002 (Calbiochem) or carrier alone. Cells were fixed with 4% paraformaldehyde in PBS and rinsed in PBS prior to applying mounting solution containing DAPI (4′,6-diamido-2-phenylindole-2HCl (Vector Laboratories). GFP-tagged proteins and DAPI-stained nuclei were visualized with a Zeiss confocal laser scanning 510 microscope in the University of Illinois at Chicago Research Resource Center. For quantitative analysis, the percentage of total fluorescence in the nuclear and cytoplasmic compartments was analysed after subtracting background fluorescence using ImageJ software provided by the National Institutes of Health. Results are reported as the mean ± S.E.M. from at least 12 cells expressing each construct.

For immunoprecipitation and Western blotting studies, HEK-293 cells were plated in 100 mm dishes and allowed to grow in Opti-MEM with 5% BCS for 2 days. Cells were re-fed with DMEM plus 10% BCS, then transfected with calcium phosphate precipitates containing 10 μg of DNA, as previously reported [26]. Transfected cells were washed twice with HBSS, and then re-fed with DMEM/BCS for 24 h. Cells were washed twice with HBSS and stabilized in DMEM/BSA for 18 h before studies. Following initial results suggesting that the ability of fusion proteins containing amino acids 1–50 of FOXO1 to bind 14-3-3 proteins may be impaired, subsequent immunoprecipitation and Western blotting studies were performed after cells were treated with 100 ng/ml recombinant human IGF-I (insulin-like growth factor-I) for 20 min to optimize the activation of PKB and related PI 3-kinase-dependent kinases, phosphorylation of FOXO1 and 14-3-3 binding. IGF-treated cells were washed twice with ice-cold PBS and lysed by the addition of 800 μl of lysis buffer (1% Nonidet P-40, 20 μg/ml aprotinin, 1 mM PMSF, 1 mM sodium orthovanadate, 50 mM NaF and 5 mM sodium-EDTA in PBS). Lysates were cleared by 20 min centrifugation at 14000 ***g*** at 4 °C. Protein concentration was determined by the Bradford assay (Bio-Rad) and lysates were stored at − 80 °C until analysis.

Immunoprecipitation and Western blotting

To evaluate interactions between 14-3-3 and GFP fusion proteins, cell lysates (1500 μg of protein) were incubated with 3 μl of polyclonal antibody against full-length GFP (Clontech) at 4 °C for 4 h prior to addition of 20 μl of a 50% (v/v) Protein A/G plus–agarose bead slurry (Santa Cruz Biotechnology) and incubation for 1.5 h at 4 °C. To evaluate interactions with FLAG-tagged proteins, lysates were incubated with anti-FLAG antibody conjugated to agarose beads (anti-FLAG® M2 affinity gel; Sigma) for 1.5 h. Beads were pelleted by centrifugation for 2 min at 1000 ***g***, and then washed three times with lysis buffer, and twice with PBS. Proteins were eluted and prepared for analysis by heating in reduced Laemmli sample buffer at 100 °C for 5 min. Proteins were resolved by 4–20% gradient SDS/PAGE and transferred on to PVDF membranes for Western blotting.

Membranes were blocked with 10% (w/v) non-fat dried milk in Tris-buffered saline, then probed either with antibody–horseradish peroxidase conjugates containing antibody against GFP (Clontech) or 14-3-3 proteins (Santa Cruz Biotechnology), or with anti-FLAG M2 monoclonal antibody (Sigma) and then anti-mouse IgM–horseradish peroxidase conjugate (Santa Cruz Biotechnology). The antibody against 14-3-3 proteins recognizes human 14-3-3β, γ, θ, ε, ζ, η and σ isoforms. Phosphospecific antibodies used to evaluate the phosphorylation of Thr-24 and Ser-256 were from Cell Signalling. Control studies with proteins prepared from cells treated with IGF-I or LY294002, and with phospho- and non-phospho-peptide competitors, confirmed that the antibodies interact specifically with phospho-FOXO1. Antibody–antigen complexes were identified by chemiluminesence (ECL + System; Amersham Biosciences).

RESULTS AND DISCUSSION

Multiple elements contribute to nuclear/cytoplasmic shuttling of FOXO1

As shown in Figure 1(A), previous studies indicate that the phosphorylation of Thr-24, Ser-256 and Ser-319 (and nearby residues), an NLS located at the C-terminal end of the DBD (L1) and an NES located further downstream (E1), all contribute to the regulation of the cellular distribution of FOXO1. However, the mechanisms by which the phosphorylation of these residues alters the cellular trafficking are not understood. Also, it is not known whether other nuclear localization or export signals also contribute to nuclear/cytoplasmic shuttling of FOXO1.

To address these questions, we examined the localization of fusion proteins expressing FOXO1 in-frame with GFP in HEK-293 cells. As shown in Figure 1(B), fusion proteins containing full-length FOXO1 in-frame with GFP are located primarily in the cytoplasmic space of HEK-293 cells (Figure 1B, panel 1) and replacing Thr-24, Ser-256 and Ser-319 with alanine residues results in nuclear targeting of the fusion protein (Figure 1B, panel 2), confirming that phosphorylation at these sites is critical for nuclear exclusion of FOXO1 in HEK-293 cells, as previously reported [17]. To determine whether the NLS located at the C-terminal end of the DBD is required for nuclear targeting of FOXO1, we replaced a cluster of arginine residues (Arg-251, Arg-252 and Arg-253) located within this NLS with neutral residues (serine, alanine and serine), which is known to disrupt the function this NLS [17,18]. As shown in panel 3 of Figure 1(B), disrupting the function of this NLS impairs, but does not completely prevent, nuclear targeting of full-length FOXO1–GFP. This result confirms that the NLS located at the C-terminal end of the DBD contributes to nuclear localization, and also indicates that some other element(s) also contributes to nuclear targeting of FOXO1.

As shown in panel 4 of Figure 1(B), treatment with leptomycin B, which disrupts the function of leucine-rich NESs, results in nuclear localization of the full-length fusion protein containing wild-type FOXO1 in HEK-293 cells, as previously reported [17]. To determine whether the leptomycin-sensitive NES located downstream from the DBD is required for cytoplasmic localization of FOXO1 in HEK-293 cells, we replaced Leu-378 with alanine, which disrupts the function of this NES [21]. As shown in panel 5 of Figure 1(B), mutation of Leu-378 impairs, but does not completely disrupt, nuclear exclusion of the FOXO1–GFP fusion protein. Replacing all five leucine residues in the region of this NES (Leu-374Ala, Leu-375Ser, Leu-378Ala, Leu-380Ser and Leu-381Ala) yields a similar result (not shown). These results confirm that this NES contributes to nuclear exclusion, and also indicate that some other element(s) also promotes cytoplasmic localization of FOXO1 through a leptomycin B-sensitive mechanism.

To identify elements involved in determining the cellular distribution of FOXO1, we created a series of constructs expressing fragments of FOXO1 in-frame with GFP. As shown in Figure 2(A), GFP, which is small enough to diffuse through nuclear pores, is distributed in both the nuclear and cytoplasmic compartments when it is expressed by itself in HEK-293 cells, as reported in [17]. In contrast, the fusion protein containing the first 149 amino acids of FOXO1 in-frame with GFP is localized predominantly in the cytoplasmic space (Figure 2B). To our knowledge, this result provides the first direct evidence that the region N-terminal to the DBD is sufficient to promote cytoplasmic localization.

The fusion protein containing the DBD and flanking sequences (amino acids 150–280) of FOXO1 is effectively localized in the nucleus (Figure 2C), while the fusion protein containing amino acids 280–400 is effectively localized in the cytoplasmic space (Figure 2D), indicating that elements in these regions contribute to nuclear and cytoplasmic localization, respectively. In contrast, fusion proteins containing amino acids 389–500 or 491–655 of FOXO1 are distributed evenly throughout the cell (Figures 2E and 2F), indicating that these regions do not promote either nuclear or cytoplasmic localization of fusion proteins. Based on these findings, we focused our attention on the role that elements located within the region of the DBD (amino acids 150–280) play in nuclear localization, and the role that elements located between amino acids 1–149 and 280–400 play in cytoplasmic localization of fusion proteins.

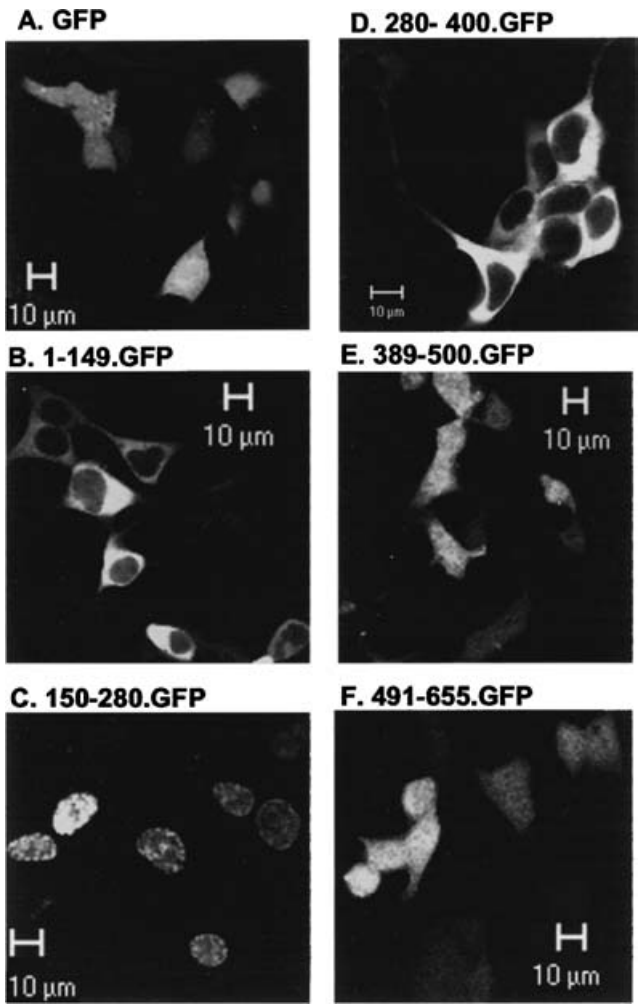

Figure 2 Effect of different regions of FOXO1 on the nuclear/cytoplasmic distribution of GFP fusion proteins

GFP (**A**) or fusion proteins containing amino acids 1–149 (**B**), 150–280 (**C**), 280–400 (**D**), 389–500 (**E**) or 491–655 (**F**) of FOXO1 in-frame with GFP were expressed in HEK-293 cells and visualized by laser confocal microscopy. Scale bars, 10 μm.

Multiple elements in the FOXO1 DBD contribute to nuclear localization

As shown Figure 3(A), amino acids 150–280 containing the DBD and flanking sequences are sufficient to promote nuclear localization of fusion proteins (panel 1). Also, disrupting the function of the NLS located at the C-terminal end of the DBD, either by replacing Arg-251, Arg-252 and Arg-253 with neutral amino acids (Figure 3A, panel 2), or by deleting these residues together with other basic residues located at the C-terminal end of this NLS (Figure 3A, panel 3), does not disrupt this effect. This suggests that some other element(s) located in the DBD also contributes to nuclear localization.

We considered the possibility that DNA-binding activity may be required for nuclear localization by the DBD after the function of C-terminal NLS is disrupted. Previous studies have shown that basic residues at the C-terminal region of the DBD play an important for DNA binding through electrostatic interactions, and replacing Arg-251, Arg-252 and Arg-253 with neutral residues greatly reduces DNA-binding activity [17]. The observation that replacing these residues with arginines in the fusion protein

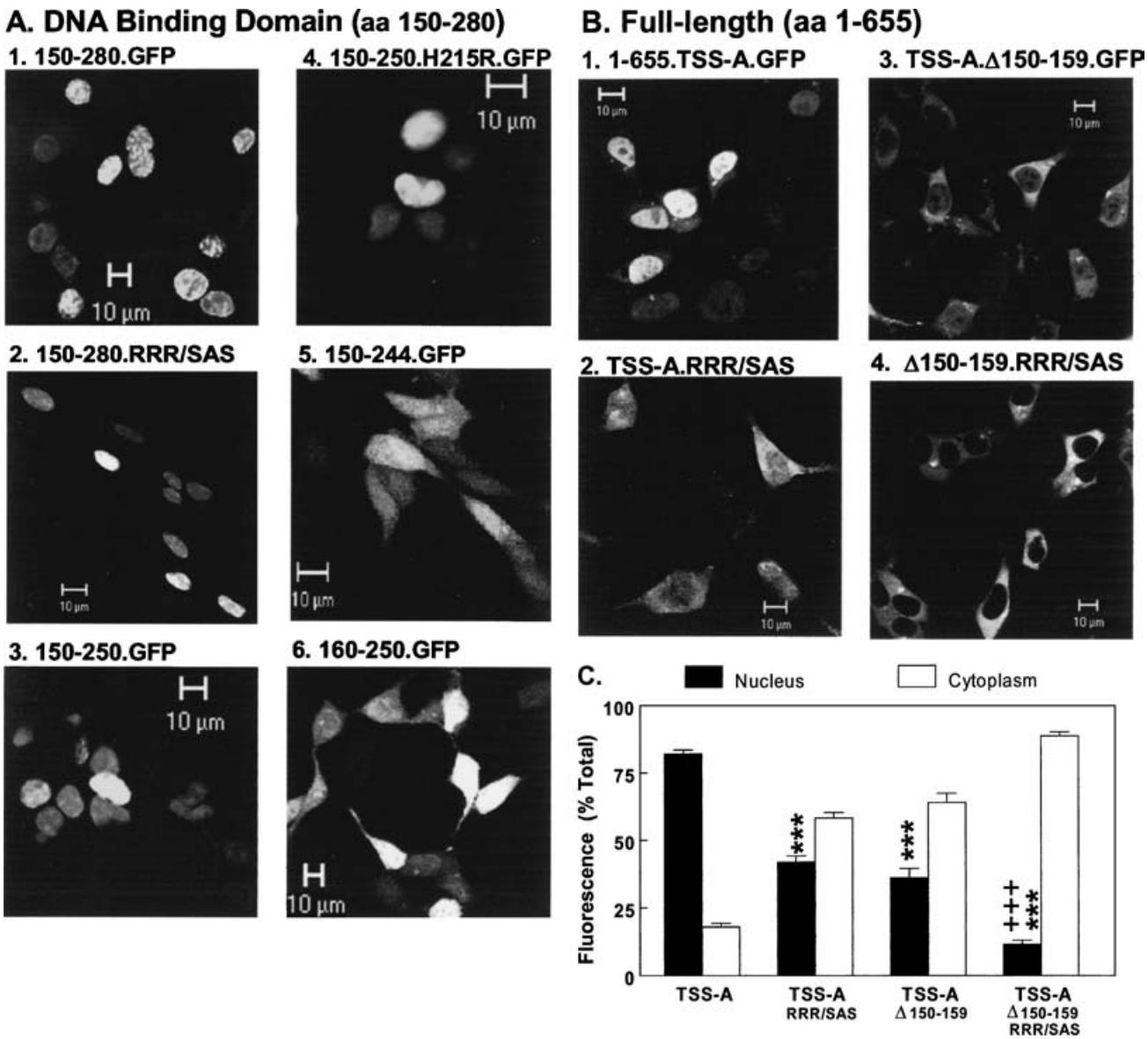

Figure 3 N- and C-terminal residues in the FOXO1 DBD contribute to nuclear localization

(**A**) Fusion proteins containing the DBD. Cells are shown expressing fusion proteins containing amino acids located in the region of the FOXO1 DBD in-frame with GFP (150-280.GFP; panel 1), the DBD with mutation of Arg-251, Arg-252 and Arg-253 within the known NLS (150-280.RRR/SAS.GFP; panel 2), the DBD with deletion of these residues and other C-terminal basic residues (150-250.GFP; panel 3), the DBD with deletion of these residues and mutation of H215 in helix 3 (150-250.H215R.GFP; panel 4), the DBD with the deletion of an additional six amino acids at the C-terminal end (150-244.GFP; panel 5) and the DBD with the deletion of 10 amino acids at the N-terminal end of the DBD (160-250.GFP; panel 6). (**B**). Contribution of N- and C-terminal ends of the DBD to nuclear localization of full-length FOXO1. Cells are shown expressing fusion proteins containing full-length FOXO1 with mutation of Thr-24, Ser-256 and Ser-319 in-frame with GFP (1-655.TSS-A.GFP; panel 1); mutation of Thr-24, Ser-256 and Ser-319 together with the mutation of Arg-251, Arg-252 and Arg-253 in the NLS located at the C-terminal end of the DBD (TSS-A.RRR/SAS; panel 2); mutation of Thr-24, Ser-256 and Ser-319 with deletion of 10 amino acids (150–159) at the N-terminal end of the DBD (TSS-A.Δ150-159.GFP; panel 3) or mutation of Thr-24, Ser-256 and Ser-319 with both deletion of amino acids 150–159 and mutation of Arg-251, Arg-252 and Arg-253 (Δ150-159.RRR/SAS; panel 4). (**C**) Nuclear/cytoplasmic distribution of full-length fusion proteins. Full-length FOXO1–GFP fusion proteins shown in (**B**) were visualized by confocal microscopy and the percentage of total cellular fluorescence in the nuclear (solid bars) or cytoplasmic (open bars) compartments was determined by analysis with ImageJ software. The mean ± S.E.M. is shown for at least 12 cells for each construct. $^{***}P < 0.001$ versus 1-655.TSS-A.GFP; $^{+++}P < 0.001$ versus TSS-A.RRR/SAS.GFP and TSS-A.Δ150-159.GFP.

containing amino acids 150–280 does not disrupt nuclear targeting suggests that DNA-binding activity is not required for nuclear localization by the DBD. Previous studies have shown that replacing His-215 in helix 3 of the FOXO1 DBD with arginine also disrupts DNA binding by the FOXO1 DBD ([27] and X. Zhang and T. Unterman, unpublished work). As shown in panel 4 of Figure 3(A), replacing His-215 with arginine does not disrupt the ability of amino acids 150–250 to localize fusion proteins to the nucleus, where the C-terminal NLS has been deleted. Together, these results indicate that DNA binding is not required for nuclear localization by the region of the DBD, even when the C-terminal NLS has been disrupted or deleted.

We performed additional studies to identify residues required for this nuclear localization function. Deleting an additional six residues from the C-terminal end of the DBD disrupts the ability of this region to localize fusion proteins in the nucleus (Figure 3A, panel 5; 150-244.GFP). Two basic residues (Lys-245 and Lys-248) are present in this region; however, replacing these residues with

alanines does not disrupt the ability of amino acids 150–250 to localize fusion proteins in the nucleus (results not shown), indicating that they are not required for nuclear localization.

Based on early studies indicating that residues located at both the N- and C-terminal ends of the DBD function together to promote nuclear localization of some Forkhead transcription factors [28], we asked whether this also is true for FOXO1. NLSs often are enriched in basic residues [29], and the N-terminal region of the FOXO1 DBD is enriched in basic amino acids (Arg-150, Lys-151, Arg-156, Arg-157). As shown in Figure 3(A), panel 6, deleting amino acids at the N-terminal end of the DBD (160-250.GFP) disrupts the ability of this region to localize fusion proteins to the nucleus. Replacing basic residues in this region with neutral amino acids also disrupts the ability of this region to localize fusion proteins in the nucleus (results not shown). These results suggest that, in addition to the NLS at the C-terminal end of the DBD, residues at the N-terminal end of the DBD may function together with residues located near the C-terminal end of the DBD (between amino acids 244 and 250) to promote nuclear localization of FOXO1, similar to other Forkhead transcription factors.

We next evaluated the role of these elements in localizing full-length FOXO1–GFP fusion proteins in the nucleus (Figure 3B). Full-length FOXO1–GFP fusion proteins are targeted to the nucleus of HEK-293 cells when all three predicted PKB phosphorylation sites are mutated (Figure 3B, panel 1), and disrupting the function of the NLS located at the C-terminal end of the DBD only partially impairs nuclear localization (Figure 3B, panel 2), as previously noted. Deleting amino acids located at the N-terminal end of the DBD also partially disrupts nuclear localization of the full-length fusion protein (Figure 3B, panel 3, TSS-A.Δ150-159), and replacing basic residues in this region with neutral amino acids has a similar effect (results not shown), confirming that these residues contribute to nuclear localization of full-length FOXO1. As shown in panel 4 of Figure 3(B), deleting amino acids at the N-terminal end of the DBD in combination with mutation of the NLS located at the C-terminal end of the DBD (Δ150-159.RRR/SAS) effectively disrupts nuclear localization of the full-length fusion protein in HEK-293 cells.

These results were quantified by morphometric analysis. As shown in Figure 3(C), disrupting the function of the NLS at the C-terminal end of the DBD by mutating the cluster of arginine residues located in this region (TSS-A.RRR/SAS) significantly reduces the amount of fusion protein that is localized in the nucleus. Deleting basic residues at the N-terminal end of the DBD (Δ150-159) has a similar effect, and the effects are additive where these mutations are combined (TSS-A.Δ150-159.RRR/SAS). These results support the concept that elements at both the N- and C-terminal ends of the DBD contribute to nuclear localization of full-length FOXO1.

To our knowledge, these results provide the first evidence that multiple elements contribute to nuclear localization of FOXO transcription factors, and that residues located at the N-terminal region of the DBD are important for this function, similar to other Forkhead proteins [28]. The presence of multiple elements promoting nuclear localization helps to explain our previous report that phosphorylation of Ser-256, which is located within the NLS at the C-terminal end of the DBD, is not sufficient to prevent nuclear localization of full-length FOXO1 [17].

Ser-319 and nearby residues enhance the function of an NES

We performed additional studies regarding the mechanism(s) by which elements outside the known NES contribute to nuclear exclusion of FOXO1. Recent studies indicate that the phosphorylation of Ser-319 and nearby residues may promote nuclear export of FOXO1 [19]. Here, we asked whether this cluster of phosphorylation sites is sufficient to promote cytoplasmic localization of fusion proteins, or whether this effect requires the presence of the nearby NES.

As shown in Figure 4(A), the ability of amino acids 280–400 to promote cytoplasmic localization of fusion proteins is disrupted by treatment with leptomycin B (Figure 4A, panel 2; 280-400.GFP + LPMB) or by disrupting the function of the known NLS either by replacing Leu-378 with alanine (Figure 4A, panel 3; 280-400.L378A.GFP) or by deletion of the NES (Figure 4A, panel 4; 280-374.GFP). These results indicate that the ability of this region to promote cytoplasmic localization is mediated through a leptomycin B-sensitive mechanism that requires the presence of this NES, and that Ser-319 and the nearby phosphorylation sites are not sufficient to promote cytoplasmic localization in the absence of this NES.

At the same time, deletion of Ser-319 and nearby residues reduces the ability of this region to promote cytoplasmic localization of the fusion protein (Figure 4A, panel 5; 330-400.GFP), and replacing Ser-319 and nearby phosphorylation sites (including Ser-322, Ser-325 and Ser-329) with alanine residues has a similar effect (Figure 4A, panel 6; 280-440.SSSS-A.GFP). Quantitative analysis demonstrates that deletion and mutation of this cluster of phosphorylation sites reduces nuclear exclusion by this region to a similar extent (Figure 4B). Together, these results suggest the possibility that Ser-319 and nearby residues may function co-operatively with the nearby NES to promote nuclear exclusion.

Rena et al. [19] have reported that phosphorylation in this region enhances the formation of a complex with Ran, a transport protein which can interact with Crm-1 to promote nuclear export. It is interesting to speculate that phosphorylation of Ser-319 and nearby residues may enhance the function of the NES by stabilizing the formation of a complex with Ran and Crm-1. However, we cannot exclude the possibility that phosphorylation in this region also may promote cytoplasmic localization by other mechanisms, e.g. by interaction with cytoplasmic factors once proteins have been exported from the nucleus.

Multiple elements in the N-terminal region of FOXO1 promote cytoplasmic localization

We next examined elements in the N-terminal region of FOXO1 which promote cytoplasmic localization. Thr-24 is phosphorylated in response to activation of PI 3-kinase-dependent pathways [11,12,30], and mutation of this residue limits cytoplasmic localization of FOXO proteins [12,15,22,30]. As shown in panel 1 of Figure 5(A), amino acids 1–50 of FOXO1 are sufficient to promote cytoplasmic localization of fusion protein. This effect is not disrupted by treatment with leptomycin B (Figure 5A, panel 2), but it is disrupted completely by treatment with LY294002, a specific inhibitor of PI 3-kinase (Figure 5A, panel 3), or by replacing Thr-24 with alanine (Figure 5A, panel 4). To our knowledge, this result provides the first direct evidence that amino acids 1–50 of FOXO1 are sufficient to promote cytoplasmic localization. These results also indicate that phosphorylation of Thr-24 through a PI 3-kinase-dependent pathway is required for this effect, and that this effect is not mediated through a leptomycin B-sensitive mechanism.

Interestingly, subsequent studies revealed that the N-terminal region of FOXO1 also promotes cytoplasmic localization by another mechanism. As shown in Figure 5(B), the ability of amino acids 1–149 of FOXO1 to promote cytoplasmic localization of fusion proteins (panel 1) is not disrupted by mutation of Thr-24

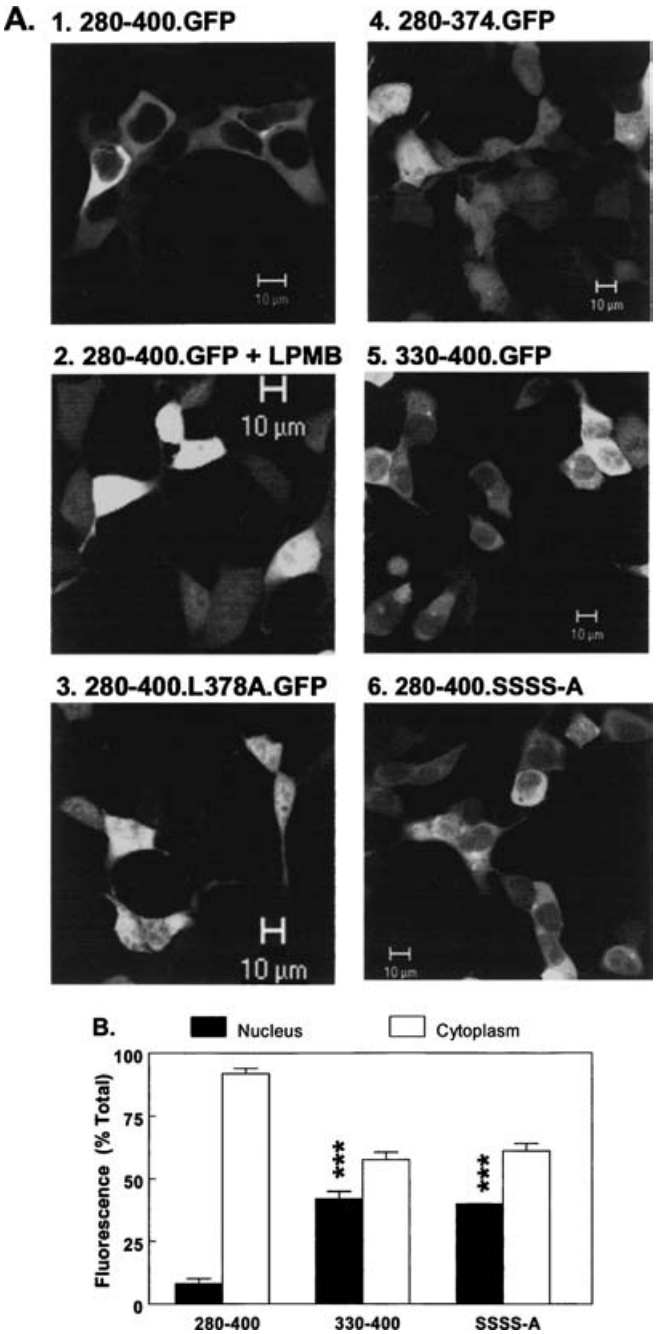

Figure 4 Role of NES and Ser-319-associated phosphorylation sites in cytoplasmic localization

(**A**) Localization of fusion proteins by amino acids 280–400. Cells are shown expressing fusion proteins containing amino acids 280–400 in-frame with GFP (280-400.GFP; panel 1) with treatment with leptomycin B (280-400.GFP + LPMB; panel 2) or mutation of Leu-378 (280-400.L378A.GFP; panel 3), deletion of amino acids 375–400 (280-374.GFP; panel 4), deletion of amino acids 280–329 (330-400.GFP; panel 5) or mutation of Ser-319, Ser-322, Ser-325 and Ser-329 (280-400.SSSS-A.GFP; panel 6). (**B**) Nuclear/cytoplasmic distribution of fusion proteins. GFP fusion proteins containing amino acids 280–400 with or without mutation of Ser-319, Ser-322, Ser-325 and Ser-329 (SSSS-A), or amino acids 330–400 of FOXO1 were visualized by confocal microscopy and the percentage of total cellular fluorescence in the nuclear (solid bars) or cytoplasmic (open bars) compartments was determined as in Figure 3(C). $^{***}P < 0.001$ versus 280-400.GFP.

(panel 2) or by treatment with LY294002 (results not shown), indicating that this effect is mediated through a mechanism that does not require phosphorylation of Thr-24. As shown in panel 3 of Figure 5(B), this Thr-24-independent ability is disrupted by treatment with leptomycin B (1-149.Thr-24A.GFP + LPMB), suggesting that it may be mediated through a Crm-1-dependent mechanism.

Studies with serial deletions suggested that amino acids located between residues 70–84 contribute to this Thr-24-independent effect (results not shown). This region of FOXO1 contains a cluster of hydrophobic residues (Met-70, Leu-74, Leu-76 and Leu-77). Leptomycin B is known to disrupt the function of NESs which are enriched in hydrophobic residues and interact with Crm1 [31]. Accordingly, we asked whether these residues contribute to the ability of this region to promote cytoplasmic localization. As shown in panel 4 of Figure 5(B), replacing Leu-74, Leu-76 and Leu-77 with alanine residues disrupts the ability of this region to promote cytoplasmic localization through a Thr-24-independent effect (1-149.T24A.L3.GFP), supporting the concept that these leucine residues function as part of a leptomycin B-sensitive NES. To our knowledge, these findings provide the first evidence that residues N-terminal to the DBD contribute to the cytoplasmic localization of FOXO1 through a leptomycin B-sensitive mechanism. Additional studies will be required to determine whether related leucine-rich sequences contribute to cytoplasmic localization of other FOXO proteins.

We next asked whether these elements also contribute to cytoplasmic localization of fusion proteins containing full-length FOXO1. As shown in Figure 5(C), mutation of leucine residues in the region of the NES located C-terminal to the DBD (1-655.L5.GFP) partially disrupts cytoplasmic localization and increases the nuclear accumulation of fusion proteins containing full-length FOXO1-GFP (panel 1), and treatment with leptomycin B further enhances nuclear accumulation (panel 2). This supports the concept that some other element contributes to cytoplasmic localization of full-length FOXO1 through a leptomycin B-sensitive mechanism. Mutation of Leu-74, Leu-76 and Leu-78 together with the mutation of the NES located C-terminal to the DBD has a similar effect (Figure 5C, panel 3, 1-655.L3.L5.GFP), supporting the concept that this cluster of leucine residues contributes to cytoplasmic localization of full-length FOXO1 through a leptomycin B-sensitive mechanism.

Mutation of Thr-24 also enhances nuclear accumulation of the fusion protein when the NES located C-terminal to the DBD is disrupted (Figure 5C, panel 4, 1-655.T24A.L5.GFP). Brunet et al. [22] have suggested that Thr-24 may promote nuclear exclusion through a mechanism involving one or more NESs located C-terminal to the DBD in FOXO3a. Here we find that Thr-24 contributes to cytoplasmic localization of full-length FOXO1 through a mechanism that does not require the function of the NES located C-terminal to the DBD in FOXO1.

14-3-3 proteins interact with Thr-24 and the C-terminal region of the DBD in FOXO1

Thr-24 is located within a consensus binding site for 14-3-3 proteins (Arg-Ser-Xaa-pThr/pSer-Xaa-Pro) and Thr-24 phosphorylation is required for the ability of FOXO1 to bind 14-3-3 proteins [15]. However, the role of 14-3-3 in modulating the function of FOXO proteins has been controversial [15,22,32]. Here we asked whether interactions with 14-3-3 proteins contribute to the ability of amino acids 1–50 and Thr-24 phosphorylation to promote cytoplasmic localization.

Studies with GFP fusion proteins containing amino acids 1–50 of FOXO1 revealed that replacing residues which are expected to contribute to 14-3-3 binding (Thr-24, Ser-22 or Pro-26) disrupts the ability of amino acids 1–50 to promote cytoplasmic

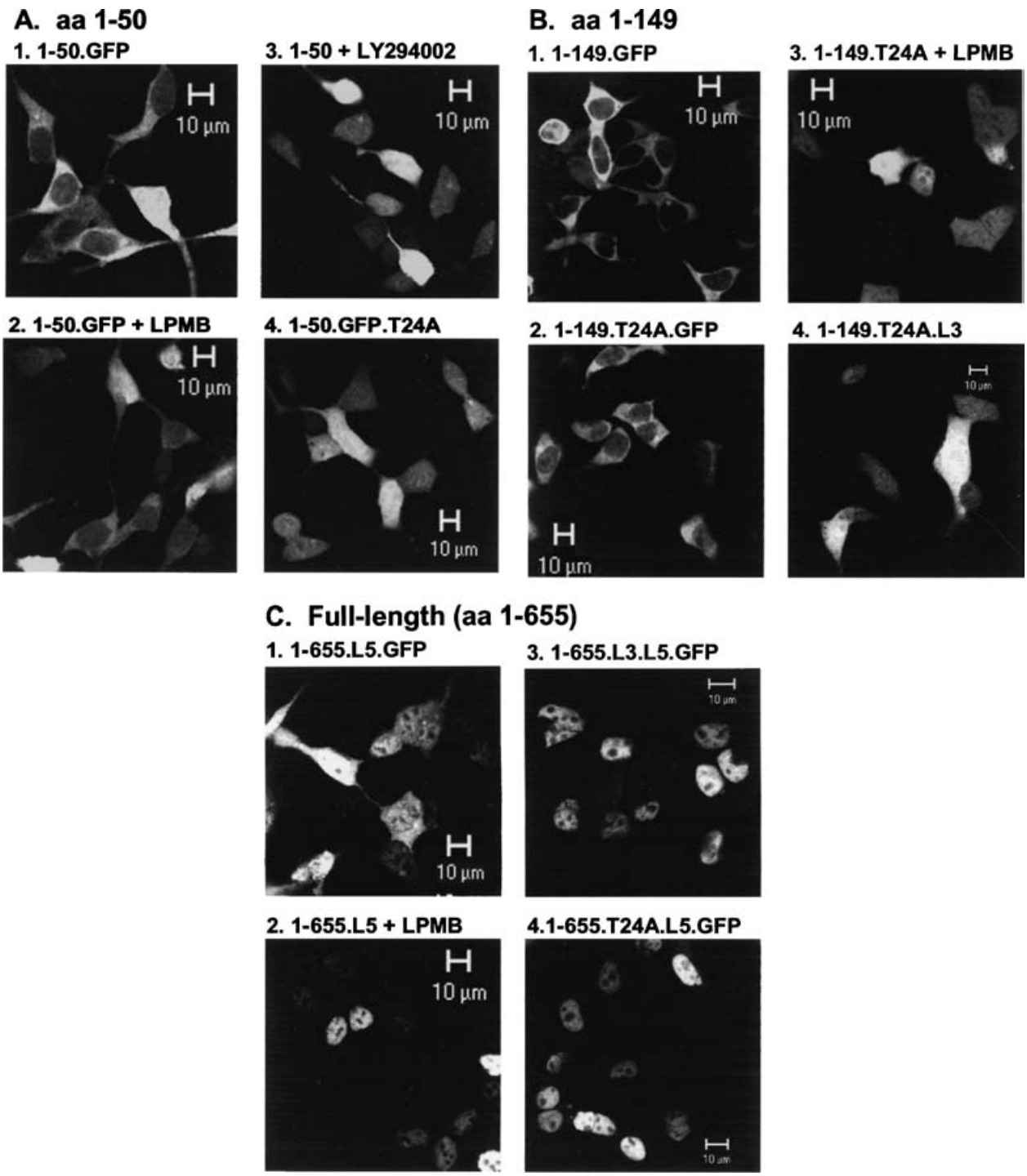

Figure 5 Role of N-terminal elements in promoting cytoplasmic localization of fusion proteins

(**A**) Role of amino acids 1–50 and Thr-24 phosphorylation. Cells are shown expressing GFP fusion proteins containing amino acids 1–50 (1-50.GFP; panel 1) with or without treatment with leptomycin B (LPMB; panel 2) or LY294002 (panel 3), or after replacing Thr-24 with alanine (1-50.T24A.GFP; panel 4). (**B**) Thr-24-independent effects of amino acids 1–149. Cells are shown expressing fusion proteins containing amino acids 1–149 (1-149.GFP; panel 1), amino acids 1–149 with mutation of Thr-24 (1–149.T24A.GFP; panel 2), amino acids 1–149 with mutation of Thr-24 and treatment with leptomycin B (panel 3), or amino acids 1–149 with mutation of Thr-24 together with Leu-74, Leu-76 and Leu-77 (1-149.T24A.L3.GFP; panel 4). (**C**) Role of N-terminal elements in cytoplasmic localization of full-length FOXO1. Cells are shown expressing fusion proteins containing full-length FOXO1 in-frame with GFP where five leucine residues in the region of the NES located C-terminal to the DBD have been mutated (1-655.L5.GFP; panel 1) with or without treatment with leptomycin B (LPMB; panel 2), or where the C-terminal NES has been mutated together with either Leu-74, Leu-76 and Leu 77 (1-655.L3.L5.GFP; panel 3) or with mutation of Thr-24 (1-655.T24A.L5.GFP; panel 4).

localization (results not shown), while replacing other residues in this region (Pro-18, Pro-20, Pro-28 or Pro-30) does not disrupt this effect (results not shown). This is consistent with the concept that interactions with 14-3-3 proteins may contribute to the ability of amino acids 1–50 to promote cytoplasmic localization.

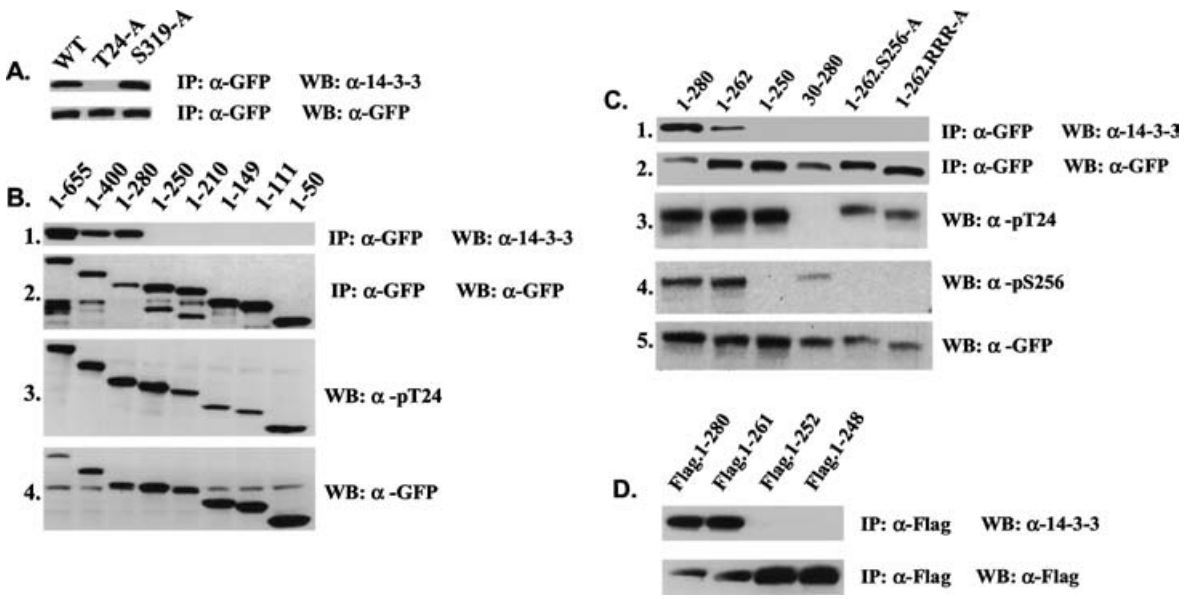

Figure 6 Interaction of FOXO1–GFP fusion proteins with 14-3-3 proteins in HEK-293 cells

(**A**) Interaction with full-length FOXO1–GFP fusion proteins. GFP fusion proteins containing full-length FOXO1 with or without mutation of Thr-24 or Ser-319 were expressed in HEK-293 cells, then precipitated with anti-GFP antibody. Precipitates were loaded for SDS/PAGE and transferred for Western blotting with anti-14-3-3 or anti-GFP antibodies. (**B**) Effect of C-terminal truncations on 14-3-3 binding. GFP fusion proteins containing amino acids 1–665, 1–400, 1–280, 1–250, 1–210, 1–149, 1–111 or 1–50 of FOXO1 were expressed in HEK-293 cells, then immunoprecipitated with anti-GFP antibody and loaded for SDS/PAGE and transfer for Western blotting. Membranes were probed with anti-14-3-3 (row 1) or anti-GFP (row 2) antibodies. Crude lysates also were analysed by Western blotting to assess the phosphorylation of Thr-24 using a phosphospecific antibody (row 3), and the level of fusion protein expressed using anti-GFP antibody (row 4). (**C**). Role of Ser-256 in interactions with 14-3-3 proteins. GFP fusion proteins containing amino acids 1–280, 1–262, 1–250, 30–280, or amino acids 1–262 with mutation of Ser-256 (1-262.S256-A) or Arg-251, Arg-252 and Arg-253 (1-262.RRR-A) were expressed in HEK-293 cells. Proteins were precipitated with anti-GFP antibody prior to Western blotting with anti-14-3-3 (row 1) or anti-GFP (row 2) antibodies. Crude lysates also were loaded for Western blotting with phosphospecific antibodies to assess the phosphorylation of Thr-24 (row 3) or Ser-256 (row 4) or the expression of the GFP fusion protein (row 5). (**D**) 14-3-3 interaction with FLAG-tagged fusion proteins. An N-terminal FLAG tag was expressed in-frame with amino acids 1–280, 1–261, 1–252 and 1–248 of FOXO1 in HEK-293 cells. Proteins were precipitated with anti-FLAG antibody, and analysed by Western blotting with antibodies against 14-3-3 proteins or the FLAG epitope.

To examine interactions with 14-3-3 proteins directly, we performed co-immunoprecipitation studies with fusion proteins expressed in cells. As shown in Figure 6(A), full-length FOXO1 effectively binds 14-3-3 proteins and replacing Thr-24 with alanine prevents this interaction, consistent with previous studies [15]. Previously, *in vitro* binding studies performed with DAF-16 (the FOXO protein expressed in *C. elegans*) in the presence of an excess of recombinant 14-3-3 proteins suggested that residues corresponding to Thr-24 and Ser-319 may bind 14-3-3 proteins co-operatively [32]. Here we find that replacing Ser-319 with alanine does not disrupt the ability of FOXO1 to bind endogenous 14-3-3 proteins (Figure 6A). This indicates that the phosphorylation of Ser-319 is not critical for FOXO1 to interact effectively with endogenous 14-3-3 proteins, consistent with other studies with full-length mammalian FOXO proteins [12,15].

We next asked whether amino acids 1–50 are sufficient for 14-3-3 binding. As shown in Figure 6(B), 14-3-3 proteins interact with fusion proteins containing amino acids 1–400 or 1–280 of FOXO1, but not with proteins containing only amino acids 1–250 or less. Western blotting of immunoprecipitates (Figure 6B, row 2) and crude lysates (Figure 6B, rows 3 and 4) confirms that this result is not due to differences in the expression level of fusion proteins or Thr-24 phosphorylation. These results indicate that, in addition to Thr-24, amino acids at the C-terminal end of the DBD also contribute to 14-3-3 binding.

As shown in Figure 6(C), GFP fusion proteins containing amino acids 1–280 and 1–262 bind 14-3-3 proteins effectively, while the protein containing only amino acids 1–250 does not, indicating that residues between amino acids 250 and 262 are important for 14-3-3 binding. Deletion of amino acids 1–29 also disrupts 14-3-3 binding, confirming that the N-terminal region containing Thr-24 is required for 14-3-3 binding.

Ser-256 resides within a consensus PKB phosphorylation motif (Arg-Xaa-Arg-Xaa-Xaa-Ser/Thr) [33] located between residues 250 and 262, and phosphorylation of Ser-256 is critical for the ability of insulin and growth factors to suppress transactivation by FOXO1 [14]. The phosphorylation of Ser-256 is required for effective phosphorylation of Thr-24 (and Ser-319) in full-length FOXO1 [15,16], but not when FOXO1 is truncated [16]. As shown in Figure 6(C), replacing Ser-256 with alanine disrupts the ability of fusion proteins containing amino acids 1–262 to bind 14-3-3 proteins (Figure 6C) without preventing phosphorylation of Thr-24. Disrupting the consensus sequence for PKB phosphorylation by replacing arginines 251–253 with neutral amino acids also disrupts 14-3-3 binding without interfering with Thr-24 phosphorylation (Figure 6C). These results indicate that, in addition to Thr-24, phosphorylation of Ser-256 also is important for 14-3-3 binding.

Since these studies were all performed with constructs containing a GFP tag at the C-terminal end of fusion proteins, we also performed studies with constructs tagged at the N-terminal end of FOXO1 with the FLAG epitope (Figure 6D). FLAG-tagged wild-type FOXO1 binds 14-3-3 proteins, and mutation of all three PKB phosphorylation sites, disrupts 14-3-3 binding (results not shown). As shown in Figure 6(D), FLAG-tagged proteins containing amino acids 1–280 and 1–261 bind 14-3-3 proteins effectively, while proteins containing only amino acids 1–252 or 1–248 do not. This result confirms that amino acids between 252 and 261 are important for stable interactions with 14-3-3 proteins.

Together, these results suggest that phosphorylation of Thr-24 may alter the distribution and function of FOXO1 through several mechanisms. On the one hand, we find that amino acids 1–50 can promote cytoplasmic localization, that the phosphorylation of Thr-24 is critical for this effect, and that mutation of residues in this region that are expected to be important for 14-3-3 binding (Thr-24, Ser-22 and Pro-26) disrupts the ability of this region to promote cytoplasmic localization. Previous studies have shown that 14-3-3 proteins form dimers that bind co-operatively with high affinity to target sites [24]. Although amino acids 1–50 FOXO1 are not sufficient to bind 14-3-3 proteins effectively in co-immunoprecipitation assays, recent findings indicate that 14-3-3 proteins may interact with target sites as monomers in cells [34]. We cannot exclude the possibility that 14-3-3 proteins may interact weakly with fusion proteins containing only amino acids 1–50 of FOXO1 in cells and promote cytoplasmic localization, possibly by tethering to other cytoplasmic proteins, as has been suggested [12].

In the context of full-length FOXO1, interactions with residues at the C-terminal end of the DBD also contribute to 14-3-3 binding, and the binding of 14-3-3 proteins to the C-terminal end of the DBD may have important effects on critical functions of FOXO1. Recent studies by Brunet et al. [22] indicate that 14-3-3 proteins may be rapidly shuttling in and out of the nucleus and bind to FOXO proteins in the nuclear compartment. We have reported that the basic region at the C-terminal end of the FOXO1 makes an important contribution to DNA binding through electrostatic interactions [17]. Based on the results of the present study, it is interesting to speculate that interactions between 14-3-3 proteins and the C-terminal end of the DBD in the nucleus may limit DNA binding by FOXO1. Since residues in this region of the DBD also contribute to the function of two NLSs (Figure 3), it is also reasonable to speculate that, once the complex has been exported out of the nucleus, 14-3-3 binding at this site may mask these NLSs and interfere with transport back into the nucleus. In addition, studies from other laboratories indicate that this region may also participate in protein–protein interactions with other nuclear transcription factors [35,36], suggesting that 14-3-3 binding at this site may also modify these interactions and thereby alter the function of other transcription factors.

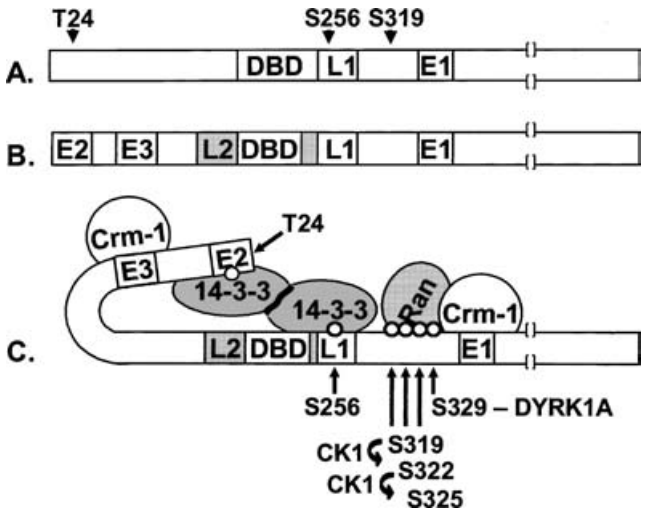

Figure 7 Nuclear/cytoplasmic localization elements in FOXO1

(**A**) Previous studies identified an NLS at the C-terminal end of the DBD (L1) and an NES located C-terminal to the DBD (E1). (**B**) Residues at the N- and C-termini of the DBD (L2, stippled regions) also contribute to nuclear localization even after the function of L1 has been disrupted. Amino acids 1–50 of FOXO1 are able to promote cytoplasmic localization (E2) and a leucine-rich region (E3) located in the N-terminal region of FOXO1 also contributes to cytoplasmic localization of fusion proteins. (**C**) The function of NESs E1 and E3 is inhibited by leptomycin B, indicating that they interact with Crm-1 or related proteins. The phosphorylation of Ser-319 and nearby residues enhances the function of the nearby export signal (E1) and the association of FOXO1 with Ran [19], suggesting that the phosphorylation of these residues may promote nuclear export by stabilizing the formation of a FOXO1–Ran–Crm-1 complex together with the nearby NES. The phosphorylation of Thr-24 and Ser-256 bind 14-3-3 proteins co-operatively. Interactions with 14-3-3 binding in the C-terminal region of the DBD may impair DNA binding, mask NLS(s) L1 and/or L2, and possibly alter interactions with other nuclear factors involved in the regulation of gene expression.

Summary of mechanisms involved in nuclear/cytoplasmic shuttling of FOXO1

Figure 7 summarizes the findings of this study. As shown in Figure 7(A), previous studies have identified an NLS located in the C-terminal region of the DBD (L1), and a leucine-rich NES located C-terminal to the DBD and Ser-319 (E1). As shown in Figure 7(B), the results of the present study indicate that residues at both the N- and C-termini of the DBD also function together to promote nuclear localization (L2; Figure 7B, stippled regions). We also find that two elements in the N-terminal region of FOXO1 promote cytoplasmic localization, including amino acids 1–50 (E2) and a leucine-rich region that promotes nuclear exclusion through a leptomycin B-sensitive mechanism (E3).

As indicated in Figure 7(C), NESs E1 and E3 are both enriched in leucine residues and the function of these signals is inhibited by leptomycin B, indicating that they interact with Crm-1. Phosphorylation of Ser-319, and subsequent phosphorylation of Ser-322 and Ser-325 by CK1, together with the phosphorylation of Ser-329 by DYRK1A, may enhance the effectiveness of the nearby NES (E1). Rena and co-workers [15,19] have indicated that the phosphorylation of Ser-319 and nearby residues promotes the association of FOXO1 with Ran [19], a nuclear transport protein that interacts with Crm-1 and can promote nuclear export [15]. Based on the present study, we speculate that these phosphorylation sites may function co-operatively with the NES to stabilize the formation of a complex with Ran and Crm-1, and thereby promote nuclear export.

The phosphorylation of Thr-24 can promote cytoplasmic localization through both a mechanism that is mediated through amino acids 1–50 (E2), and interactions with 14-3-3 proteins. The phosphorylation of both Thr-24 and Ser-256 contributes co-operatively to the binding of 14-3-3 proteins, and interaction of 14-3-3 proteins with the C-terminal region of the DBD in the nucleus may impair DNA binding, limit interactions with other transcription factors in the nucleus, and, once FOXO1 is exported from the nucleus, mask NLSs (L1 and/or L2) and thereby limit transport back into the nucleus.

Together, these studies indicate that multiple elements contribute to the regulation of nuclear/cytoplasmic shuttling of FOXO1, and that the phosphorylation of Thr-24, Ser-256 and Ser-319 alters the cellular trafficking and function of FOXO1 through multiple mechanisms. The presence of multiple and redundant mechanisms to regulate the nuclear/cytoplasmic distribution and function of FOXO proteins supports the concept that these transcription factors play an important role in mediating critical effects of insulin and growth factors on gene expression.

Note added in proof (received 16 January 2004)

While in publication, Obsil et al. [37] have reported that the phosphorylation of sites corresponding to Thr-24 and Ser-256 is also required for high affinity binding of 14-3-3 proteins to

recombinant FOXO4 *in vitro*, which is in agreement with our findings with FOXO1 in cells.

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