

AMPK and AKT protein kinases hierarchically phosphorylate the N-terminus of the FOXO1 transcription factor, modulating interactions with 14-3-3 proteins

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Forkhead box protein O1 (FOXO1) is a transcription factor involved in various cellular processes such as glucose metabolism, development, stress resistance, and tumor suppression. FOXO1's transcriptional activity is controlled by different environmental cues through a myriad of posttranslational modifications. In response to growth factors, the serine/threonine kinase AKT phosphorylates Thr²⁴ and Ser²⁵⁶ in FOXO1 to stimulate binding of 14-3-3 proteins, causing FOXO1 inactivation. In contrast, low nutrient and energy levels induce FOXO1 activity. AMP-activated protein kinase (AMPK), a master regulator of cellular energy homeostasis, partly mediates this effect through phosphorylation of Ser³⁸³ and Thr⁶⁴⁹ in FOXO1. In this study, we identified Ser²² as an additional AMPK phosphorylation site in FOXO1's N terminus, with Ser²² phosphorylation preventing binding of 14-3-3 proteins. The crystal structure of a FOXO1 peptide in complex with 14-3-3 σ at 2.3 Å resolution revealed that this is a consequence of both steric hindrance and electrostatic repulsion. Furthermore, we found that AMPK-mediated Ser²² phosphorylation impairs Thr²⁴ phosphorylation by AKT in a hierarchical manner. Thus, numerous mechanisms maintain FOXO1 activity via AMPK signaling. AMPK-mediated Ser²² phosphorylation directly and indirectly averts binding of 14-3-3 proteins, whereas phosphorylation of Ser³⁸³ and Thr⁶⁴⁹ complementarily stimulates FOXO1 activity. Our results shed light on a mechanism that integrates inputs from both AMPK and AKT signaling pathways in a small motif to fine-tune FOXO1 transcriptional activity.

The forkhead box O (FOXO)⁷ family of transcription factors is well-conserved throughout evolution, and they control the expression of a multitude of genes involved in various cellular processes. FOXO1 (FKHR), one of the family members, has been shown to regulate cellular homeostasis, insulin and glucose metabolism, immune responses, cell cycle progression, apoptosis, and senescence (1). Moreover, FOXO1-null mice are not able to complete embryonic development, confirming its essential role (2).

The transcriptional activity of FOXO1 is tightly regulated through numerous posttranslational modifications, such as phosphorylation, methylation, acetylation, ubiquitination, and glycosylation (reviewed in Refs. 3, 4). Multiple kinases are known to reduce FOXO1 activity via phosphorylation (*e.g.* AKT (5); CDK5 (6), PKA (7), CDK2 (8), CDK1 (9), and DYRK1, GSK3, and CK1 (10)). Of these, FOXO1 phosphorylation by the PI3K/AKT pathway is extensively studied. In response to a range of extracellular stimuli like growth factors, insulin, and cytokines, AKT phosphorylates Thr²⁴, Ser²⁵⁶, and Ser³¹⁹ in FOXO1 (11, 12). This allows binding of 14-3-3 scaffolding proteins to FOXO1 and consequent inhibition of transcriptional activity through export from the nucleus to the cytoplasm. 14-3-3 proteins are ubiquitously expressed in mammalian cells, and they mainly regulate the function of binding partners by affecting their subcellular localization (13). Notably, a triple mutant of FOXO1 (T24A/S256A/S319A), which cannot be phosphorylated by AKT, is predominantly nuclear and constitutively active because interactions with 14-3-3 proteins are abolished (14).

In contrast, various kinases activate the transcriptional activity of FOXO1 (AMPK (15), MST1 (16), and extracellular signal-regulated kinase and p38 (17)). AMP-activated protein kinase (AMPK) is a major regulator of cellular energy homeostasis and is activated under conditions such as nutrient deprivation or oxidative stress (18–22). Yun *et al.* (15) showed that AMPK

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This article contains Figs. S1–S9.

The atomic coordinates and structure factors (codes 6QZR and 6QZS) have been deposited in the Protein Data Bank (<http://www.pdb.org/>).

The NMR data reported in this paper have been submitted to the BMRB under accession numbers 27336 (NTDΔ), 27337 (NTDΔ-pSer²²), and 27320 (NTDΔ-pThr²⁴).

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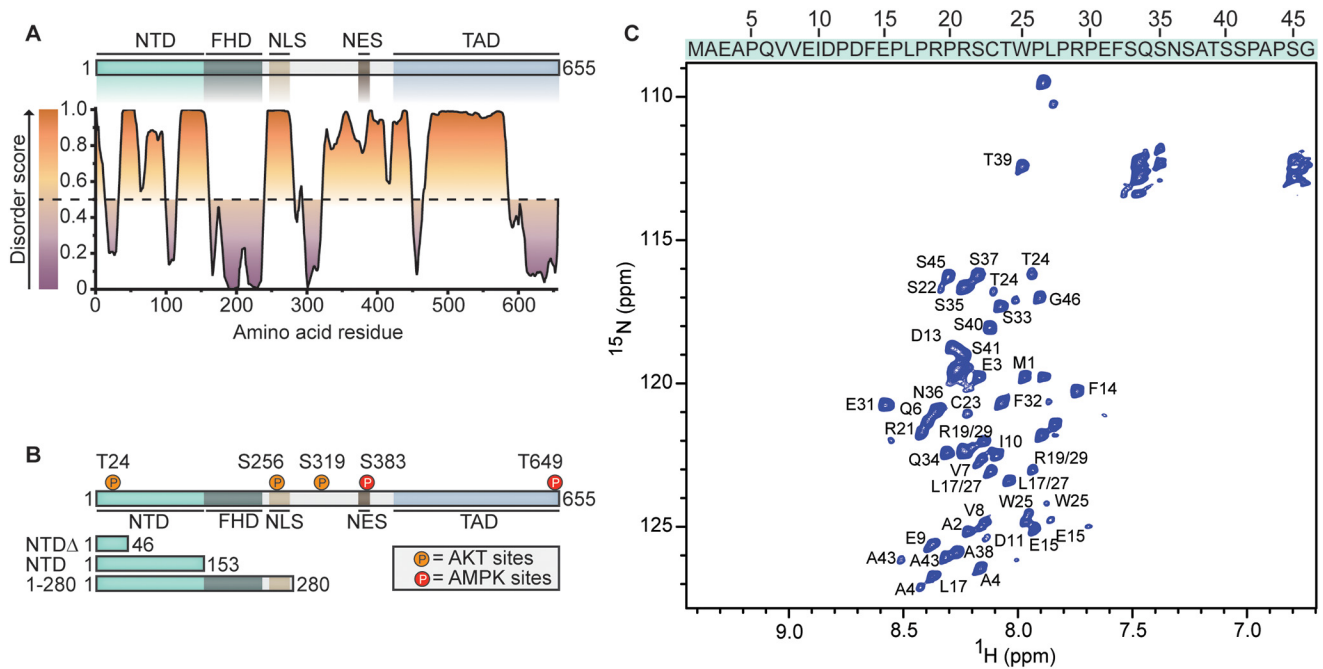
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⁷ The abbreviations used are: FOXO, forkhead box O; FKHR, forkhead in rhabdomyosarcoma; AMPK, AMP-activated protein kinase; FHD, forkhead box domain; TAD, transcription activation domain; NTD, N-terminal domain; SSP, secondary structure propensity; SPR, surface plasmon resonance; TCEP, tris(2-carboxyethyl)phosphine; HMQC, Heteronuclear Multiple Quantum Coherence; RU, resonance units; SOFAST-HMQC, Selective Optimized Flip-Angle Short-Transient heteronuclear multiple quantum coherence.



phosphorylates FOXO1 on residues Ser³⁸³ and Thr⁶⁴⁹; however, the molecular mechanism for increased FOXO1 activity upon phosphorylation of these residues remains elusive. Moreover, the authors suggested that additional AMPK sites might exist that could further explain the regulation of the transcriptional activity of FOXO1 by AMPK.

In this work, we identified Ser²² in FOXO1 as an additional AMPK phosphorylation site. Structural and biophysical data elucidated that phosphorylation of Ser²² reduces binding of 14-3-3 proteins to FOXO1 through steric hindrance and electrostatic repulsion. At the same time, Ser²² phosphorylation indirectly prevented 14-3-3 binding by negatively regulating Thr²⁴ phosphorylation by AKT. Interestingly, phosphorylation of Thr²⁴ did not affect phosphorylation of Ser²² by AMPK to a similar extent, suggesting hierarchical cross-talk between both signaling pathways. Ser²² phosphorylation both directly and indirectly abrogates complex formation with 14-3-3 proteins and plays a central role in controlling the transcriptional activity of FOXO1.

Results

The N-terminal domain of FOXO1 is intrinsically disordered

The FOXO1 protein consists of three major functional domains: the Forkhead box domain (FHD), which is responsible for DNA binding; the C-terminal transcription activation domain (TAD); and the less characterized N-terminal domain (NTD). Moreover, the protein contains signals that mediate nuclear localization and nuclear export (Fig. 1A). Although the FHD and TAD have been extensively studied, little is known

about the contribution of the NTD regarding transcriptional activity of FOXO1.

To better understand the structural features of the different domains of FOXO1, we first performed *in silico* predictions of the disorder tendency (23, 24). This analysis suggested a folded FHD (154–244) that is flanked by predominantly disordered regions (disorder scores ≥ 0.5, Fig. 1A). Of note, crystal structures have been solved for the FOXO1 FHD (25, 26), confirming a structured organization of this region. Next, we focused on the NTD and purified two recombinant proteins for structural examination using 2D NMR. One protein encompassed the full NTD (1–153) and the other a N-terminal region of the NTD (1–46), hereafter referred to as NTD and NTDΔ, respectively (Fig. 1B). The ¹H-¹⁵N Heteronuclear Single Quantum Coherence (HSQC) NMR spectra of NTDΔ (Fig. 1C) and the NTD (Fig. S1) showed narrow dispersions of ¹H-¹⁵N chemical shifts in the ¹H dimension (7.7–8.6 ppm), a characteristic of proteins lacking secondary structure elements. Although backbone resonances of NTDΔ were fully assigned, individual assignments of amino acids in the entire NTD could not be obtained because of peak overlaps. Nevertheless, both spectra showed a high similarity in chemical shifts and line widths, indicating an intrinsically disordered nature of the NTD. A secondary structure propensity (SSP) analysis (27) further confirmed a predominantly random coil conformation of NTDΔ based on the Cα and Cβ chemical shifts from the 3D ¹³C-¹⁵N HNCαCβ experiment (Fig. S2). However, residues 21–25 and 35–40 had positive average SSP scores, 0.19 and 0.08, respectively, suggesting a weak partial α-helical propensity of those regions.

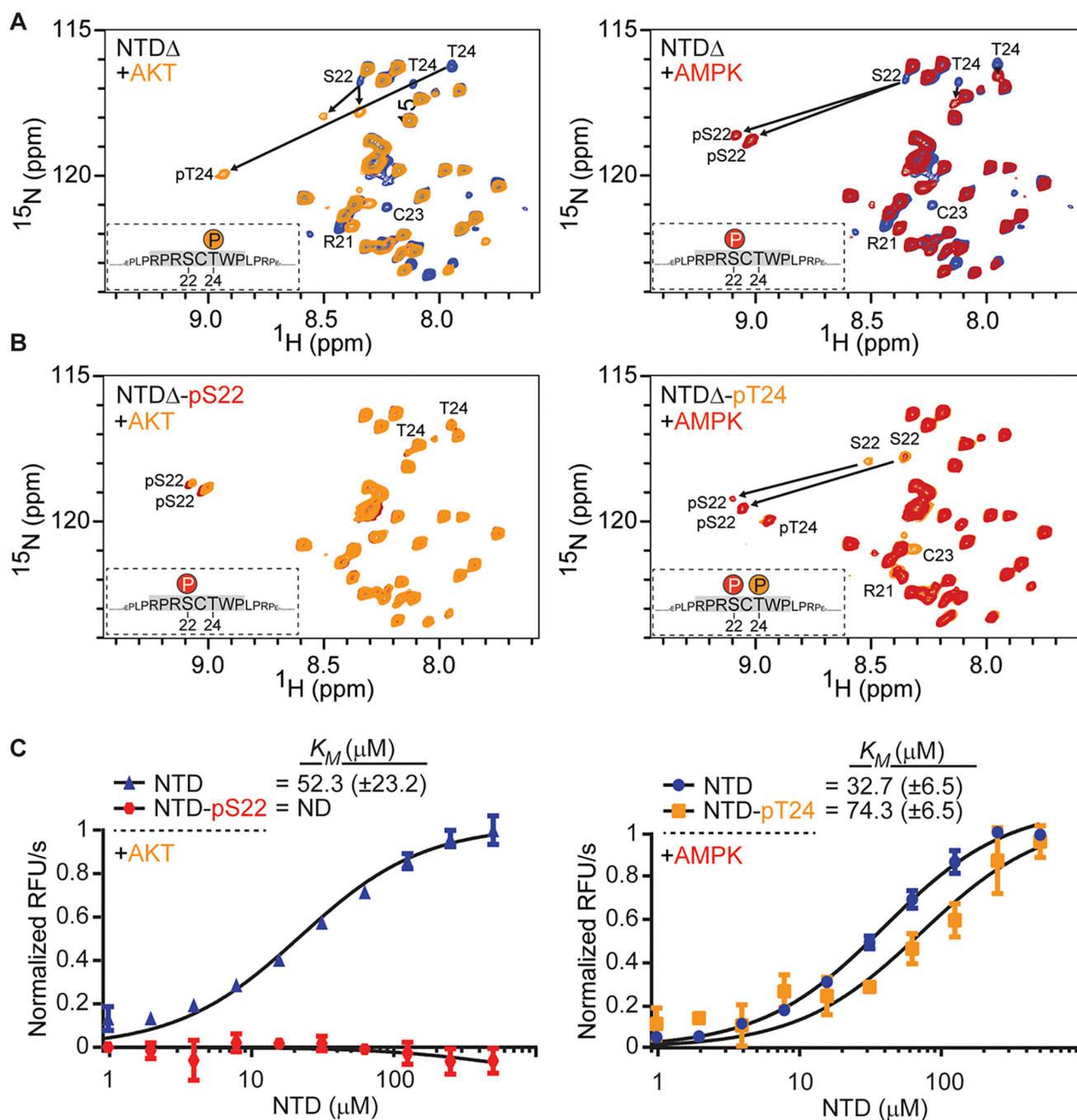


Figure 2. Phosphorylation of the NTD by AKT and AMPK. A, 2D ¹H-¹⁵N HMQC NMR spectra of NTDΔ incubated for 3 h with purified AKT (orange) or AMPK (red) to assess Thr²⁴ and Ser²² phosphorylation by AKT and AMPK, respectively. Chemical shift changes of phosphorylated amino acids are indicated by arrows. Doubling of peaks for pSer²² suggests dual conformations. B, 2D ¹H-¹⁵N HMQC NMR spectra of prephosphorylated NTDΔ incubated with the indicated kinases for 4.5 h: NTDΔ-pSer²² with AKT to monitor Thr²⁴ phosphorylation (orange) and NTDΔ-pT24 with AMPK to analyze Ser²² phosphorylation (red). Chemical shift changes of phosphorylated amino acids are indicated by arrows. C, the activities of both AKT and AMPK kinases on different substrates were analyzed using a fluorescence-based ADP biosensor. The unphosphorylated NTD as substrate for AKT and AMPK is shown in blue. The activity of AKT on NTD-pSer²² is shown in red. The activity of AMPK on NTD-pThr²⁴ is shown in orange. K_m values were determined based on the initial reaction rates. Data are mean \pm S.D. from three independent experiments. ND, not detectable.

The NTD retains unstructured properties upon Thr²⁴ phosphorylation by AKT

The NTD of FOXO1 contains one of the two 14-3-3 binding motifs (amino acids 19–26), and phosphorylation of Thr²⁴ by AKT is required to mediate interactions with 14-3-3 proteins. Interestingly, residues 21–25 showed a weak α -helical propensity (Fig. S2), and, therefore, we hypothesized that phosphory-

lation of Thr²⁴ might induce structural changes that could influence 14-3-3 binding.

To measure phosphorylation of Thr²⁴ in time-resolved 2D ¹H-¹⁵N Heteronuclear Multiple Quantum Coherence (HMQC) NMR experiments, either NTDΔ or the full NTD were incubated with purified AKT. Over the course of 3 h, a single chemical shift appeared at 8.95/119.89 (¹H/¹⁵N) ppm (Fig. 2A,

Fig. S3A), a region typical for phosphorylated residues (28). Simultaneously, two chemical shifts assigned to Thr²⁴ gradually disappeared from 7.97/116.45 (¹H/¹⁵N) and 8.13/116.82 (¹H/¹⁵N) ppm, highlighting strong phosphorylation of Thr²⁴ by AKT, as expected. The appearance of two distinct individual chemical shifts might be caused by specific *cis* and *trans* conformations of the flanking proline residue, as observed with other intrinsically disordered proteins (29). NTDA contains 10 prolines, and the presence of 15 Hα/N peaks in a ¹⁵N-¹³C Hα(Cα)N NMR spectra revealed that five prolines occur in both *cis* and *trans* conformations (Fig. S4). Apart from the changes in chemical shifts for Thr²⁴, only minor differences were detected for residues in proximity to the phosphorylation site: Leu¹⁷/Leu²⁷, Arg¹⁹/Arg²⁹, Arg²¹, Ser²², Cys²³, and Tyr²⁵.

Finally, the SSP scores for both the unphosphorylated and Thr²⁴ phosphorylated NTDA were comparable (Fig. S2). This suggested that Thr²⁴ phosphorylation by AKT does not influence the local structural organization of the NTD.

AMPK phosphorylates Ser²² in the NTD

AMPK has been shown to phosphorylate Ser³⁸³ and Thr⁶⁴⁹ in FOXO1, but it was suspected that additional AMPK phosphorylation sites might exist (30). Several AMPK phosphorylation motifs have been described (15, 22, 31, 32), and central elements are two to three basic and/or hydrophobic residues at position 3–7 N-terminally together with one hydrophobic residue at position 3–4 C-terminally of the phosphorylated residue. Interestingly, Ser²² in the NTD of FOXO1 fulfills these criteria and was predicted by the PhosphoNET software (33) as a potential AMPK site.

To analyze whether AMPK can phosphorylate Ser²² *in vitro*, we first performed an LC-MS measurement and identified one major and one minor phosphorylation site by the occurrence of a +80-Da and +160-Da peak. Subsequent peptide digestion and LC-MS/MS suggested Ser²² as an AMPK phosphorylation site in the NTD of FOXO1 (Fig. S5).

To confirm phosphorylation of Ser²² by AMPK, we incubated NTDA as well as the full NTD with purified AMPK and evaluated the chemical shifts in time-resolved 2D ¹H-¹⁵N HMQC NMR experiments. Within 2 h, gradual disappearance of the Ser²² chemical shift at 8.36/116.64 (¹H/¹⁵N) ppm was observed in combination with the emergence of two new peaks at 9.04/118.80 (¹H/¹⁵N) ppm and 9.09/118.65 (¹H/¹⁵N) ppm, indicating that Ser²² was phosphorylated by AMPK (Fig. 2A and Fig. S3A). Flanking residues next to the phosphorylation site were shifted in the NMR spectra (Leu¹⁷/Leu²⁷, Arg¹⁹/Arg²⁹, Arg²¹, Cys²³, Thr²⁴, and Tyr²⁵), as observed for the phosphorylation of Thr²⁴ by AKT within the same region. There was no substantial difference between the SSP scores of the unphosphorylated and Ser²²-phosphorylated NTDA (Fig. S2). Hence, neither Ser²² nor Thr²⁴ phosphorylation changed the secondary structure of this region of the NTD.

Phosphorylation of Ser²² by AMPK decreases phosphorylation of Thr²⁴ by AKT

The proximity of Ser²² and Thr²⁴ led us to explore whether the phosphorylation status of one of those residues affects the sub-

sequent phosphorylation of the other site or whether phosphorylation occurs independently. Time-resolved 2D NMR experiments on the prephosphorylated NTDA were conducted. NTDA-pThr²⁴ was incubated with purified AMPK, and over the course of 4.5 h, two peaks assigned to Ser²² at 8.53/117.87 (¹H/¹⁵N) ppm and 8.37/117.71 (¹H/¹⁵N) ppm disappeared (Fig. 2B, red). In parallel, two new peaks appeared at 9.07/119.49 (¹H/¹⁵N) ppm and 9.11/119.14 (¹H/¹⁵N) ppm, illustrating successful phosphorylation of Ser²². Full phosphorylation of Ser²² in NTDA-pThr²⁴ was achieved after about 4.5 h, approximately twice the time compared with the unphosphorylated protein as a substrate. Next, we incubated NTDA-pSer²² with AKT. No peaks were shifted in the NMR spectra after 4.5 h, highlighting that AKT was unable to phosphorylate Thr²⁴ within this time scale (Fig. 2B, orange). After 24 h, a weak signal appeared for pThr²⁴, with a concomitant decrease in intensity of the Thr²⁴ peak, suggesting very weak phosphorylation by AKT (data not shown). Comparable results were observed with the full NTD (Fig. S3B). Taken together, the prior phosphorylation status of Ser²² drastically affected subsequent phosphorylation of Thr²⁴ by AKT, but pThr²⁴ only moderately reduced the phosphorylation rate of Ser²² by AMPK.

To further substantiate this observation, Michaelis–Menten kinetics (*K_m*) were determined for AMPK and AKT with the unphosphorylated and prephosphorylated NTD. For AMPK, the *K_m* of the unphosphorylated NTD and NTDA-pThr²⁴ as a substrate was 32 μM and 74 μM, respectively (Fig. 2C, right panel). This statistically significant (*p* = 0.0016) 2.3-fold increase of *K_m* was in a similar range as observed in the NMR measurements. For AKT, the *K_m* was 52 μM for the unphosphorylated NTD, whereas the *K_m* could not be determined for the NTD-pSer²², implying that it is a poor AKT substrate (Fig. 2C, left panel). Overall, time-resolved 2D NMR measurements combined with the biochemical assay demonstrate a hierarchical order where the AMPK-mediated phosphorylation of Ser²² in the NTD has a strong regulatory effect on Thr²⁴ phosphorylation by AKT but not vice versa.

Phosphorylation of Ser²² prevents interactions of FOXO1 with 14-3-3 proteins

A major mechanism to control FOXO1 transcriptional activity is the binding of 14-3-3 proteins. Different 14-3-3 interaction motifs have been described in the literature, and FOXO1 contains two mode I motifs, RXX(pS/pT)XP (34). A prerequisite for complex formation is phosphorylation of one residue in each of the 14-3-3 interaction motifs by AKT: Thr²⁴ and Ser²⁵⁶ (11). Interestingly, Ser²² is located in the N-terminal 14-3-3 binding interface (Fig. S8B). To examine the impact of Ser²² phosphorylation on 14-3-3 binding, we first studied the interaction of the NTD and 14-3-3 proteins *in vitro* using surface plasmon resonance (SPR) direct binding assays. 14-3-3 ζ was immobilized to the SPR sensor surface, and binding affinities of the unphosphorylated NTD or the NTD phosphorylated on Ser²², Thr²⁴, or both were measured. As expected, an AKT-phosphorylated NTD (pThr²⁴) caused a strong interaction with 14-3-3 ζ (*K_d*, 5.1 μM), confirming the central role of pThr²⁴ as binding platform for

Phosphorylation of Ser²² in FOXO1 attenuates 14-3-3 binding

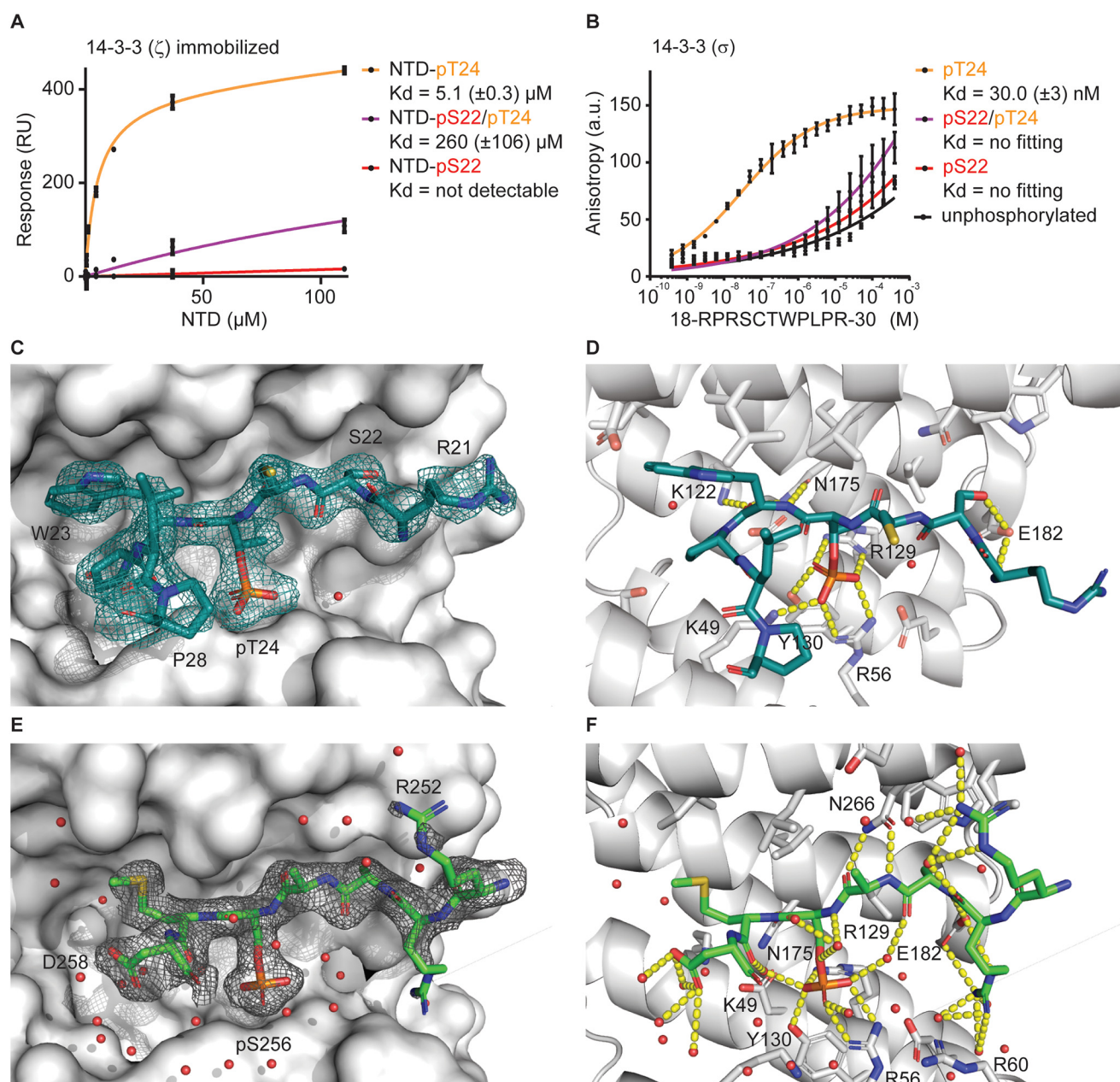


Figure 3. Phosphorylation of Ser²² in the NTD of FOXO1 hinders 14-3-3 interactions *in vitro*. **A**, surface plasmon resonance binding analysis of 14-3-3 ζ and the NTD of FOXO1 phosphorylated at the indicated residues. NTD-pThr²⁴ served as positive control, and the unphosphorylated NTD was used as a linear component for data correction. Data are expressed as mean \pm S.D. of technical triplicates from one representative experiment. **B**, fluorescence anisotropy binding analysis of interactions between 14-3-3 σ and the FOXO1 peptide (1⁸RPRSTWPLPR³⁰) in different phosphorylation states. Data are expressed as means \pm S.D. from three independent experiments and fitted with Prism (specific binding with the Hill slope). **C**, representative FOXO1/14-3-3 complex with displayed electron density (final 2Fo-Fc, contoured at 1 σ) with 14-3-3 as a gray surface, FOXO1-pThr²⁴ as sticks, and water as a red sphere. Amino acids of the FOXO1-pThr²⁴ peptide are indicated in one-letter code. **D**, details of the interactions between FOXO1-pThr²⁴ and 14-3-3 σ . Amino acids of 14-3-3 σ are indicated in one-letter code, and hydrogen bonds are illustrated by yellow dashed lines. **E**, FOXO1-pSer²⁵⁶/14-3-3 complex with displayed electron density (Final 2Fo-Fc, contoured at 1 σ) with 14-3-3 as a gray surface, FOXO1-pSer²⁵⁶ as sticks, and water as a red sphere. Amino acids of the FOXO1-pSer²⁵⁶ peptide are indicated in one-letter code. **F**, details of the interactions between FOXO1-pSer²⁵⁶ and 14-3-3 σ . Amino acids of 14-3-3 σ are indicated in one-letter code, and hydrogen bonds are illustrated by yellow dashed lines.

14-3-3 proteins (Fig. 3A). An AMPK-phosphorylated NTD (pSer²²) showed a very weak affinity to 14-3-3 ζ . Interestingly, the double-phosphorylated NTD (pSer²²/pThr²⁴) also possessed a reduced affinity for 14-3-3 ζ of more than 50-fold (K_d , 260 μ M). Similar results were observed with the θ isoform of 14-3-3 (Fig. S6A), and it appeared that phosphorylation of Ser²² strongly blocked 14-3-3 binding even in the context of phosphorylated Thr²⁴.

Crystal structures of pThr²⁴ and pSer²⁵⁶ peptides in complex with 14-3-3 σ

To fully understand the consequence of Ser²² phosphorylation on 14-3-3 binding, we aimed to crystallize the interface of the NTD and 14-3-3. However, no crystals could be obtained with the full NTD, and, therefore, peptides were used to represent the different phosphorylated 14-3-3 recognition sites of

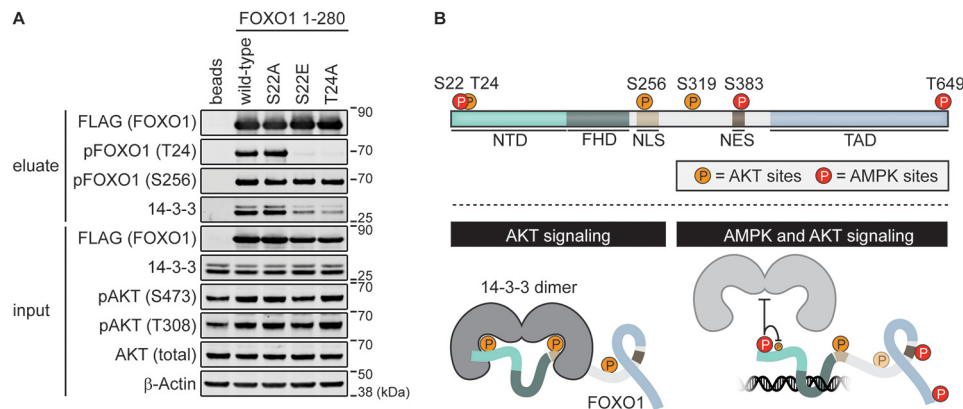


Figure 4. The phosphorylation state of Ser²² regulates binding of 14-3-3 proteins to FOXO1 in HEK293 cells. A, FLAG-FOXO1(1–280)–EGFP fusion proteins were expressed in HEK293 cells for 20 h. After cell lysis, complexes were immunoprecipitated using anti-FLAG-agarose beads and analyzed by Western blotting with the indicated antibodies. Eluates were adjusted according to amounts of bait proteins (anti-FLAG). The T24A construct served as a positive control for a reduced 14-3-3 interaction. The activity of AKT kinase was monitored with pAKT (Ser⁴⁷³/Thr³⁰⁸) antibodies. B, schematic overview of the regulation of transcriptional activity of FOXO1 by the AKT and AMPK signaling pathways. Optimal growth conditions result in active AKT, and phosphorylation of Thr²⁴ as well as Ser²⁵⁶ in FOXO1 stimulates binding of 14-3-3 proteins to inhibit its transcriptional activity. On the other hand, phosphorylation of Ser²² by AMPK is likely to increase the activity of FOXO1 by preventing 14-3-3 binding both directly through steric hindrance and electrostatic repulsion and indirectly by attenuating Thr²⁴ phosphorylation by AKT.

FOXO1. The peptide representing the pThr²⁴ site consists of 11 amino acids with the sequence ¹⁸RPRSCWPLPR³⁰.

First, Fluorescein amidite (FAM)-labeled versions of the peptides in different phosphorylation states were used in fluorescence anisotropy measurements to test whether they reflect the binding behavior between the NTD and 14-3-3 proteins observed in SPR experiments. The highest affinity was measured between 14-3-3 σ and the pThr²⁴ peptide (30 nM), whereas the pSer²² and double-phosphorylated peptides had weaker binding affinities (Fig. 3B). For 14-3-3 ζ , the pThr²⁴ peptide had an affinity of 18.3 nM, whereas the double-phosphorylated peptide had a binding affinity of 12.9 μ M (Fig. S6B). Moreover, the binding of the pSer²² and unphosphorylated peptide could not be quantified. Together, these results are in line with the findings of the SPR experiments employing the entire NTD.

Next, unlabeled peptides were used for structure analysis. The pThr²⁴ peptide in complex with 14-3-3 σ produced crystals of the space group P2₁ that diffracted to 2.3 Å and harbored eight 14-3-3/peptide molecules in the asymmetric unit (Fig. S7A). Up to nine residues of the 11 amino acids comprising the pThr²⁴ peptide could be built into the electron density (Fig. 3C). The peptide binds into the amphipathic groove of the 14-3-3 protein, where the side chain of Trp²⁵ establishes an extensive hydrophobic contact with the “hydrophobic roof” (35) of the 14-3-3 monomer binding channel. The two prolines (Pro²⁶ and P28) induce a strong turn in the peptide, guiding the chain away from the Trp²⁵ position toward the 14-3-3 dimer interface and out of the central binding channel. The pThr²⁴ is coordinated by Lys⁴⁹, Arg⁵⁷, Arg¹²⁹, and Tyr¹³⁰ of the protein. Additional polar contacts were mainly established via the peptide backbone and polar residues of 14-3-3 (carbonyl of Trp²⁵ with Lys¹²², nitrogen of Trp²⁵ with Asn¹⁷⁵, and nitrogen of Arg²¹ with Glu¹⁸²) (Fig. 3D). Interestingly, the hydroxyl group of Ser²² forms a polar contact with Glu¹⁸², locking this residue in all eight monomers in a similar conformation (Fig. S7A). A close-up of that conformation explains how a phosphorylation of this residues disrupts the binding of the double-phosphory-

lated peptide and the NTD. The serine fills a flat pocket of the 14-3-3 surface; any extension of that residue will cause a steric clash with the protein and impair binding.

To complete the picture of FOXO1 binding to 14-3-3, the structure was also solved of the complex between pSer²⁵⁶ and the C-terminal 14-3-3 binding site to 1.9 Å and in the space group P2₁2₁2₁, with two molecules found in the asymmetric unit (Fig. S7C). Seven residues (Arg²⁵²–Asp²⁵⁸) of the 12 amino acids comprising the FOXO1-pSer²⁵⁶ peptide (²⁵¹RRRAASMDNNSK²⁶²) could be built into the electron density (Fig. 3E). The phosphorylated serine (pSer²⁵⁶) is found in the same pocket as pThr²⁴, coordinated by Arg⁵⁷, Arg¹²⁹, and Tyr¹³⁰ of 14-3-3 (Fig. 3F). The hydrophobic roof of the 14-3-3 monomer binding channel is occupied by the side chain of Met²⁵⁷, whereas residues Asp²⁵⁸ and Asn²⁵⁹ extend out of the binding groove. The small side chain of the x-2 residue, Ala²⁵⁴, is in a similar position as the corresponding residue Ser²²; however, it lacks the opportunity to engage in a polar contact with Glu¹⁸² in 14-3-3. These results emphasize a distinct feature of the N-terminal 14-3-3 binding site, where interactions can be directly regulated through phosphorylation of both Thr²⁴ and Ser²².

Ser²² phosphorylation hinders 14-3-3 interactions in cells

To further investigate the significance of Ser²² and Thr²⁴ phosphorylation regarding 14-3-3 interactions in a cellular context, we used FOXO1 (1–280) constructs (Fig. 1B) bearing either phosphodeficient (S22A and T24A) or phosphomimetic (S22E) mutations for immunoprecipitation in HEK293 cells. Amino acids 1–280 of FOXO1 contained both 14-3-3 interaction motifs, but it was observed that a single point mutation (T24A) in the N-terminal binding site is sufficient to strongly reduce the overall 14-3-3 interactions because of loss of avidity (13). The WT construct was phosphorylated on Thr²⁴ and precipitated, as expected, various isoforms of 14-3-3 proteins (Fig. 4A). Phosphorylation of Thr²⁴ was a consequence of continuous AKT activity under optimal growth conditions, confirmed with pAKT antibodies (Thr³⁰⁸ and Ser⁴⁷³). For our experi-

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ments, we selected a HEK293 cell line with low endogenous FOXO1 levels; therefore, we could ensure that the signal of the pFOXO1 (Thr²⁴) antibody represented the phosphorylation status of the ectopically expressed proteins. The T24A mutation that prevented phosphorylation by AKT strongly reduced 14-3-3 interactions, as described previously (13, 36). The S22E mutation reflected an intracellular state of continuous AMPK activity, where Ser²² is phosphorylated, and strikingly showed attenuated 14-3-3 binding comparable with the T24A mutation. The structural data propose that mimicking the Ser²² phosphorylation directly prevents 14-3-3 binding through steric hindrance and electrostatic repulsion in the binding pocket. Moreover, despite active AKT, Thr²⁴ phosphorylation was not detectable, and it seemed that Ser²² phosphorylation additionally controls 14-3-3 binding indirectly by preventing AKT from accessing its phosphorylation site. This is consistent with our *in vitro* data showing that NTD-pSer²² is a poor AKT substrate. Overall, our results substantiate the central role of the phosphorylation state of Ser²² to control binding of 14-3-3 proteins, thus regulating the transcriptional activity of FOXO1.

Discussion

FOXO transcription factors orchestrate the expression of genes involved in central cellular processes such as apoptosis, cell cycle progression, and stress resistance. Among the plethora of posttranslational modifications that regulate FOXO1 transcriptional activity, phosphorylation by AKT and the energy-sensing AMPK act in an opposing fashion. AKT-mediated phosphorylation of FOXO1 induces binding of 14-3-3 proteins, its nuclear export, and consequent transcriptional inactivation (13), whereas phosphorylation by AMPK has been shown to increase FOXO1 activity (37). In this work, we expand the interaction network of AKT and AMPK on FOXO1 by demonstrating that AMPK can phosphorylate the NTD of FOXO1 on Ser²² to affect 14-3-3 interactions.

Attenuated binding of 14-3-3 proteins to FOXO1, caused by phosphorylation of Ser²², could be explained by conformational changes within the NTD of FOXO1, steric and electrostatic effects decreasing the binding affinity between FOXO1 and 14-3-3, or a reduction in AKT-mediated phosphorylation of Thr²⁴ in FOXO1.

Conformational switching driven by posttranslational modification of intrinsically disordered proteins has been shown, for example, for Nucleophosmin (NPM1), where successive multiple phosphorylations induce a change from an ordered pentamer to a disordered monomeric state to control its function (30). Another example includes 4EBP2, where phosphorylation by ERK2 induces a conformational change from a disordered to a folded structure to form a regulatory switch (38). Our NMR results showed that no conformational changes occur in the NTD of FOXO1 by Ser²² or Thr²⁴ phosphorylation and exclude this as a probable cause to modulate 14-3-3 binding.

Another possibility could be that phosphorylation of Ser²² indirectly prevents 14-3-3 interactions by diminishing Thr²⁴ phosphorylation by AKT. In a cellular context, we observed that AKT was unable to phosphorylate Thr²⁴ in the phosphomimetic S22E FOXO1, whereas the phosphodeficient S22A as well as WT FOXO1 were unaffected. Moreover, time-resolved

2D NMR and measuring the Michaelis–Menten kinetics also showed that pSer²² impedes Thr²⁴ phosphorylation by AKT. Interestingly, the crystal structure of AKT with bound substrate (39) suggests that a phosphoserine in the –2 position of the AKT phosphorylation site would be unfavorable electrostatically, leading to steric hindrance and obstructing binding of AKT. A similar sensitivity to mutations in the AKT binding motif was observed in patients with diffuse large B cell lymphoma (36). It was shown that the presence of point mutations at Arg¹⁹ and Arg²¹ in the NTD of FOXO1 diminished phosphorylation of Thr²⁴ by AKT, as we observed for pSer²². Together, this emphasizes that phosphorylation of Ser²² renders the NTD a poor substrate for AKT and diminishes Thr²⁴ phosphorylation. This, in turn, will reduce 14-3-3 interactions and activate FOXO1.

Besides the effect on phosphorylation of Thr²⁴ by AKT, we observed that phosphorylation of Ser²² directly reduces the affinity for the ζ and θ isoforms of 14-3-3 in SPR measurements. Our structure of 14-3-3 σ and a pT24 FOXO1 peptide further showed that phosphorylation of Ser²² at the –2 position of the canonically phosphorylated serine or threonine in the 14-3-3 motif would lead to both electrostatic repulsion and steric hindrance. Comparable alleviation of 14-3-3 binding through phosphorylation at the same position within the binding surface has been reported previously for RGS18 (40) and kinesin-14 (41). The effect of steric hindrance at the –2 position is additionally illustrated by a mutation in C-Raf, where a mutation of serine to a leucine (S257L) reduces 14-3-3 binding (42). Together, the phosphorylation status of Ser²² will translate into regulatory effects on 14-3-3 binding through steric and electrostatic forces in the binding pocket.

A sequence alignment of the human FOXO proteins shows full conservation of the serine corresponding to Ser²² in FOXO1 within the N-terminal 14-3-3 recognition motif (Fig. S8B). This offers the possibility that other FOXO family members might be subjected to a similar modulation of 14-3-3 binding through phosphorylation of the conserved serine. However, the kinases involved could vary between different FOXO family members because of sequence differences in the proximity of the phosphorylation site. AMPK is capable to recognize promiscuous substrates, but a hydrophobic residue N-terminal of its phosphorylation site is a core feature in the recognition motif. Strikingly, this hydrophobic residue in position –7 (Leu¹⁷ in FOXO1) is only present in FOXO1, whereas FOXO3, FOXO4, and FOXO6 have a polar glutamine residue in the corresponding position that is likely to prevent AMPK binding (Fig. S8B). In fact, upon inhibition of AMPK, decreased transcriptional activity of FOXO1 was observed, whereas the activity of FOXO3 was unaffected (19). Whether AKT-mediated binding of 14-3-3 proteins can be modified through cross-talk between diverse kinases in different FOXO proteins awaits further analysis. The key components of both PI3K/AKT and AMPK signaling pathways are well-conserved throughout evolution. Interestingly, sequence alignments of different FOXO1 homologs between various species suggest that the additional regulation of 14-3-3 binding by AMPK might have evolved in vertebrates, as indicated by a conserved AMPK recognition motif. In *Drosophila melanogaster*, two residues of the AMPK recognition motif are substituted with amino acids that contain

different chemical properties. This makes it unlikely that dFoxO is subjected to a comparable regulation by AMPK. Finally, the direct impairment of 14-3-3 binding through phosphorylation at the −2 position by specific kinases may be a general regulation mechanism among 14-3-3 substrates because a bioinformatic analysis revealed a serine as the most prevalent residue at this position (34).

AMPK and AKT pathways are heavily intertwined by negative feedback loops. This makes it challenging to quantify the specific contribution of Ser²² phosphorylation regarding FOXO1 regulation in a cellular context. Furthermore, interpretation of data and literature is complicated, as tool compounds used to influence AMPK activity (*i.e.* resveratrol and AICAR) can lead to secondary effects. Cell- and tissue-related differences in signaling pathways, and particularly AMPK and AKT activity, further hamper interpretation of the data. Even though AMPK and AKT act antagonistically on each other and FOXO1, cellular conditions have been described where both kinases are simultaneously active (43–45). Zou *et al.* (45) suggested that metformin activates FOXO1 via AMPK signaling through impeding phosphorylation by AKT and concomitantly increasing its nuclear localization. Although phosphorylation of FOXO1 was only analyzed on Ser²⁵⁶ and not Thr²⁴ in that study, the mechanism where phosphorylation of Ser²² by AMPK leads to loss of 14-3-3 interactions could in part explain the described nuclear retention of FOXO1. Another report described enhanced FOXO1 transcriptional activity after H₂O₂ treatment, which is known to activate AMPK. Paradoxically, the increased transcriptional activity was accompanied by a higher level of AKT-phosphorylated pFOXO1 (46). This could be a consequence of increased AMPK activity under these conditions that might overrule the AKT-mediated export of FOXO1 from the nucleus to the cytoplasm. Although further studies are required to fully understand the observed cross-talk between AMPK and AKT signaling to regulate FOXO1 activity as well as the relevant cellular context, we demonstrate a hierarchy in its kinase regulation. Thereby, AKT and AMPK pathways directly integrate on FOXO1 with the potential to fine-tune its transcriptional activity through interactions with 14-3-3 proteins.

Experimental procedures

Vector construction

FOXO1 constructs were synthesized by GeneArt and codon-optimized for expression in respective host cells. FOXO1 constructs (NTDΔ and NTD) contained an N-terminal 6× His-Asn tag as well as a tobacco etch virus protease cleavage site for purification of recombinant proteins and were cloned into a pET-24 vector. FOXO1 (1–280) constructs (WT, S22A, S22E, and T24A) that were used in cell experiments carried an N-terminal 3×FLAG-tag as well as C-terminal enhanced GFP and were cloned into a pcDNA3.1 vector.

Expression and purification of FOXO1 proteins

NTDΔ and NTD were expressed in *Escherichia coli* BL21 Star (DE3). Terrific broth (TB) medium was supplemented with kanamycin (100 mg/l), 3 mM MgCl₂, 0.8% glycerol, 0.02% glucose, and 0.2% lactose for autoinduction (47). The cells were grown at 37 °C for 4 h, followed by 20-h growth at 20 °C before

lysis in HEPES buffer (pH 7.4), 150 mM NaCl, 1 mM TCEP, and 1× protease inhibitors (Roche, 26733200) using a cell disruptor at 25 kilopound per square inch (ksi) (Constant Systems). The protein was captured on a HisTrap FF column (GE Healthcare) and subsequently eluted in increasing imidazole concentrations (10–400 mM). The protein was further purified on a size exclusion S75 column (GE Healthcare) that was pre-equilibrated in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM TCEP, and 1× protease inhibitors.

The double-isotopically labeled protein (¹³C/¹⁵N) was expressed in *E. coli* BL21 Star (DE3) and grown in TB medium to an optical density of 2. Then cells were harvested by centrifugation at 4500 × *g* for 5 min and resuspended in half of the volume of optimized high cell density minimal medium (48) supplemented with ¹⁵NH₄Cl (Cambridge Isotope Lab, NLM-467-PK) and ¹³C glucose (Cambridge Isotope Lab, CLM-1396-PK). After 1 h of adaptation at 37 °C, the temperature was lowered to 20 °C, and protein expression was induced with 0.5 mM isopropyl 1-thio-β-D-galactopyranoside for 16–48 h. Labeled proteins were purified as described above, with exception of the final buffer that was for the NTD (50 mM NaPi (pH 6.8), 120 mM NaCl, 1 mM TCEP, and 10% D₂O) and for NTDΔ (20 mM HEPES (pH 6.7), 100 mM NaCl, 1 mM TCEP, and 10% D₂O).

Peptides

EPLPRRSCTWPLPRPE, EPLPRPR(pS)CTWPLPRPE (pSer²²), and EPLPRPRSC(pT)WPLPRPE (pThr²⁴) were purchased from Cambridge Research Biochemicals and diluted in H₂O. AKT1-PKB-α S473D (amino acids 118–480, referred to as AKT) and 14-3-3 proteins (isoforms θ and ζ) were produced by the Reagents and Services Division of the MRC Protein Phosphorylation and Ubiquitylation Unit of the University of Dundee and stored at −80 °C. AMPK (His₆-α2, S108A-β2, γ1) was expressed and purified as described previously (18) and used at a stock concentration of 6 mg/ml in 120 mM Tris-HCl (pH 8.0), 200 mM NaCl, and 10% glycerol. The ADP biosensor 5-ATR-ParM (tetramethylrhodamine-labeled ParM protein) was expressed, purified, and labeled as described previously (49).

Phosphorylation of FOXO1

The NTD of FOXO1 in 20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1 mM TCEP, 1× protease inhibitor (Roche, 26733200), and 0.1 mM Na₃VO₄ was supplemented with AKT or AMPK to a final concentration of 0.19 μM and 0.33 μM, respectively. For sequential phosphorylation of FOXO1, AKT was added overnight before the reaction could proceed with AMPK for one additional night at 20 °C. Prior to surface plasmon resonance experiments, the FOXO1 preparations were buffer-exchanged to 10 mM HEPES (pH 7.4), 350 mM NaCl, 1 mM TCEP, and 0.05% (v/v) Tween 20 using a NAP-10 column. Samples were separated with Novex 4–12% BisTris gels (Invitrogen) in 1× MES running buffer, and phosphorylation was assessed by staining with Pro-Q Diamond Phosphoprotein Gel Stain (Invitrogen) followed by SYPRO Ruby Gel Stain (Invitrogen) and Phos-tag gels (Wako Pure Chemical Industries Ltd.) stained with Instant Blue (Expediton). Further confirmation of phosphorylation was performed by MS, where the molecular mass of

Phosphorylation of Ser²² in FOXO1 attenuates 14-3-3 binding

each FOXO1 sample was measured by Electrospray Ionisation-Mass Spectrometry (ESI-MS) micromass ZQ analysis (Waters). Samples were diluted in an equal volume of buffer A (0.1% formic acid and 3% acetonitrile) and separated over a C4 column (Dionex) in a chromatography from 100% buffer A to 100% buffer B (0.1% formic acid and 90% acetonitrile). Mass spectroscopic data were analyzed with the MassLynx software.

Surface plasmon resonance

Experiments were performed on a Biacore 3000 (GE Healthcare) and either 14-3-3 θ or ζ were covalently immobilized on a low-density CM5 chip (Xantec) using standard amine-coupling procedures (1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC)/N-hydroxysulfosuccinimide (NHS) activation) with proteins in acetate buffer (pH 5.5). The various FOXO1 preparations were injected at a concentration of 330 μ M to 17 nM with 10 $3\times$ dilution steps. The resonance units (RU) at steady state were recorded, and data were adjusted for nonspecific binding observed with unphosphorylated FOXO1. Steady-state data were fitted to a model with R_{\max} fixed at 600 RU or 400 RU for 14-3-3 θ or ζ , respectively, using nonlinear regression in Prism (GraphPad). The kinetic parameters K_{on} and K_{off} of the SPR raw data were extracted using curve fitting in the BiaEvaluation 4.1 software.

NMR

Measurements were recorded on an 800-MHz Bruker Avance III spectrometer equipped with a cryogenic probe (Bruker) at 20 °C with 330- μ l sample in medium-walled tubes. Spectral assignment was obtained by using 2D ¹H-¹⁵N Selective Optimized Flip-Angle Short-Transient heteronuclear multiple quantum coherence (SOFAST-HMQC) HMQC and a standard Bruker pulse sequence for triple resonance spectra 3D ¹H-¹⁵N-¹³C HNC α C β , HNC α , and HNC α C α C β . All spectra were processed in TopSpin (Bruker), and Collaborative Computational Project for NMR (CCPNMR) (50) was used for sequential assignment of chemical shifts.

To follow the phosphorylation of the NTD by AKT or AMPK, a series of 2D ¹H-¹⁵N SOFAST HMQC experiments were used for either 100 μ M uniformly labeled ¹³C-¹⁵N NTD or 400 μ M uniformly labeled ¹³C-¹⁵N NTD Δ . After complete phosphorylation, a 3D HNC α C β spectrum was recorded for assignment, followed by addition of the second kinase to obtain the other series of 2D ¹H-¹⁵N SOFAST HMQC measurements. At completion of the phosphorylation, or after 24 h, a 3D HNC α C β spectrum was recorded for assignment of chemical shifts. ¹⁵N-¹³C H α (C α)N NMR spectra were recorded to assess conformation of prolines in the NTD, and H α /N peaks were identified. The integrity of all proteins was monitored by SDS-PAGE after NMR analysis to assure that no proteolytic cleavage occurred during the measurements.

Secondary structure propensity analysis

The secondary chemical shifts ($\Delta\delta$) were calculated as $\Delta\delta = \delta - \delta_{\text{rc}}$, where δ is the observed chemical shift, and δ_{rc} is the random coil chemical shift (27, 51). The presented SSP score is a weighted average of the $\Delta\delta$ shifts over five residues. Negative SSP values indicate a preference for the β -strand or extended

structure, whereas positive values indicate an α -helical propensity. A weighted SSP score of 1 represents a full secondary structure. Prolines as well as residues preceding prolines were excluded from analysis because prolines do not have the backbone amide proton. This makes them invisible in 2D ¹H-¹⁵N HMQC, and they strongly affect the chemical shifts of flanking residues. The phosphorylated residues are not part of the reference database; hence, a correct comparison could not be made. These residues were excluded from the SSP analysis.

Biochemical enzymatic assay

The ADP-sensing 5-ATR-ParM (49) was used to measure the kinase activity of AKT and AMPK. The assay was carried out in 30 mM Tris-HCl (pH 7.5), 25 mM KCl, and 10 mM MgCl₂ buffer supplemented with fresh 1 mM TCEP, 5 μ M BSA, and 1 \times protease inhibitors (Roche, 26733200) on the day of the experiment. FOXO1 peptides were dissolved in H₂O to a concentration of 20 mM. The assay was performed with a total assay volume of 12 μ l in a microtiter plate (Greiner, 784900). Under the assay conditions, the biosensor showed a linear response to ADP in the range of 1–400 μ M ADP (Ultra-Pure, Promega). First, 0.05 μ M 5-ATR-ParM, 1 mM ATP, and varying concentrations of FOXO1 peptide (0.5 mM to 0.25 μ M) were diluted in running buffer, centrifuged at 200 $\times g$ for 1 min, and incubated on ice. After 1 h, enzyme was added as well as AKT (5 nM to 200 nM) for the NTD or NTD-pSer²² or AMPK (5 nM to 25 nM) for the NTD or NTD-pThr²⁴. Substrates were pre-equilibrated. The plates were centrifuged at 200 $\times g$ for 1 min, followed by kinetics measurements. The accumulation of ADP was assessed as an increased fluorescent signal with excitation at 540 nm and an emission filter at 590 nm using a PheraStar microplate reader (BMG Labtech). Data were recorded with an interval of 120 s for 1.5 h and analyzed with the MARS software (BMG Labtech). The initial linear velocity was extracted and plotted against substrate concentration in Prism (GraphPad). The initial velocity was normalized to the maximum initial velocity, except for AKT, with NTD-pSer²². No activity could be measured; therefore, data were scaled using normalization parameters from the measurement of AKT with the NTD. All experiments were performed in triplicate at least three separate times. Data were analyzed using a nonlinear regression Michaelis–Menten model. A K_m for one run in a single experiment (AKT with the NTD, third of four runs) was flagged as an outlier by a Grubb's outlier test and excluded from further analysis. Differences in K_m values were tested for significance with one-way analysis of variance. The K_m shows a significant difference between AMPK with NTD and NTD-pThr²⁴ as substrates ($p = 0.0016$); the K_m differences are nonsignificant between AKT NTD and AMPK NTD or NTD-pThr²⁴ ($p = 0.0795$ and $p = 0.1592$, respectively). The K_m value could not be determined for NTD-pSer²² with AKT.

Crystallography

The 14-3-3 σ Δ C protein (C-terminally truncated 14-3-3 σ , amino acids 1–231) was mixed in a 1:1 ratio with either the FOXO1-pThr²⁴ or the FOXO1-pSer²⁵⁶ peptide to a final concentration of 12 mg/ml of protein in buffer (20 mM HEPES (pH 7.5), 2 mM MgCl₂, and 2 mM β -mercaptoethanol). The 14-3-

3 σ ΔC/FOXO1-pThr²⁴ mixture was crystallized using the sitting drop method in a 1:1 ratio of 14-3-3 σ ΔC/FOXO1-pThr²⁴ complex to crystallization buffer (0.1 M BisTris propane (pH 7), 0.2 M sodium nitrate, 28% (v/v) PEG 400, and 5% (w/v) glycerol) and incubated at 4 °C. The 14-3-3 σ ΔC/FOXO1-pSer²⁵⁶ mixture was crystallized using the sitting drop method in a 1:1 ratio of 14-3-3 σ ΔC/FOXO1-pSer²⁵⁶ complex to crystallization buffer (0.1 M BisTris propane (pH 7), 0.2 M sodium citrate tribasic dehydrate, 20% (w/v) PEG 3350, and 10% (w/v) glycerol) and incubated at 4 °C.

Diffraction data for the crystals were collected at the synchrotron (PETRA III, Hamburg, Germany) and processed with Diffraction Integration for Advanced Light Sources (DIALS) using xia2. The structures were solved by molecular replacement with a previously solved 14-3-3/peptide complex (PDB code 3MHR), using molrep for the 14-3-3 σ ΔC/FOXO1-pThr²⁴ structure and Phaser-MR for the 14-3-3 σ ΔC/FOXO1-pSer²⁵⁶ structure. Model building and refinement of both structures were performed using Coot, REFMAC5, and phenix.refine.

Cell culture and plasmid DNA transfection

HEK293 cells were free of mycoplasma and authenticated by STR DNA profiling analysis. Cells were grown in DMEM (Gibco, 31966) supplemented with 10% FBS (Gibco, 10270-106) without antibiotics at 37 °C in 5% CO₂. Plasmid DNA was transfected with FuGENE HD transfection reagent (Promega) according to the manufacturer's protocol.

FLAG immunoprecipitation

Anti-FLAG M2-agarose affinity gel (A2220) was purchased from Sigma-Aldrich, and immunoprecipitations were performed according to the manufacturer's instructions. In brief, HEK293 cells were transfected in 10-cm dishes with 10 μ g of FLAG-tagged fusion proteins. 20 h after transfection, cells were washed with ice-cold PBS and resuspended in 1 ml of lysis buffer (20 mM Tris-HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100 (v/v), 2 mM EDTA, 25 mM NaF, 1 mM Na₃VO₄, 1 \times cOmplete protease inhibitor mixture (Roche, 26733200)). After incubation on ice for 20 min, lysates were sonicated and cleared by centrifugation (12,000 \times g for 10 min) before cell extracts were incubated with anti-FLAG M2 beads for 90 min while rotating. Beads were washed with 20 mM Tris-HCl (pH 7.4), 150 mM NaCl and protein complexes were eluted with SDS sample buffer (without DTT). DTT was added to 50 mM final concentration before SDS-PAGE. All steps were performed at 4 °C.

Western blotting and antibodies

Equal amounts of proteins were separated on 4%–12% Bis-Tris gels (Invitrogen) in MES buffer and transferred to nitrocellulose membranes with a Trans-Blot Turbo system (Bio-Rad).

pFOXO1-Thr²⁴ (9464), pFOXO1-Ser²⁵⁶ (9461), 14-3-3 (8312), AKT (2938), pAKT-Ser⁴⁷³ (4060), and pAKT-Thr³⁰⁸ (13038) antibodies were purchased from Cell Signaling Technology. β -Actin (A1978) and FLAG (F3165) antibodies were from Sigma-Aldrich. IRDye secondary antibodies for Western blotting were from LI-COR, and membranes were scanned using an Odyssey CLx imaging system (LI-COR).

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AMPK and AKT protein kinases hierarchically phosphorylate the N-terminus of the FOXO1 transcription factor, modulating interactions with 14-3-3 proteins

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