Press Release

Keio University

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Keio University

Shedding New Light on Cancer Therapy:

Elucidation of the Mechanism by Which a Tumor Suppressor Is Inactivated

— Toward the Development of Novel Anticancer Drugs That Induce Cancer Cell Death —

A research group led by Shota Enomoto (Master's Program, 2nd year) and Professor Masanori Osawa of the Division of Physics for Life Functions, Graduate School of Pharmaceutical Sciences, Keio University, in collaboration with Dr. Toshiya Senda, Director of the Structural Biology Research Center at the Institute of Materials Structure Science, High Energy Accelerator Research Organization (KEK), and Professor Hideyuki Saya, Director of the Cancer Research Center at Fujita Health University, has elucidated the molecular mechanism by which the cancer suppressor protein FOXO3a is functionally inactivated through its interaction with the 14-3-3ζ protein (hereafter 14-3-3ζ), a key regulator of aberrant cancer cell proliferation.

In cancer cells, phosphorylation signaling is aberrantly enhanced, leading to uncontrolled cell proliferation. One of the transcription factors regulated by phosphorylation signaling is FOXO3a. Under normal conditions, FOXO3a functions as a tumor suppressor by inducing apoptosis (programmed cell death). However, in cancer cells, FOXO3a becomes phosphorylated, binds to 14-3-3ζ, and is forcibly dissociated from DNA, resulting in loss of its function. Therefore, elucidating the mechanism by which 14-3-3ζ binding causes FOXO3a to dissociate from DNA—and identifying compounds that specifically inhibit this interaction—could lead to the development of novel anticancer drugs that restore FOXO3a-mediated apoptosis.

Although the interaction mode between 14-3-3ζ and phosphorylated FOXO3a has been studied, the molecular mechanism by which 14-3-3ζ bound to the phosphorylation site induces DNA dissociation from a spatially distant DNA-binding domain has remained unclear. In this study, to clarify this "competitive inhibition mechanism mediated through distinct binding sites," we quantified DNA dissociation using fluorescence-detection size-exclusion chromatography (FSEC)[*1] and analyzed the interaction between phosphorylated FOXO3a and 14-3-3ζ at atomic resolution using nuclear magnetic resonance (NMR)[*2]. As a result, we demonstrated that 14-3-3ζ directly interacts not only with the phosphorylation sites of FOXO3a but also with its DNA-binding domain (DBD)[*3], competitively displacing DNA.

These findings were published in the international scientific journal *Nature Communications* on Feb 16, 2026.

1. Key Points of the Study

- Using FSEC, the study quantitatively demonstrated that binding of 14-3-3ζ to phosphorylated FOXO3a results in complete dissociation of DNA even under equimolar conditions.
- NMR analysis revealed for the first time that 14-3-3ζ directly interacts not only with the phosphorylation sites of FOXO3a but also with its DNA-binding domain (DBD), thereby competing with DNA for binding.
- The study proposes a novel molecular mechanism in which 14-3-3ζ is "tethered" to FOXO3a via phosphorylation sites, leading to a marked increase in effective local concentration near the DNA-binding domain and enabling complete DNA dissociation despite intrinsically weak individual

interactions.

2. Research Background

In cancer cells, excessive phosphorylation signaling promotes abnormal cell proliferation. One of the key proteins regulating this signaling pathway is 14-3-3ζ. By binding to phosphorylated motifs in target proteins, 14-3-3ζ regulates their function and intracellular localization, thereby sustaining oncogenic signaling. One such target protein is the tumor suppressor FOXO3a.

Normally, FOXO3a binds DNA in the nucleus and activates transcription of apoptosis-related genes, suppressing tumorigenesis. However, in cancer cells, phosphorylation promotes its binding to 14-3-3ζ, causing dissociation from DNA and loss of apoptotic function.

A longstanding question has been the molecular mechanism underlying DNA dissociation. FOXO3a binds DNA through its DNA-binding domain (DBD), whereas the two phosphorylation sites recognized by 14-3-3ζ are spatially distant from the DBD. Although 14-3-3ζ is known to function as a dimer and recognize phosphorylated motifs through its binding groove, additional interactions had not been reported. Thus, it remained unclear how 14-3-3ζ binding leads to DNA dissociation despite distinct binding sites. This study aimed to clarify how competition occurs between spatially separated binding sites.

3. Research Methods and Findings

In this study, phosphorylated FOXO3a (dpFOXO3a) and full-length 14-3-3ζ were prepared using an *E. coli* expression system and analyzed using purified proteins.

• 14-3-3ζ Exhibits Overwhelming Competitive Advantage over DNA

To quantitatively evaluate how strongly 14-3-3ζ competes with DNA, the researchers employed fluorescence-detection size-exclusion chromatography (FSEC). Fluorescein-labeled DNA was detected via fluorescence (Fig. 1a), while proteins were simultaneously detected via tryptophan fluorescence (Fig. 1b).

FSEC analysis revealed that stepwise addition of 14-3-3ζ to the dpFOXO3a–DNA complex led to a decrease in the complex and an increase in free DNA, accompanied by formation of a dpFOXO3a–14-3-3ζ complex. Remarkably, addition of just one equivalent of 14-3-3ζ resulted in nearly complete DNA dissociation. This indicates that under competitive conditions, 14-3-3ζ has more than a 100-fold advantage over DNA. However, the dissociation constants (Kd) for dpFOXO3a–DNA and dpFOXO3a–14-3-3ζ differ by only about twofold, indicating that binding affinity alone cannot explain this striking competitive dominance.

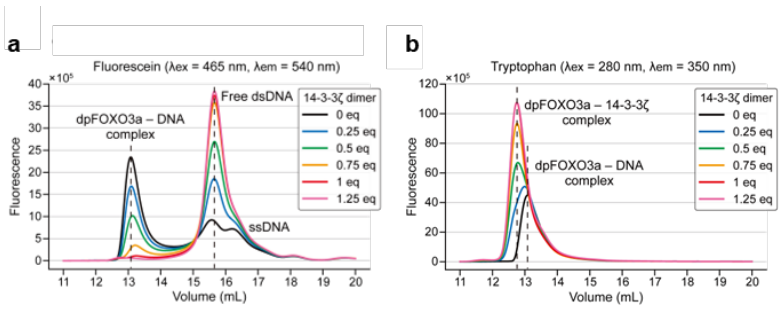

Fig. 1. 14-3-3ζ binds to dpFOXO3a and completely dissociates DNA.

Mixtures containing dpFOXO3a (10 μM), DNA (10 μM), and 0–1.25 equivalents of the 14-3-3ζ dimer were analyzed.

a Elution profiles of fluorescein-labeled DNA detected by fluorescence.

b Elution profiles of proteins detected by tryptophan fluorescence.

- **NMR Reveals Direct Interaction between 14-3-3ζ and the DNA-Binding Domain**

To uncover the molecular basis of this competitive advantage, NMR-based interaction analyses were performed. NMR does not require crystallization and allows residue-level analysis even for proteins containing extensive intrinsically disordered regions, such as dpFOXO3a.

Analysis of NMR spectra of [^{2}H, ^{15}N]-labeled dpFOXO3a upon addition of 14-3-3ζ revealed clear signal changes not only at phosphorylation sites but also at residues within the DNA-binding domain (DBD) (Fig. 2**a**, **b**). These residues clustered on one face of the DBD, coinciding with the known DNA-binding interface (Fig. 2**c**, **d**). These results demonstrate for the first time that 14-3-3ζ directly interacts with the DBD of FOXO3a and competes with DNA for binding.

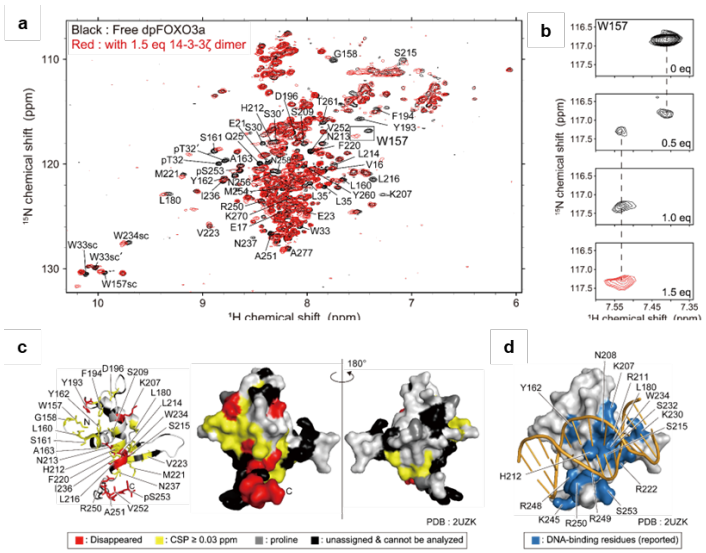

Fig. 2. 14-3-3ζ interacts not only with the phosphorylated sites of dpFOXO3a but also with the DNA-binding domain (DBD), directly competing with DNA.

a Overlay of the NMR spectra of [^{2}H, ^{15}N]-labeled dpFOXO3a alone (black) and in the presence of 1.5 equivalents of the 14-3-3ζ dimer (red).

b Chemical shift changes of the W157 signal upon addition of 14-3-3ζ.

c Mapping of residues showing significant signal perturbations onto the structure of the DBD.

d Previously reported crystal structure of the DBD–DNA complex.

- **Identification of the DBD-Binding Interface on 14-3-3ζ**

To identify the corresponding interface on 14-3-3ζ, a FOXO3a construct containing the DBD and the C-terminal intrinsically disordered region (CTD) was prepared and added to [^{2}H, ^{15}N]-labeled 14-3-3ζ for NMR analysis (Fig. 3**a-c**).

Significant signal changes were observed in residues located above the canonical phospho-motif binding

groove of 14-3-3ζ, indicating that this region interacts with the DBD.

Fitting of signal changes as a function of CTD concentration yielded a dissociation constant of 10–20 μM for the DBD–14-3-3ζ interaction, revealing that this interaction is intrinsically weaker than DNA binding (Fig. 3**b**).

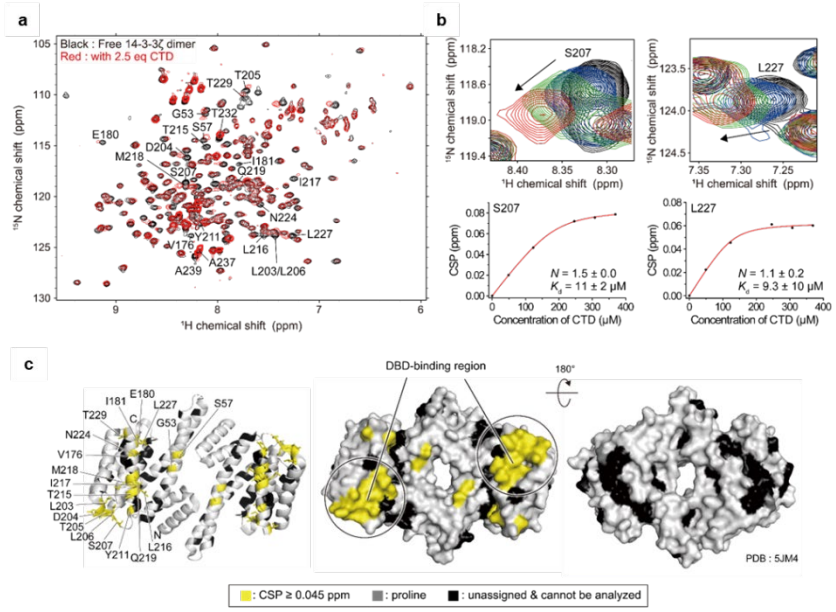

Figure 3. The DBD binds to the upper region of the 14-3-3ζ groove.

a Overlay of the NMR spectra of the [²H, ¹⁵N]-labeled 14-3-3ζ dimer alone (black) and in the presence of 2.5 equivalents of the CTD (red).

b Chemical shift changes of S207 and L227 upon addition of the CTD, together with the fitted binding curves.

c Mapping of residues showing significant signal perturbations onto the structure of the 14-3-3ζ dimer.

- **A Tethering[*5]-Based Model for Competitive DNA Dissociation**

Based on these findings, the researchers propose a model in which 14-3-3ζ binds not only to the phosphorylation sites but also directly to the DBD of FOXO3a, competing with DNA for the same binding surface (Fig. 4**a**, **b**).

Although the DBD–14-3-3ζ interaction is weak on its own, tethering of 14-3-3ζ via phosphorylation sites dramatically increases its effective local concentration near the DBD, enabling frequent access and ultimately complete DNA dissociation (Fig. 4**a**).

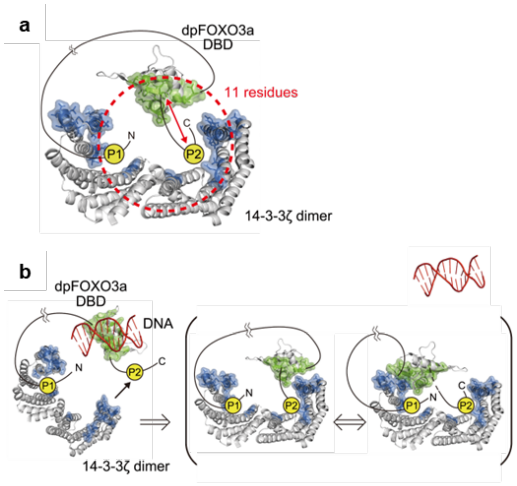

Figure 4. Competitive DNA dissociation mechanism mediated by 14-3-3ζ binding to dpFOXO3a.

a Tethering of 14-3-3ζ to dpFOXO3a via phosphorylated motifs.

b Proposed model in which tethered 14-3-3ζ dynamically competes with DNA for the FOXO3a DNA-binding domain, leading to complete DNA dissociation.

4. Future Perspectives

This study demonstrates that FOXO3a inactivation cannot be explained solely by binding of 14-3-3ζ to phosphorylation sites. Instead, it critically depends on a tethering-enhanced, dynamic competitive interaction at the DNA-binding domain. Unlike many protein–protein interactions that rely on large, high-affinity interfaces, FOXO3a engages 14-3-3ζ through multiple weak interactions that cooperatively yield strong functional inhibition.

Importantly, the moderate affinities of individual interactions (Kd in the micromolar range) fall within a range that is realistically targetable by small or medium-sized molecules. Thus, selectively disrupting the interaction between 14-3-3ζ and the phosphorylation motifs of FOXO3a to release tethering may reduce competitive inhibition at the DNA-binding domain and restore FOXO3a's transcriptional and tumor-suppressive activity.

The tethering-based, FOXO3a-specific regulatory mechanism elucidated in this study provides a foundation for a new drug discovery concept targeting 14-3-3ζ–phosphorylation motif interactions, with the potential to develop novel anticancer therapies that reactivate FOXO3a-mediated tumor suppression.

5. Publication Information

Title:

14-3-3ζ interacts with DNA-binding domain of FOXO3a and competitively dissociates DNA by dual-motif tethering

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Glossary

***1 FSEC (Fluorescence-Detection Size-Exclusion Chromatography):**
A size-exclusion chromatography technique in which analytes are detected by fluorescence. By exploiting the distinct excitation and emission wavelengths of different fluorophores, multiple components can be simultaneously and selectively detected.

***2 NMR (Nuclear Magnetic Resonance):**
A spectroscopic method that observes nuclear resonance phenomena in a strong magnetic field, enabling atomic-level analysis of molecular structure, dynamics, and interactions.

***3 DNA-Binding Domain (DBD):**
A protein domain that contains at least one structural motif capable of recognizing double-stranded or single-stranded DNA. A DBD (DNA-binding domain) may bind specifically to a particular DNA sequence (recognition sequence) or exhibit affinity for DNA more broadly. Some DNA-binding domains also recognize nucleic acids that adopt folded structures.

***4 Intrinsically Disordered Region:**
Also referred to as a non-structured region, this term describes protein regions that do not form stable secondary structures. While intrinsically disordered regions are highly dynamic and difficult to analyze by X-ray crystallography or cryo-electron microscopy, they can be effectively studied by NMR, which detects residue-specific dynamics in solution.

***5 Tethering:**
In intermolecular interactions—particularly protein–DNA or protein–protein interactions—the phenomenon in which two molecules are connected by a flexible linker (tether), increasing the probability of encounter and thereby enhancing the efficiency and specificity of their interaction.

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