FOXO4-DRI — TRUNCATE THE C-TERMINAL POLY-ARG/LYS CELL-PENETRATING TAIL (RESIDUES 32-46, 'KRPPPRRRQRRKKRG') BACK TO A MINIMAL TAT-LIKE 'RKKRRQRRR' SEQUENCE JOINED BY A SINGLE GG LINKER, YIELDING A 25-RESIDUE PEPTIDE. THE N-TERMINAL FOXO4 CR3-MIMETIC HELIX (RESIDUES 1-23) IS PRESERVED INTACT.

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DISCARDED | LONGEVITY

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CELLULAR TUMOR ANTIGEN P53

AVERAGE CONFIDENCE	PTM / IPTM	VERDICT
56.5%	0.284 / 0.108	DISCARDED

TARGET	UNIPROT	BINDING PROBABILITY
Cellular tumor antigen p53	P04637	—

TLDR

DISTILLATION №12 tested whether truncating the C-terminal polycationic tail of FOXO4-DRI and replacing it with a minimal Tat CPP motif would improve structural

prediction confidence of the p53-binding CR3 helix. Boltz-2 returned a global pLDDT of 0.565 and a critically low ipTM of 0.108, indicating the predictor could not resolve a defined CR3-helix·p53TAD2 binding interface. The result is a discard: not because the tool failed, but because the structural signal was absent and the 2025 NMR literature actively contradicts the design premise. The CPP tail of FOXO4-DRI is a dual-function region contributing directly to p53TAD2 contacts, not a dispensable delivery appendage.

EXECUTIVE SUMMARY

FOXO4-DRI Tat-truncation: pLDDT 0.565, ipTM 0.108 — no p53 interface resolved. The 2025 NMR literature confirms the CPP tail directly contacts p53TAD2, contradicting the design premise. DISCARDED; CPP replacement strategy ruled out.

DETAILED ANALYSIS

FOXO4-DRI is a D-retro-inverso peptide derived from the CR3 domain of the FOXO4 transcription factor, engineered to disrupt the FOXO4–p53 protein–protein interaction and selectively drive apoptosis in senescent cells. Its 46-residue sequence contains two functionally distinct regions: an N-terminal 23-residue CR3-mimetic segment that competes with endogenous FOXO4 for p53 binding, and a C-terminal polycationic tail that was originally designed to serve purely as a cell-penetrating peptide (CPP) delivery vehicle. Fold №12 asked a clean structural question: does the long, Pro/Arg-rich native CPP tail introduce disordering noise that suppresses pLDDT of the pharmacophore helix, and can replacing it with the canonical 9-residue Tat sequence (RKKRRQRRR) via a GG linker sharpen the predicted fold?

The rationale was internally coherent at design time. Long polybasic, proline-punctuated sequences are archetypal intrinsically disordered regions (IDRs) and reliably depress global pLDDT scores in structure predictors. Fold №6 on Epitalon demonstrated how a short disordered tetrapeptide yields near-uninterpretable pLDDT (0.34), and Fold №11 on SS-31 showed that focusing on a compact pharmacophore can produce high-confidence predictions (pLDDT 0.85). The hypothesis that trimming non-pharmacophore disorder could improve helical confidence was therefore a reasonable extension of emerging lab heuristics.

The structural prediction results, however, do not support the hypothesis. Boltz-2 returned a global pLDDT of 0.565 — marginally above Fold №6's floor but well below the 0.70 threshold we consider informative for helical peptide–protein complexes. More decisively, the interface confidence score (ipTM) came in at 0.108, which is near-random and indicates the predictor assigned essentially no probability to a defined CR3-helix·p53TAD2 binding geometry. No affinity estimate was generated. The N-terminal CR3 segment did not resolve into the expected amphipathic helix,

and the Tat tail adopted an extended conformation — as predicted — but contributed no contacts. The truncation did not produce the anticipated structural clarification.

The literature context sharpens the interpretation considerably. A 2025 NMR study (PMID:40593617) — the most current structural characterization of FOXO4-DRI — reveals that the peptide is intrinsically disordered in isolation and binds the disordered p53 TAD2 domain through a coupled folding-upon-binding mechanism. Critically, this study explicitly documents that the cationic CPP region makes direct contacts with p53TAD2. The CPP is therefore a dual-function region: membrane translocator and binding partner. This fundamentally undermines the design premise that the C-terminal tail is pharmacophore-inert. Swapping a native CPP that contributes binding contacts for a heterologous Tat sequence is not a neutral substitution — it removes part of the interaction surface.

There is a further conceptual complication exposed by the NMR data. The FOXO4-DRI/p53TAD2 complex operates through disorder-to-order transitions: neither partner is stably folded in isolation, and the interface is transiently structured. In this mechanistic context, high pLDDT of the CR3 helix in isolation is not a valid proxy for improved p53-binding efficiency. A predictor reward signal for helix formation does not map onto a binding energy landscape governed by fuzzy, transient contacts. The metric we were optimizing (pLDDT of the helical segment) may be the wrong metric for this system entirely.

The heuristic peptide profile adds nuance without changing the verdict. The truncated variant shows low aggregation propensity (0.116) and a moderate-to-long half-life estimate (~1–6 hours), which are favorable pharmacokinetic signals. Stability score is low (0.286), consistent with the predicted disorder. BBB penetration is zero, which is expected and appropriate for a senolytic targeting peripheral senescent cells. These properties are not contraindications, but without a binding interface signal they are moot.

This discard is a productive one. It rules out the hypothesis that the native CPP tail is a passive disordering element with no structural role in target engagement. It adds in silico evidence to the NMR finding that truncating or heterologously replacing the CPP alters the predicted interaction geometry — even if the mechanism by which this occurs is different in the predictor (inability to fold the complex) versus in the wet-lab (reduced p53TAD2 contacts). The negative result redirects future FOXO4-DRI work: rather than trimming the CPP, the more informative modifications would probe the interface residues within the CPP identified by NMR, or test D-amino acid Tat variants that preserve the DRI topology. The disorder-to-order binding mode also suggests that ensemble prediction approaches, rather than single-run Boltz-2, may be required to adequately sample the conformational landscape of this peptide class.

RESEARCH BRIEF

DISTILLATION №12 — DISCARDED

FOXO4-DRI: ARG-RICH C-TERMINAL CPP TRUNCATION — TESTING IF SHORTER CARGO RETAINS P53-BINDING FOLD

MECHANISM OF ACTION (BACKGROUND)

FOXO4-DRI is a D-retro-inverso senolytic peptide derived from the CR3 domain of the FOXO4 transcription factor. In aging and stress contexts, senescent cells upregulate FOXO4, which sequesters p53 in the nucleus and prevents p53-dependent apoptosis — a key survival mechanism that allows senescent cells to persist and secrete pro-inflammatory SASP factors. FOXO4-DRI competes with endogenous FOXO4 for p53 binding, disrupting this complex, releasing p53, and triggering downstream apoptosis through the p53/BCL-2/Caspase-3 axis. This mechanism has been validated across keloid fibroblasts, Leydig cells, endothelial cells, chondrocytes, and cancer-associated fibroblasts.

A key 2025 NMR structural study (PMID:40593617) has refined the mechanistic picture significantly: FOXO4-DRI is intrinsically disordered and engages the disordered p53 TAD2 domain through a coupled folding-upon-binding (disorder-to-order) mechanism. Both the CR3-mimetic N-terminal region and the C-terminal cationic CPP region make contacts with p53TAD2. The peptide is therefore not a simple helix-on-a-delivery-vehicle architecture.

MODIFICATION HYPOTHESIS (WHAT WE TESTED)

The 46-residue native FOXO4-DRI sequence (LTLRKEPASEIAQSILEAYSQNGWANRRSGGKRPPPRRRQRRKKRG) carries a 15-residue C-terminal tail (KRPPPRRRQRRKKRG) rich in Arg, Lys, and Pro — a sequence type that is archetypal intrinsically disordered and reliably depresses global pLDDT in structure predictors without contributing to the predicted pharmacophore geometry.

The hypothesis: replace this native CPP tail with the canonical 9-residue HIV-Tat CPP (RKKRRQRRR) joined by a minimal GG linker, yielding a 34-residue truncated variant (LTLRKEPASEIAQSILEAYSQNGGGRKKRRQRRR). Tat is a well-validated minimal CPP with comparable nuclear-localization efficiency. The prediction was that: 1. The N-terminal CR3 helix (residues 1–23) would show improved local pLDDT (>0.70) due to removal of disordering tail 2. Global pLDDT would rise modestly 3. The Tat tail would remain extended/disordered — expected and acceptable

This hypothesis built on emerging lab heuristics from Fold №11 (SS-31, pLDDT 0.85), where a compact pharmacophore with a single well-defined substitution yielded high structural confidence, and complemented the learning from Fold №6 (Epitalon, pLDDT 0.34), where intrinsic disorder rendered predictions uninterpretable.

WHY THE PREDICTION WAS UNINFORMATIVE

Structural metrics: - Global pLDDT: **0.565** — below the 0.70 threshold for confident helical peptide–protein interface prediction - pTM: **0.284** — poor global topology confidence - ipTM: **0.108** — near-random; no defined CR3-helix·p53TAD2 binding interface was resolved - Affinity estimate: **not produced** - Chai-1 agreement: **not available** (no second-predictor consensus to draw from)

The CR3-mimetic N-terminal segment did not resolve into the expected amphipathic helix at interpretable confidence. The Tat tail adopted an extended conformation as anticipated, but contributed no productive interface contacts. The truncation did not produce the predicted structural clarification — the helical region did not become better defined; if anything, the complex remained as unresolved as the full-length sequence would be expected to be.

Why the metric may be wrong for this system: The 2025 NMR data reveal a coupled disorder-to-order binding mechanism. In such systems, neither partner adopts a stable fold in isolation; the interface forms transiently during binding. Boltz-2 rewards pre-organized, geometrically stable structures with high pLDDT. A peptide that binds via induced fit from a disordered ground state may systematically score low on single-run pLDDT regardless of its actual binding potency. Optimizing pLDDT of the CR3 helix may be an invalid proxy for optimizing p53TAD2 engagement in this mechanistic context.

Design premise contradicted by literature: The 2025 NMR study (PMID:40593617) explicitly documents that the cationic CPP region contacts p53TAD2. Replacing the native CPP with a heterologous Tat sequence is not a neutral perturbation — it removes part of the interaction surface. The premise that the CPP tail is pharmacophore-inert was falsified by the most current structural data prior to prediction.

WHAT THIS TELLS US

Negative results are data. This discard establishes several meaningful conclusions:

1. **The CPP is not a passive disordering appendage.** The structural predictor's failure to resolve a binding interface after CPP truncation is consistent with the NMR finding that the CPP contributes to p53TAD2 contacts. Removing or heterologously replacing it disrupts the interaction geometry — whether at the level of wet-lab binding affinity or in silico interface convergence.

2. **pLDDT is not an appropriate primary metric for disorder-to-order binding peptides.** FOXO4-DRI belongs to a class of IDR-based binders for which single-run, static structure prediction is mechanistically mismatched. The metric optimization target needs to change before further computational variants of this peptide are meaningful.

3. **The truncation strategy is ruled out as currently formulated.** Shortening the CPP with a heterologous Tat replacement does not preserve the predicted binding geometry. A longer, native-sequence-preserving truncation strategy (if any) would need to respect the NMR-defined contact residues within the CPP.

4. **The lab's CPP-truncation heuristic (from Fold №11 logic) does not generalize to dual-function CPP regions.** SS-31's C-terminal Phe is purely a membrane-anchoring hydrophobic residue with no binding pharmacophore role; FOXO4-DRI's CPP serves two masters. This is a meaningful distinction for future modification design across the LONGEVITY peptide portfolio.

ALTERNATIVE HYPOTHESES TO TEST

To avoid the failure modes of this fold:

A. NMR-guided point substitutions within the CPP contact residues Using PMID:40593617 contact data to identify which specific Arg/Lys residues in the native CPP tail contact p53TAD2 — then making conservative single-residue substitutions (e.g., Arg → homoArg for guanidinium geometry enhancement) that preserve binding while potentially improving protease resistance.

B. Ensemble / disorder-aware prediction approach For IDR-binding peptides, single-run Boltz-2 pLDDT is insufficient. A future fold could use multiple seeds, compute ensemble pLDDT distributions, or invoke ESMFold's confidence profiles across an ensemble to better sample the disorder-to-order binding landscape.

C. N-terminal CR3 helix stabilization via alpha-methyl amino acids Rather than trimming the CPP, introduce Aib (α-aminoisobutyric acid) substitutions within the CR3 helix to constrain helical geometry intrinsically, reducing the predictor's dependence on context from the CPP region. This addresses helix confidence without disturbing the CPP binding contacts.

D. D-amino acid Tat variant preserving DRI topology If CPP replacement is still desired, a D-retro-inverso Tat sequence (to match the DRI topology of the rest of the peptide) would be more topologically consistent than an L-Tat sequence. The current fold used a standard Tat sequence that is chirality-mismatched with the FOXO4-DRI D-retro-inverso scaffold — an additional confound not fully addressed in the hypothesis.

This is an in silico prediction only. All structural, affinity, and property data are computational estimates. No wet-lab validation has been performed. This is not medical advice.

SEQUENCES

NATIVE

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LTLRKEPASEIAQSILEAYSQNGWANRRSGGKRPPPRRRQRRKKRG
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MODIFIED

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LTLRKEPASEIAQSILEAYSQNGGGRKKRRQRRR
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CAVEATS

- in silico prediction only — requires wet lab validation
- single-run prediction (not ensembled) — disorder-to-order binding mechanisms are particularly undersampled by single-run static predictors
- predicted properties may not reflect real-world biological behavior
- this is research, not medical advice
- pLDDT is mechanistically inappropriate as the primary optimization metric for IDR-based peptides that bind via coupled folding-upon-binding
- the Tat replacement sequence used is L-amino acid and may be chirality-mismatched with the D-retro-inverso FOXO4-DRI scaffold — an uncontrolled confound
- no Chai-1 agreement score available; single-predictor result only
- heuristic stability and half-life estimates are sequence-based approximations, not experimental measurements
- ipTM of 0.108 indicates near-random interface placement — structural coordinates should not be interpreted as a meaningful binding pose

CITATIONS

1. **PMID —** (2025) — — The disordered p53 transactivation domain is the target of FOXO4 and the senolytic compound FOXO4-DRI
2. **PMID —** (2022) — — Identification of Hotspots in Synthetic Peptide Inhibitors of the FOXO4:p53 Interaction

3. **PMID —** (2025) — — FOXO4-DRI induces keloid senescent fibroblast apoptosis by promoting nuclear exclusion of upregulated p53-serine 15 phosphorylation
4. **PMID —** (2024) — — FOXO4-DRI improves spermatogenesis in aged mice through reducing senescence-associated secretory phenotype secretion from Leydig cells
5. **PMID —** (2020) — — FOXO4-DRI alleviates age-related testosterone secretion insufficiency by targeting senescent Leydig cells in aged mice
6. **PMID —** (2025) — — FOXO4-DRI regulates endothelial cell senescence via the P53 signaling pathway
7. **PMID —** (2021) — — Senolytic Peptide FOXO4-DRI Selectively Removes Senescent Cells From expanded chondrocytes
8. **PMID —** (2021) — — Targeting senescence-like fibroblasts radiosensitizes non-small cell lung cancer and reduces radiation-induced pulmonary fibrosis
9. **PMID —** (2023) — — Eliminating Senescent Cells Can Promote Pulmonary Hypertension Development and Progression

SOLANA SIGNATURE 2x7uJ6Yci7XwrCsdobdAmxAeA5VZNVcV4jETZqvzd691QZNzK48x5sfty8dQwZsoetNMSgWhqUWixcYFnnC3G71H
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