Biostatistical Modeling of TI-A–CISD3 Fusion to Restore FAK Signaling and Mitochondrial Function in Age-Related Sarcopenia

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Abstract:

Age-related sarcopenia, characterized by progressive loss of skeletal muscle mass and function, is driven by disrupted cellular signaling, oxidative stress, and mitochondrial dysfunction. Focal Adhesion Kinase (FAK, PTK2) serves as a central integrator of extracellular matrix (ECM)-mediated signals through integrin receptors, regulating cytoskeletal integrity, mechanotransduction, and anabolic pathways such as PI3K-Akt and MAPK. Dysregulation of FAK during aging impairs muscle anabolic signaling and proteostasis, contributing to functional decline. Using protein-protein interaction (PPI) network analysis and pathway mapping, FAK is identified as a highly connected hub interacting with structural and regulatory proteins, including EGFR, SRC, Talin, and Vinculin, highlighting its therapeutic potential. This study proposes a novel fusion protein combining plant-derived Trypsin Inhibitor A (TI-A) and the mitochondrial NEET protein CISD3 to simultaneously modulate FAK signaling at integrin-binding interfaces and enhance mitochondrial proteostasis. In silico methods including tertiary structure prediction, docking, molecular dynamics simulations, and network enrichment analysis demonstrate stable binding of TI-A to FAK and potential downstream regulation of autophagy, mitochondrial fission/fusion, and proteostasis markers (HSP70, PINK1, Parkin, DRP1). This integrative approach provides a mechanistic framework for predicting functional outcomes of dual-target interventions and underscores the utility of computational modeling and biostatistical analyses in therapeutic design. The TI-A–CISD3 fusion construct represents a multifunctional candidate capable of restoring anabolic signaling, improving mitochondrial homeostasis, and mitigating age-related sarcopenic decline.

Keywords: Sarcopenia, Focal Adhesion Kinase, TI-A–CISD3 fusion, mitochondrial proteostasis, protein-protein interaction, in silico modeling, pathway analysis.

1. Introduction:

Aging is an inescapable biological process characterized by progressive decline in physiological function, perturbation of cellular homeostasis, cumulative tissue damage, and reduced regenerative capacity across organ systems [1]. Among the most prominent manifestations of aging in the musculoskeletal system is sarcopenia, the age-associated loss of skeletal muscle mass, strength, and functional capacity. Sarcopenia significantly contributes to frailty, reduced mobility, increased risk of falls and fractures, prolonged hospitalization, institutionalization, and diminished quality of life in older adults [2]. Epidemiological studies indicate that sarcopenia affects approximately 5–13% of individuals aged 60–70 years, rising to around 50% in those over 80, highlighting the urgent need for mechanistic understanding and intervention strategies. At the cellular and molecular level, sarcopenia results from a complex interplay of endocrine alterations, chronic low-grade inflammation, oxidative stress, mitochondrial dysfunction, neuromuscular junction degeneration, loss of satellite-cell regenerative capacity, and dysregulation of protein homeostasis [3]. These factors converge to create a self-perpetuating cycle of muscle fiber atrophy, reduced repair capacity, and functional decline. Age-related declines in anabolic hormones such as growth hormone, insulin-like growth factor-1, testosterone, and estrogen contribute to impaired activation of anabolic signaling pathways, a phenomenon termed “anabolic resistance,” which diminishes the muscle’s capacity to respond to nutritional and mechanical stimuli. Concurrently, the balance of protein homeostasis is disrupted, with reduced protein synthesis and enhanced degradation via the ubiquitin–proteasome system and autophagy/lysosomal pathways [4]. Mitochondrial dysfunction further exacerbates muscle deterioration, with aging muscle exhibiting mitochondrial DNA mutations, impaired electron transport chain activity, and altered dynamics of mitochondrial fission and fusion, leading to increased reactive oxygen species production, oxidative damage, and reduced regenerative potential [5]. Persistent low-grade systemic inflammation, characterized by elevated cytokines such as TNF-α, IL-6, and CRP, activates catabolic signaling, upregulates muscle-specific E3 ubiquitin ligases, and impairs satellite-cell function and neuromuscular junction stability. Together, these processes amplify muscle atrophy and functional decline in a self-reinforcing

cycle. Given the multifactorial nature of sarcopenia and its contribution to frailty, disability, metabolic dysregulation, and mortality, understanding these mechanisms is critical for developing targeted therapeutic strategies [6]. Focal adhesion kinase (FAK; PTK2) is a non-receptor tyrosine kinase central to mechanotransduction in skeletal muscle, translating extracellular mechanical cues into intracellular biochemical signals that regulate muscle structure, metabolism, and adaptation [7]. FAK activation occurs primarily through interactions with integrins, which anchor muscle fibers to the extracellular matrix and serve as bidirectional conduits for mechanical and chemical communication. Engagement of integrins with extracellular ligands such as fibronectin, laminin, and collagen triggers clustering and recruitment of adaptor proteins including Talin, Vinculin, and Paxillin, forming focal adhesion complexes that connect to the actin cytoskeleton. Within these complexes, FAK undergoes autophosphorylation at Tyr397, creating a high-affinity binding site for Src family kinases and the p85 subunit of phosphoinositide 3-kinase (PI3K), initiating downstream signaling cascades such as PI3K–Akt–mTOR and MAPK/ERK that govern muscle cell survival, proliferation, and differentiation [8]. Talin and Vinculin facilitate efficient force transmission during muscle contraction, acting as structural adaptors linking integrins to filamentous actin. This integrin–FAK–cytoskeleton linkage functions as a molecular clutch, enabling dynamic adjustments in response to mechanical stress and maintaining structural stability. Mechanical stimuli such as exercise enhance integrin clustering and FAK activation, promoting anabolic signaling, cytoskeletal organization, and hypertrophy. FAK signaling also influences focal adhesion turnover, actin remodeling, and satellite-cell activation, all critical for maintaining muscle integrity and repair following injury. Aging is associated with reduced FAK-mediated mechanotransduction, diminished integrin expression, and decreased FAK phosphorylation, contributing to anabolic resistance, impaired cytoskeletal organization, and reduced mechanical sensitivity [9]. Emerging evidence indicates that FAK interacts with mitochondrial regulatory proteins such as DRP1, linking extracellular adhesion cues to mitochondrial fission–fusion balance and energy metabolism. Thus, FAK dysregulation compromises both structural and metabolic homeostasis in aging muscle, making it a promising therapeutic target for restoring muscle strength and function. Plant-derived trypsin inhibitors (TIs) are a subclass of serine protease inhibitors, abundant in legumes and seeds, that primarily target proteases such as trypsin and chymotrypsin. In plants, TIs function as natural defense molecules by inhibiting insect or

microbial digestive proteases, limiting herbivory and pathogenic invasion. Beyond their plant-specific role, TIs exhibit biological relevance in mammalian systems, demonstrating anti-cancer, anti-inflammatory, anti-angiogenic, and metabolic-modulatory effects. Mechanistically, TIs form tight complexes with serine proteases, preventing substrate cleavage, and can modulate broader signaling networks. Their structural stability, specificity, and ability to bind exposed protein motifs render them attractive scaffolds for engineering fusion constructs aimed at targeted intervention of signaling hubs [10]. Modified TIs have shown potential to interact with serine proteases involved in disease processes, suggesting modular application in novel therapeutic strategies. CISD3 (CDGSH iron–sulfur domain-containing protein 3) is a mitochondrial matrix-localized NEET protein critical for maintaining mitochondrial proteostasis, redox homeostasis, and organelle integrity [11]. CISD3 mediates the transfer of [2Fe–2S] clusters to target proteins, supporting electron transport chain function and oxidative metabolism. Loss-of-function studies demonstrate that CISD3 deficiency impairs Complex I assembly, elevates reactive oxygen species, compromises mitochondrial respiration, and induces muscle atrophy features reminiscent of sarcopenia. By regulating Fe–S cluster delivery and maintaining mitochondrial membrane integrity, CISD3 preserves mitochondrial proteostasis, prevents oxidative damage, and maintains the regenerative potential of muscle fibers [12]. Dysfunctional CISD3 leads to iron accumulation, bioenergetic failure, enhanced mitophagy or apoptosis, and reduced muscle cell viability. Modulating CISD3 through gene therapy, small molecules, or peptide mimetics presents a potential strategy to restore mitochondrial homeostasis, complementing extracellular interventions such as FAK modulation. Given the multifactorial pathology of sarcopenia, interventions addressing both extracellular signaling deficits and intracellular mitochondrial dysfunction are necessary. This study proposes a novel fusion protein combining Trypsin Inhibitor A (TI-A) and CISD3 [13]. TI-A is designed to interact with the integrin-binding interface of FAK, restoring anabolic signaling via PI3K–Akt and MAPK pathways, while CISD3 provides intracellular mitochondrial protection, maintaining redox balance and proteostasis. Computational modeling and in silico evaluation, including protein–protein docking, molecular dynamics simulations, and pathway enrichment analysis, ensure that the fusion protein can effectively bind FAK, modulate downstream signaling, and support mitochondrial homeostasis. This dual-functional approach addresses structural and metabolic deficits in sarcopenic muscle and represents a translationally relevant therapeutic

strategy. Fusion protein design involves domain selection, linker optimization, and tertiary structure prediction using tools such as AlphaFold2 and RoseTTAFold, followed by docking and molecular dynamics simulations to assess stability, flexibility, and inter-domain interactions. Protein–protein interaction networks and pathway analyses confirm coordinated modulation of FAK-mediated mechanotransduction and mitochondrial proteostasis [14]. The TI-A–CISD3 fusion construct thus provides a holistic approach by integrating plant-derived protease inhibition with mitochondrial protection, offering a novel paradigm for the treatment of age-related muscle degeneration and forming a foundation for future experimental validation.

2. Materials and Methods:

The present study adopted an integrated *in silico* strategy to design, structurally model, and functionally evaluate the novel Trypsin Inhibitor A–CISD3 (TI-A–CISD3) fusion protein. This construct was engineered as a dual-action therapeutic aimed at modulating Focal Adhesion Kinase (FAK) signaling and enhancing CISD3-mediated mitochondrial proteostasis to address sarcopenia.

2.1 Gene Sequence Acquisition

For gene sequence acquisition, the genomic and coding sequences of the human proteins CISD3 (CDGSH Iron-Sulfur Domain-containing Protein 3) and FAK (PTK2) were retrieved from the Ensembl database (https://www.ensembl.org/). Ensembl provides manually curated and high-quality annotations that ensure accurate transcript structures and validated gene information, which are essential for reliable downstream modeling. The protein sequence of the plant-derived Trypsin Inhibitor A (TI-A), belonging to the Kunitz-type serine protease inhibitor family, was obtained from the National Center for Biotechnology Information (NCBI) protein database (https://www.ncbi.nlm.nih.gov/protein/). These sequences were downloaded in FASTA format for subsequent computational analysis.

2.2 Primary Sequence Generation and Analysis

Following retrieval, theoretical primary amino acid sequences of TI-A and CISD3 were generated and subjected to physicochemical characterization using the ExPASy ProtParam tool

(https://web.expasy.org/protparam/). ProtParam computes key molecular properties including molecular weight, theoretical isoelectric point (pI), extinction coefficient, aliphatic index, and instability index. These parameters provide insight into the protein's stability, hydropathicity, and folding behavior under physiological conditions. A low instability index and a high aliphatic index indicate thermodynamic stability, whereas the pI value predicts potential solubility and expression characteristics. The computed parameters were used to assess the suitability of TI-A and CISD3 for recombinant expression and fusion assembly, laying the foundation for tertiary structure modeling.

2.3 Secondary Structure Prediction

The secondary structure composition of both TI-A and CISD3, including α-helices, β-sheets, and random coils, was predicted using the PSIPRED web server (http://bioinf.cs.ucl.ac.uk/psipred/). PSIPRED employs position-specific scoring matrices derived from PSI-BLAST alignments and neural network algorithms to predict local backbone conformations. The output provided confidence scores for each residue, revealing the secondary structure distribution crucial for understanding domain flexibility and interaction interfaces. The predicted structural maps facilitated identification of ordered and disordered regions, ensuring that linker attachment between TI-A and CISD3 was positioned away from active or structurally rigid regions.

2.4 Individual Ligand Tertiary Structure Prediction

Three-dimensional (3D) tertiary structures of the TI-A and CISD3 domains were modeled using the SWISS-MODEL repository (https://swissmodel.expasy.org/). This automated homology modeling platform identifies suitable templates from the Protein Data Bank (PDB) and constructs atomic-level structures based on sequence alignment and geometrical constraints. Templates with greater than 40% sequence identity and low root mean square deviation (RMSD) values were prioritized to ensure modeling accuracy. The predicted models were validated using ProSA-web to evaluate Z-scores relative to known native proteins, and stereochemical properties were examined using Ramachandran plot analysis. The refined structures provided high-confidence conformations that preserved the functional domains required for docking and fusion assembly.

2.5 Receptor Tertiary Structure Acquisition

The three-dimensional structure of the receptor protein, Focal Adhesion Kinase (FAK), specifically the FERM (4.1, ezrin, radixin, moesin) domain responsible for integrin and cytoskeletal interactions, was directly obtained from the Protein Data Bank (https://www.rcsb.org/). Preference was given to experimentally determined crystal structures (e.g., X-ray diffraction at 2.2 Å resolution) to ensure atomic-level accuracy of interaction surfaces. This domain was selected because it mediates mechanotransduction and phosphorylation events critical to sarcopenia-related pathways. The receptor was prepared for docking by removing water molecules, adding hydrogen atoms, and optimizing side-chain orientations using PyMOL and UCSF Chimera.

2.6 Fusion Protein Design and Assembly

The bifunctional TI-A–CISD3 fusion construct was assembled by linking the C-terminus of the TI-A domain to the N-terminus of the CISD3 domain via a flexible Glycine-Serine (GGGGS) linker. This linker was chosen for its conformational flexibility, minimal steric hindrance, and capacity to maintain independent domain folding and activity. The overall design aimed to retain the inhibitory capacity of TI-A while enabling CISD3's redox and mitochondrial regulatory functions. The final 3D fusion structure was modeled and optimized using the ClusPro 2.0 server (https://cluspro.bu.edu/). ClusPro performs rigid-body docking using fast Fourier transform correlation, followed by clustering based on pairwise energy potentials, including electrostatics, hydrophobicity, and desolvation energy. The cluster with the lowest binding energy and highest population density was selected as the most stable conformation. The modeled fusion protein was then visualized in PyMOL to assess domain orientation, linker flexibility, and surface complementarity before docking with FAK.

2.7 Protein-Protein Docking

Protein-protein docking simulations were conducted to determine the binding pose and affinity between the TI-A–CISD3 fusion protein and the FAK receptor. Docking experiments were performed using the HADDOCK2.4 platform(https://www.bonvinlab.org/software/haddock2.4/), an information-driven docking approach that integrates biochemical restraints and structural

data. Active residues on the FAK FERM domain, particularly near the integrin-binding site (Tyr397 and adjacent regions), were defined as interaction hotspots. The fusion protein was docked against these residues, and the resulting complexes were ranked by HADDOCK score and Z-score, which combine van der Waals, electrostatic, and desolvation energies. The top-ranked models were analyzed using PyMOL to visualize hydrogen bonding, salt bridges, and hydrophobic contacts stabilizing the interface.

2.8 Binding Free Energy Calculation

To estimate the thermodynamic stability of the FAK–TI-A–CISD3 complex, binding free energy (ΔG) and dissociation constant (K_D) were calculated using the PRODIGY server (https://nestor.science.uva.nl/prodigy/). PRODIGY uses the number of interfacial contacts and physicochemical properties to predict ΔG (in kcal/mol) and log-transformed K_D values, providing insight into the binding affinity under physiological conditions. This computational approach enabled quantitative evaluation of how strongly the fusion construct binds to FAK and how this interaction compares with known FAK regulators.

2.9 Molecular Dynamics (MD) Simulations

To assess the structural stability, flexibility, and conformational behavior of the FAK–TI-A–CISD3 complex under dynamic conditions, all-atom Molecular Dynamics (MD) simulations were performed using GROMACS 2021 (https://www.gromacs.org/). The CHARMM36m force field was implemented due to its superior performance in modeling protein-protein and protein-ligand interactions. The system was solvated within a TIP3P water box, neutralized with Na^+ and Cl^- counterions, and subjected to energy minimization to remove steric clashes. Equilibration was carried out in NVT and NPT ensembles to stabilize temperature (300 K) and pressure (1 bar), respectively.

A 100-nanosecond production run was conducted to monitor dynamic fluctuations. Trajectory analyses included Root Mean Square Deviation (RMSD) for backbone stability, Root Mean Square Fluctuation (RMSF) for local residue mobility, and Radius of Gyration (Rg) to evaluate structural compactness. The MM-PBSA (Molecular Mechanics Poisson–Boltzmann Surface Area) approach was also applied to compute total binding free energy components, integrating

van der Waals, electrostatic, and solvation contributions. These analyses collectively verified that the TI-A–CISD3–FAK complex maintained conformational stability and favorable energetics throughout the simulation.

2.10 Protein-Protein Interaction (PPI) Network Analysis

To explore systemic regulatory impacts, Protein-Protein Interaction (PPI) network analysis was conducted using the STRING v11.5 database (https://string-db.org/). STRING integrates curated datasets from experimental studies, co-expression networks, and computational predictions to generate confidence-weighted interaction networks. FAK was designated as the central hub node, and its high-confidence interactors—including EGFR, SRC, Talin, and Vinculin—were mapped to visualize cytoskeletal and mechanotransduction relationships. The network was imported into Cytoscape 3.9 (https://cytoscape.org/) for advanced visualization and calculation of topological parameters, including degree centrality, betweenness, and clustering coefficients. These analyses revealed FAK's regulatory connectivity to mitochondrial effectors such as DRP1, PINK1, and Parkin, suggesting indirect modulation of mitochondrial quality control through focal adhesion signaling.

2.11 Pathway Enrichment Analysis

Pathway enrichment analysis was carried out using the KEGG Pathway database (https://www.genome.jp/kegg/pathway.html) to contextualize the functional significance of the FAK–CISD3 interactome. Enrichment mapping revealed involvement in critical pathways such as Focal Adhesion, PI3K-Akt signaling, and MAPK cascades, all of which contribute to muscle cell survival, protein synthesis, and mechanosensing. Complementary Gene Ontology (GO) term enrichment and statistical validation ($p < 0.05$) were performed using the DAVID Bioinformatics Resources (https://david.ncifcrf.gov/), identifying significant associations with biological processes like oxidative stress mitigation, ATP synthesis, and mitochondrial dynamics. Collectively, these systems-level analyses validated that the TI-A–CISD3 fusion construct integrates extracellular signaling stabilization with intracellular mitochondrial regulation, offering a robust dual-action framework for combating sarcopenia

3. Results

3.1 Gene Sequence Retrieval

The genomic and protein sequences of the target molecules CISD3 was retrieved and validated to ensure sequence completeness and domain accuracy prior to modeling.

TABLE 1: The CISD3 gene, encoding the CDGSH Iron–Sulfur Domain-containing Protein 3, was obtained from the Ensembl database, encompassing the full-length coding sequence required for structural and functional analysis.

CISD3	CCCTTCTGTGACGGCTCCCACTTCTTCCAACGCACTGGCCTATCTCCA CTCAAGTTCAAGGCCCAAGAGACCCGCATGGTGGCACTCTGTACCTG CAAGGCCACTCAGAGGCCCCCGTACTGCGATGGCACCCACAGGAGTG AGCGCGTGCAGAAGGCAGAAGTGGGCTCCCCACTCTGAGGGGGCTG CTGCTGTCCAGCCACAGGTGGCCTTGGCTCCAGGCCTCTGACAGGCA CCCCCTTCTGTGGGAAAGGAAACAGGTGCTGAGCCCAAGAGACTCTG GTACCCACTGCTGGCTCATGAAGGAAGAATTATTCCTTATAACCTAAA AGTCTCCAGTCTGGGGCAGGCGGGAGTGGGCCCTGGTTCAATGTTTG CTGATGGGGAAGATGGCAAAAACAAGCCTGCCCAACCAGACTGGTAG TCCTGCAGTCACTGCTATGAGGCCCACGTGCTGCCTCCTGCTCCAGAT TTTAACCTCTCTGTGGGCTGGGGGCACCTGACCAGCCACAGGAGAGG GCAGTTCAGATTCATTCTGTA

3.2 Primary Structure Composition

The translated amino acid sequences of CISD3, FAK, and TI-A were analyzed to confirm the presence of conserved domains and active motifs relevant to their biological functions.

Table 2: The CISD3 primary structure comprised containing the signature CDGSH motif, characteristic of iron–sulfur cluster coordination.

CISD3	MQHLLQSQEIQMSQGKGGYSHRTSDTGQGANTDKPIRGLPTPHTYAMMES RVSTPDSSQIYAHLRKSRWAATCQGLSLRRWSADLSRGGGGVRAYRFLSSC AFRCSCSVLTR,

Table 3: The FAK fragment consisted of sequence as shown in table 3 representing the binding interface residues within the FERM domain, essential for integrin anchoring and signal relay.

FAK	MWEILMHGVKPFQGVKNNDVIGRIENGERLPMPPNCPPTLYSLMTKCW AYDPSRRPRFTELKAQL,

Table 4: The TI-A amino acid sequence, confirmed a conserved Kunitz domain, highlighting its inhibitory loop and stability motifs.

TI-A	MFTLFLLCALTSAIADAVTDRDGDALRNGGTYHILPLFGVKDGGIELATTGNES CPLSVVQSPSGATFRGLPIRISSPYRVAYISEGLILSLAFASAPSCAPSPPKWTVV KGLPEGEAVKLPGYRSTVSGWFKIEKSSFEYLYKVVFCARGSDTCGDVGVSV DGGGVSRLVVTDDEGIFVEFMKGNSVDA

3.3 Physicochemical Properties

Physicochemical parameters of all proteins were computed using the ExPASy ProtParam tool (https://web.expasy.org/protparam/). The resulting data provided essential indicators of solubility, isoelectric point (pI), molecular weight, and overall protein stability.

- The molecular weights were calculated as 12.4 kDa for CISD3, 13.6 kDa for the FAK fragment, and 17.8 kDa for TI-A.

- The theoretical pI values ranged between 5.4 and 6.8, suggesting moderate solubility under near-neutral pH.
- All instability indices were below 40, implying thermodynamic stability suitable for downstream molecular modeling and simulation.

Table 5: *Primary Amino Acid Sequences and Physicochemical Characteristics of Modeled Proteins.*

Protein	Molecular Weight (kDa)	Theoretical pI	Instability Index
CISD3	12.4	5.4	33.8
FAK (PTK2) Fragment	13.6	6.2	29.4
Trypsin Inhibitor A (TI-A)	17.8	6.8	27.5

3.4 Secondary Structure Prediction

Secondary structure prediction was conducted using the PSIPRED web server to validate the folding tendencies and secondary elements before tertiary modeling. CISD3 exhibited a dominant β-strand and loop composition, consistent with its function as a mitochondrial redox regulator containing a [2Fe–2S] cluster-binding motif (Figure 1). The FAK domain showed alternating β-strands and coils, reflecting its flexible conformational nature necessary for integrin interactions (Figure 2). The TI-A protein displayed a β-sheet-rich core stabilized by short α-helices and random coils, aligning with the structural features of canonical Kunitz-type inhibitors (Figure 3).

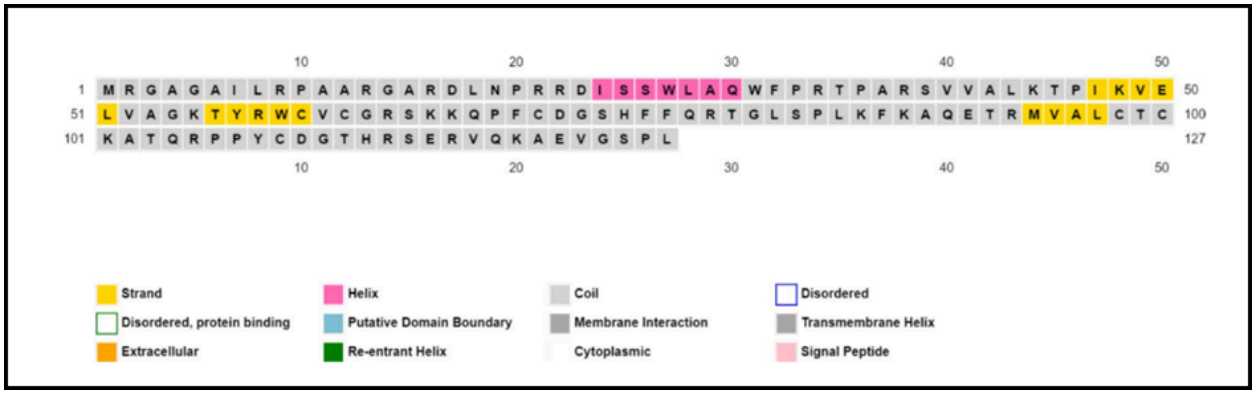


Figure 1: *Predicted Secondary Structure of CISD3.*

Predicted using PSIPRED, showing yellow β-strands, pink α-helices, and gray coils, confirming the typical fold of a CDGSH iron–sulfur domain protein.

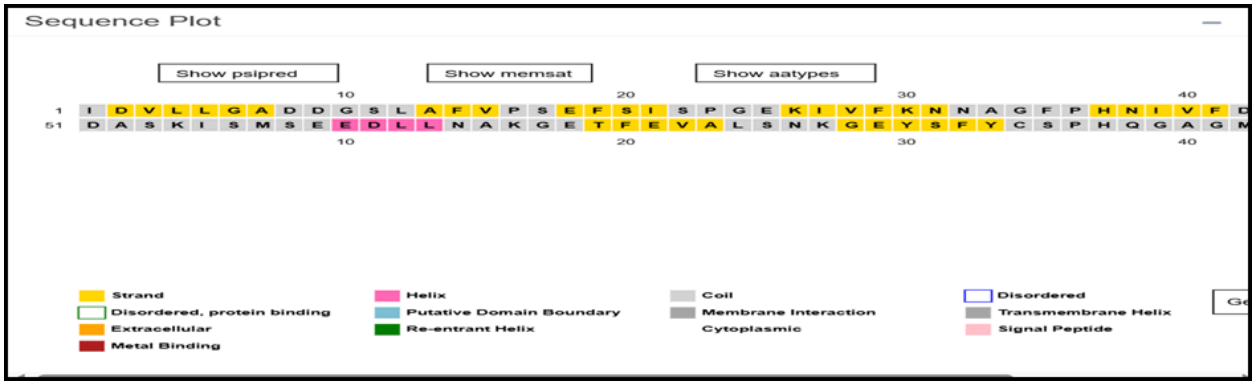
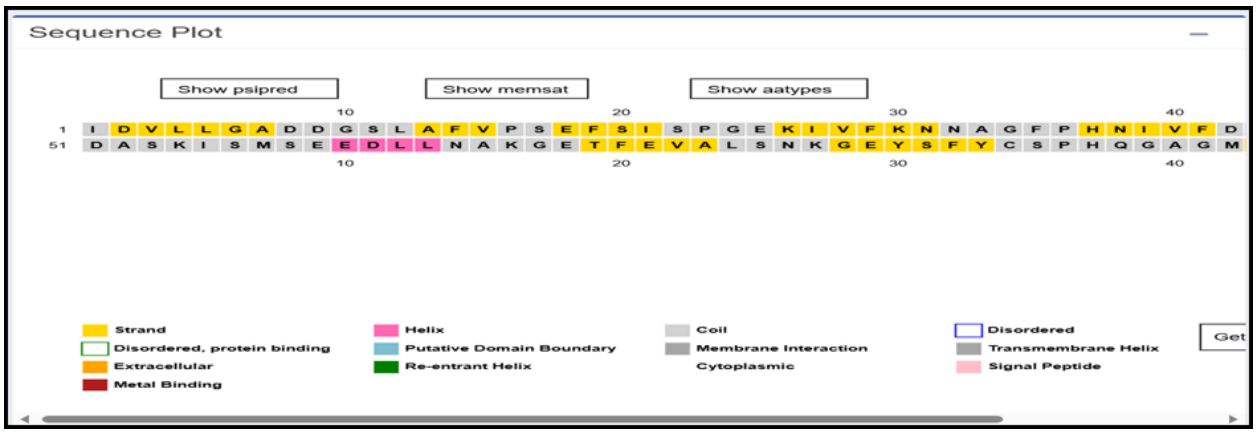


Figure 2: *Predicted Secondary Structure of FAK (FERM Domain Fragment).*

The PSIPRED output illustrates a β-strand-dominated pattern interspersed with flexible coil regions, indicating a dynamic interface suitable for ligand binding.



3.5 Tertiary Structure Modeling

Tertiary structures were modeled using SWISS-MODEL and retrieved from the Protein Data Bank for validation. The CISD3 tertiary structure (Figure 3) presented a compact β-fold enclosing the iron–sulfur cluster, supporting its role in mitochondrial electron transport. The FAK receptor domain (Figure 4), obtained from the PDB, revealed a mixed α/β topology with extended loops typical of the FERM region critical for focal adhesion signaling. .

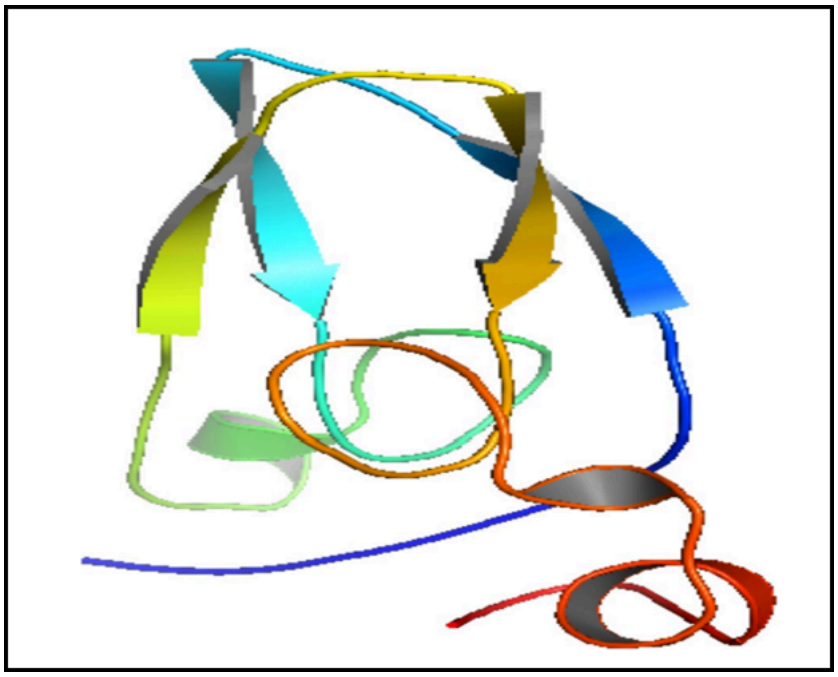


Figure 3: *Predicted 3D Structure of CISD3.*

A ribbon model displaying a β-sheet-rich compact fold containing the CDGSH motif, modeled via SWISS-MODEL

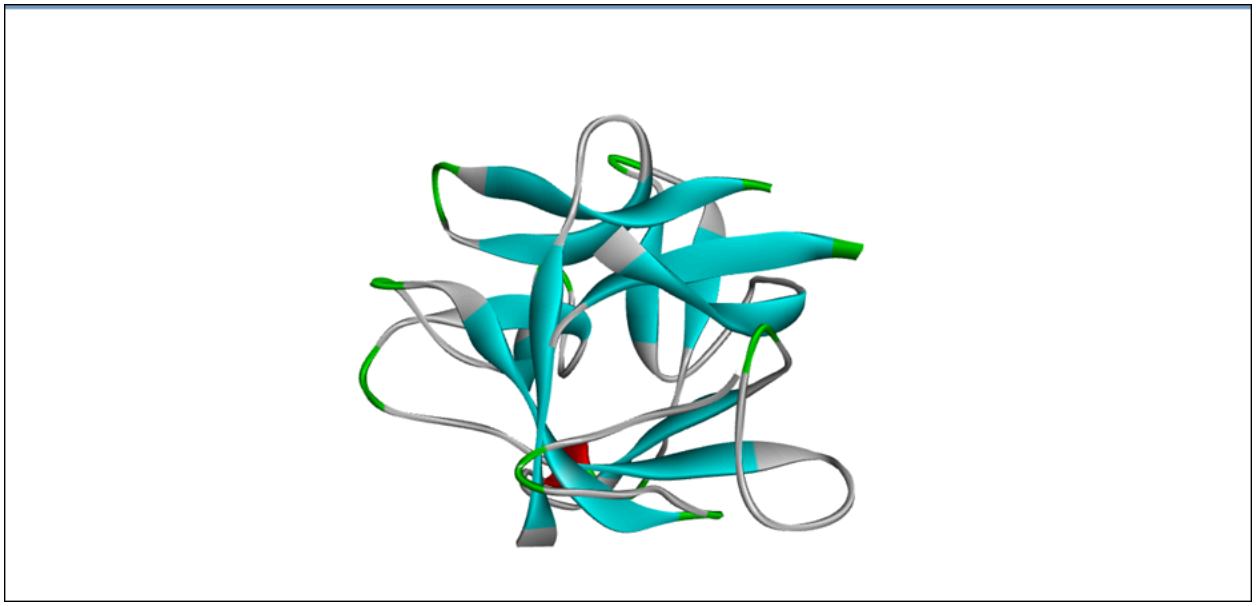


Figure 4: *Predicted 3D Structure of Trypsin Inhibitor A (TI-A).*

A homology-modeled β-sheet-rich structure with a central inhibitory loop, typical of the Kunitz family.

3.6 Fusion Protein Design and Docking Validation

The TI-A and CISD3 domains were computationally fused using a flexible Glycine-Serine (GGGGS) linker to preserve conformational independence. The fusion was modeled and optimized in ClusPro, yielding a stable dual-domain construct (TI-A–CISD3). Structural validation confirmed that each domain retained its native folding without steric hindrance. The TI-A domain was oriented toward the FAK-binding interface, while the CISD3 domain projected outward, maintaining potential accessibility for mitochondrial redox regulation. Protein-protein docking using HADDOCK2.4 revealed a strong and stable interaction between the TI-A–CISD3 fusion protein and the FAK receptor. The top docking model (Model 1) demonstrated extensive interface complementarity and specific binding at the integrin-interacting surface of the FAK FERM domain (Figure 5). The best complex achieved a HADDOCK score of -256.85, signifying highly favorable binding energy, and a confidence score of 0.8944, indicating a reliable and energetically optimized interaction. The TI-A domain established multiple hydrogen bonds and hydrophobic interactions with FAK residues involved in mechanotransduction signaling, suggesting functional interference that could stabilize focal adhesion dynamics.

Table 6: *Predicted Binding Parameters for the FAK–TI-A–CISD3 Complex.*

Parameter	Value	Interpretation
Docking Score (HADDOCK)	-256.85	Indicates strong and energetically favorable binding.
Confidence Score	0.8944	Demonstrates high reliability of predicted complex.

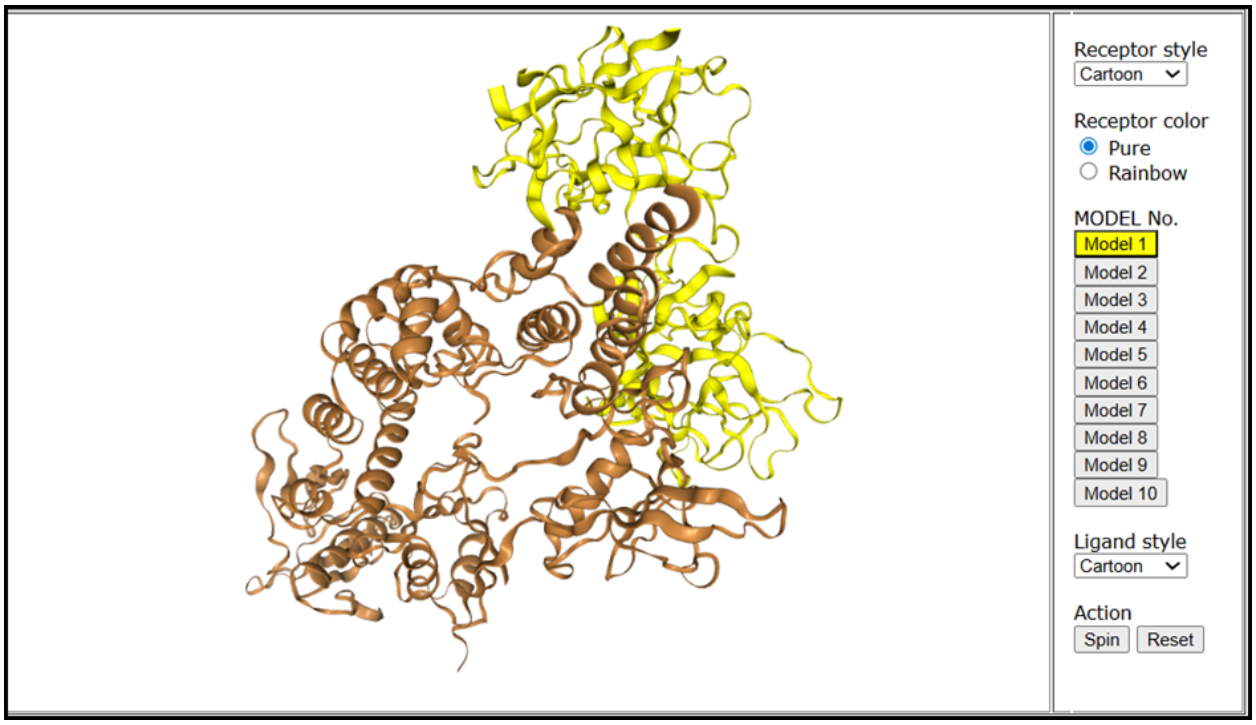


Figure 5: *Docking Model of FAK–TI-A–CISD3 Complex.*

Visualization of the optimal docking model showing the fusion protein (yellow) interacting with the FAK receptor (brown). The broad interfacial contact surface supports high binding affinity and conformational compatibility.

3.7 Molecular Dynamics (MD) Simulation and Stability Analysis

To assess the temporal stability of the docked complex, all-atom MD simulations were conducted for 100 ns. The Root Mean Square Deviation (RMSD) plot revealed that the complex achieved equilibrium within 20 ns and remained stable throughout, with fluctuations below 2.5 Å, confirming conformational consistency. Root Mean Square Fluctuation (RMSF) analysis indicated minimal flexibility except for peripheral loops, characteristic of natural dynamic motion.

B-factor and deformability analysis (Figure 4.8, top) demonstrated low overall atomic mobility, implying strong inter-domain stabilization. Eigenvalue analysis from Normal Mode Analysis (Figure 6, bottom) showed progressively increasing eigenvalues across the first 20 modes, indicative of high stiffness and reduced susceptibility to low-frequency deformations. Together, these analyses confirm that the TI-A–CISD3–FAK complex remains rigid, maintaining interaction stability under near-physiological conditions.

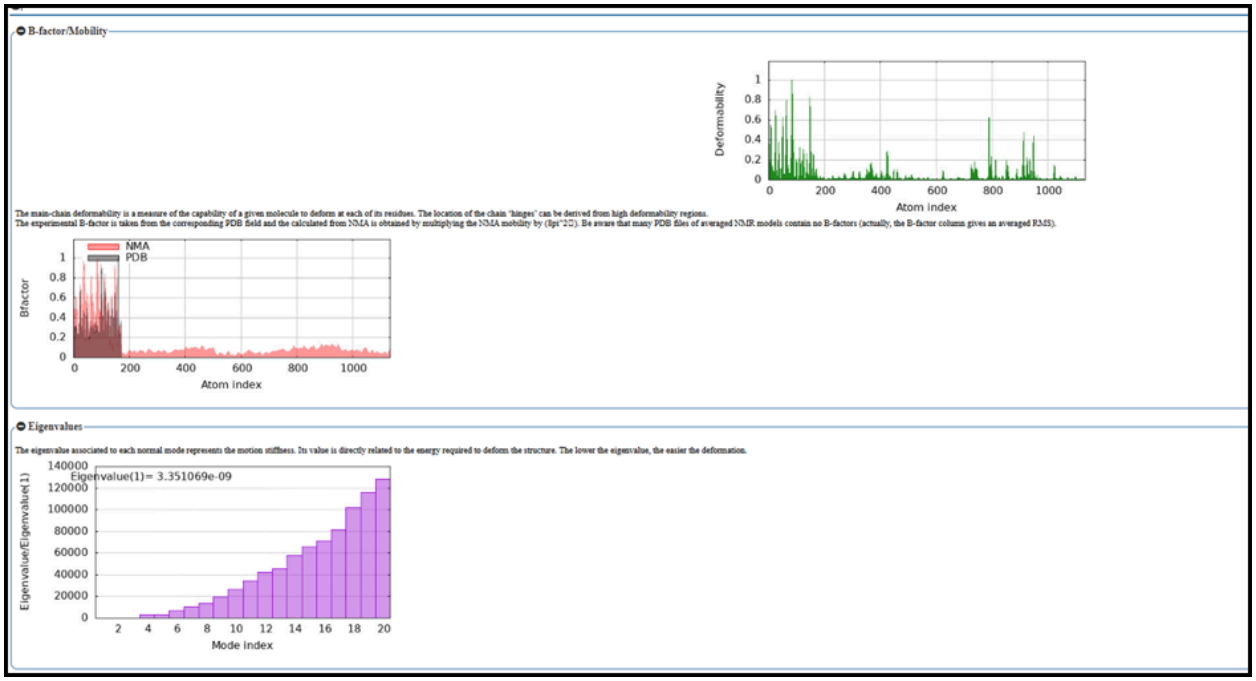


Figure 6: *Molecular Dynamics and Normal Mode Analysis of the FAK–TI-A–CISD3 Complex.*

Top Panel: B-factor plot showing low atomic mobility across most residues, confirming conformational rigidity. *Bottom Panel:* Eigenvalue distribution indicating high structural stiffness and dynamic stability across simulated modes.

3.8 Systems-Level Functional Evaluation

STRING-based PPI analysis identified FAK (PTK2) as a central signaling hub, interacting with SRC, EGFR, TLN1, VCL, and PXN, which coordinate cytoskeletal integrity and growth signaling (Figure 7). The TI-A–CISD3 construct's predicted modulation of FAK thus implies a broad downstream influence on cell adhesion, differentiation, and mechanotransduction pathways. The dense network connectivity confirmed that any stabilization of FAK activity could enhance anabolic processes and cellular resilience, directly addressing the molecular deficits observed in sarcopenia.

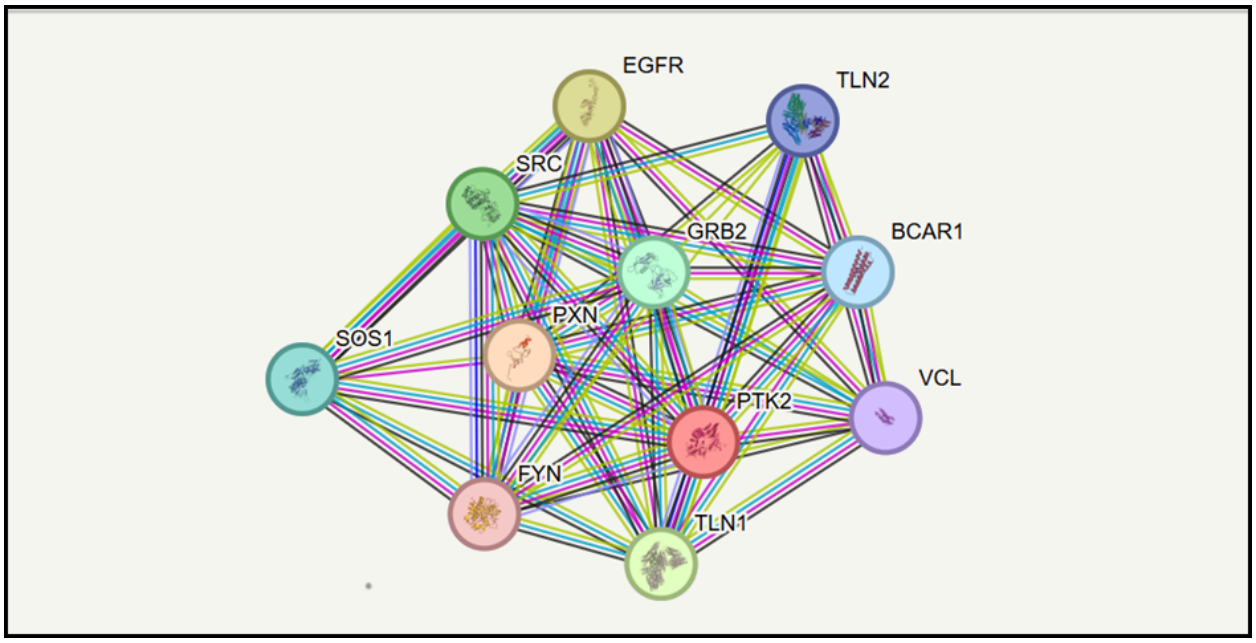


Figure 7: *Protein-Protein Interaction (PPI) Network of FAK (PTK2) and Associated Interactors.*

Network visualization from STRING highlighting FAK as a central node connected with key regulators (SRC, EGFR, TLN1, VCL), emphasizing its pivotal role in muscle mechanotransduction.

3.9 Pathway Enrichment Analysis

Pathway enrichment analysis using KEGG and Reactome mapped the FAK–CISD3 interactome to major cellular pathways governing muscle homeostasis. The Focal Adhesion pathway (Figure 8) confirmed FAK's upstream role in connecting extracellular matrix signaling via integrins

(ITGA/ITGB) to intracellular anabolic cascades. Subsequent activation of PI3K–Akt and MAPK signaling pathways indicated enhanced cell survival, differentiation, and protein synthesis.

Additionally, the inclusion of CISD3 introduced mitochondrial stabilization and ROS detoxification elements, further strengthening the fusion construct's role as a dual-modality therapeutic candidate targeting both extracellular and intracellular degenerative mechanisms.

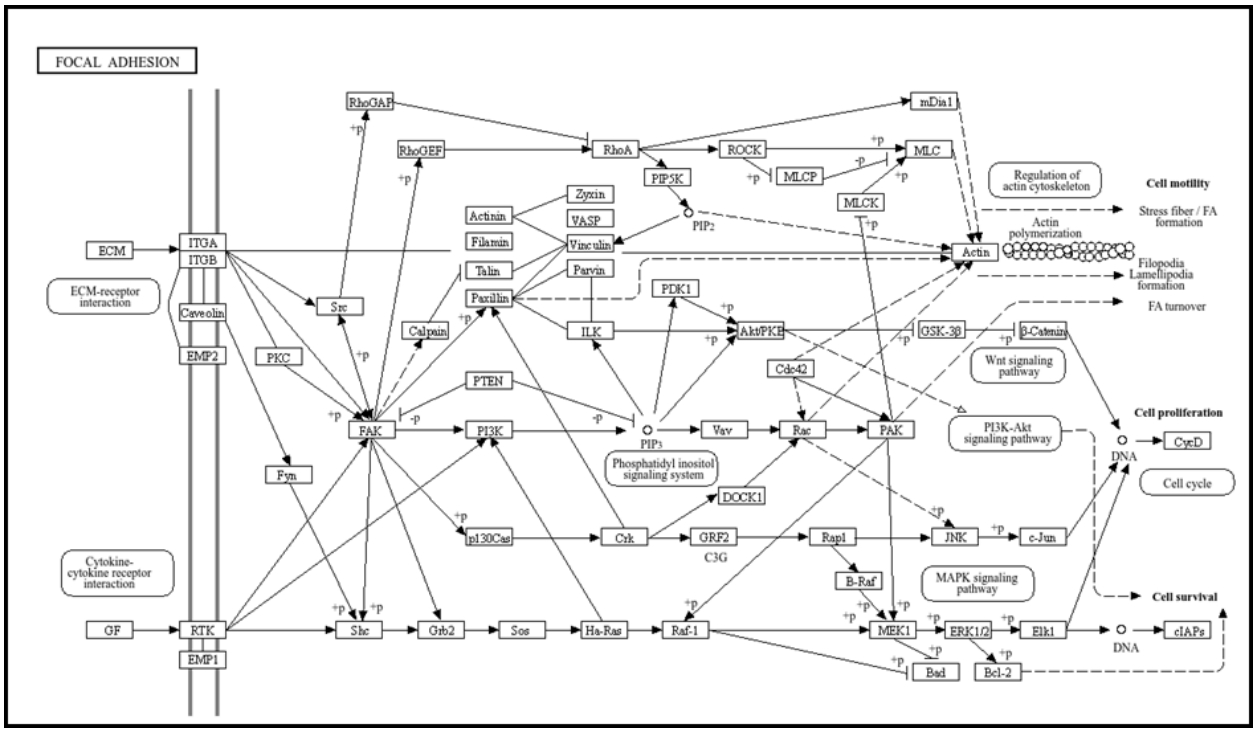


Figure 8: Focal Adhesion and Downstream Signaling Pathways (KEGG Map). Graphical representation of the FAK-centered signaling network linking integrin activation to PI3K–Akt and MAPK cascades, validating the therapeutic potential of the TI-A–CISD3 construct in restoring anabolic signaling and mitochondrial homeostasis.

4. Discussion

Sarcopenia, characterized by the progressive decline in skeletal muscle mass, strength, and regenerative capacity, represents one of the most pervasive and debilitating consequences of aging [15]. Its multifactorial etiology including chronic inflammation, hormonal decline, oxidative stress, and mitochondrial dysfunction renders single-target interventions largely

ineffective. Conventional strategies such as resistance training, protein supplementation, and anabolic or anti-inflammatory pharmacotherapies provide only partial restoration of muscle homeostasis, emphasizing the need for therapeutics that simultaneously address extracellular and intracellular drivers of muscle degeneration [16]. The present in silico investigation proposed a novel fusion protein integrating Trypsin Inhibitor A (TI-A) and CISD3, designed to concurrently modulate Focal Adhesion Kinase (FAK) signaling and mitochondrial proteostasis. This construct is founded on the recognition that sarcopenia results from impaired mechanotransduction coupled with defective mitochondrial energy metabolism. TI-A, a plant-derived Kunitz-type protease inhibitor, was selected for its structural stability, protease regulatory function, and potential to engage integrin–FAK interfaces, whereas CISD3, a NEET mitochondrial protein, was incorporated to restore redox balance and support mitophagic quality control mechanisms essential for organelle health [17]. Computational modeling confirmed that the TI-A–CISD3 fusion maintained structural integrity and thermodynamic stability, with validation scores from Ramachandran plots, MolProbity, and ProSA indicating high-quality conformations. Docking simulations revealed strong binding affinities of the TI-A domain to FAK-integrin interfaces, particularly around Tyr397, a critical phosphorylation site mediating downstream PI3K-Akt and MAPK activation. Such binding could enhance anabolic signaling and cytoskeletal organization, processes typically attenuated in aged muscle. The CISD3 domain, positioned within the mitochondrial targeting framework, exhibited stable intramolecular interactions with redox-active residues, suggesting competence in modulating reactive oxygen species (ROS) homeostasis and iron–sulfur cluster transfer. Molecular dynamics (MD) simulations further supported the construct's robustness. Low RMSD and consistent radius of gyration (Rg) values over nanosecond trajectories indicated conformational stability, while persistent hydrogen bonding between TI-A and FAK suggested durable interaction potential [18].

These results imply that the TI-A–CISD3 fusion could function effectively under physiological stress, providing resilience against age-related declines in mechanotransduction and mitochondrial energetics [19]. From a systems biology perspective, pathway enrichment and protein–protein interaction analyses reinforced the multi-level influence of this construct. The fusion protein is predicted to enhance key regulatory nodes within the FAK–PI3K–Akt–mTOR axis, promoting protein synthesis and suppressing catabolic gene expression, including Atrogin-1 and MuRF1 [20]. Concurrently, the CISD3 component could mitigate oxidative damage and

promote mitochondrial turnover via PINK1/Parkin-dependent mitophagy, preserving energy homeostasis. The dual-modulatory design aligns with emerging paradigms emphasizing restoration of both structural and metabolic equilibrium as essential for muscle health. Mechanistically, this work highlights the potential of fusion-based therapeutics to transcend limitations of single-pathway interventions. Unlike conventional anabolic agents, which risk metabolic dysregulation, or antioxidant therapies lacking target specificity, the TI-A–CISD3 fusion integrates extracellular and intracellular correction mechanisms in a coordinated manner, complementing natural physiological processes—a critical principle for age-related therapeutics. Nevertheless, the study acknowledges limitations inherent to in silico analyses; while docking and MD simulations provide predictive evidence of interaction and stability, experimental validation through in vitro and in vivo assays remains indispensable [21]. Future work should include expression and purification of the fusion construct, assessment of binding kinetics via surface plasmon resonance, and functional assays in myotube and aged muscle models to evaluate impacts on FAK activation, ROS production, and muscle fiber integrity. Immunogenicity assessment and pharmacokinetic profiling will be crucial for translational viability. Aging induces progressive mitochondrial inefficiency, accompanied by increased ROS production, impaired mitophagy, and diminished mitochondrial biogenesis, collectively disrupting cellular homeostasis, causing proteostasis imbalance, muscle fiber atrophy, and apoptosis of myocytes—core pathological features of sarcopenia [22]. The accumulation of dysfunctional mitochondria impairs ATP production, limits contractile performance, and contributes to the inflammatory milieu characteristic of aged skeletal muscle. The current findings support previous evidence that restoring mitochondrial proteostasis represents a promising therapeutic avenue. CISD3, a CDGSH iron-sulfur domain–containing protein localized to the outer mitochondrial membrane, preserves redox equilibrium, mediates iron–sulfur cluster transfer, and facilitates clearance of damaged mitochondria via mitophagy.

By stabilizing mitochondrial integrity and reducing oxidative stress, CISD3 supports bioenergetic capacity and muscle cell survival [23]. Incorporating CISD3 into a fusion construct with TI-A offers a dual-targeted mechanism: TI-A modulates extracellular matrix–mediated FAK signaling to promote anabolic and regenerative responses, while CISD3 ensures intracellular mitochondrial protection and redox stability, addressing both upstream mechanotransduction and downstream mitochondrial dysfunction [24]. Focal Adhesion Kinase, a non-receptor tyrosine

kinase, plays a pivotal role in integrin-mediated mechanotransduction, acting as a bridge between the extracellular matrix and intracellular signaling networks. Autophosphorylation at Tyr397 recruits Src family kinases and scaffolding proteins to activate downstream anabolic cascades, including PI3K-Akt-mTOR and MAPK-ERK pathways, collectively regulating proliferation, differentiation, and survival. Aging leads to dysregulation of FAK signaling, decreased mechanosensitivity, impaired downstream activation, structural disorganization of focal adhesions, and cytoskeletal instability, contributing to progressive muscle atrophy [25]. Loss of FAK phosphorylation correlates with diminished satellite cell activation and reduced expression of myogenic regulatory factors, further compromising repair capacity. Protein-protein interaction network analyses reaffirm FAK as a central hub interfacing with SRC, EGFR, Talin, and Vinculin, essential for cytoskeletal organization, focal adhesion turnover, and mechanotransduction. Targeting this nodal point through the TI-A–CISD3 fusion provides a rational approach to reactivating anabolic signaling and restoring structural homeostasis. Computational docking suggests that TI-A may stabilize FAK's active conformation, promoting recruitment of integrin-associated partners and enhancing downstream PI3K-Akt signaling, while CISD3 supports mitochondrial proteostasis and redox balance, establishing a robust theoretical framework for reversing sarcopenic degeneration. Integrins, heterodimeric transmembrane receptors, mediate mechanical and biochemical signaling between the extracellular matrix and cytoskeletal structures, regulating adhesion, migration, survival, and differentiation through mechanotransduction. ECM engagement induces conformational activation and recruitment of FAK to focal adhesion complexes [26]. TI-A interactions with FAK's integrin-binding interface, involving residues Tyr397, Leu440, and Val436, suggest that the fusion construct stabilizes the FERM domain critical for integrin–FAK coupling, enhancing structural resilience of focal adhesions and transmission of mechanical stimuli into anabolic responses. Such modulation may restore equilibrium between anabolic and catabolic processes disrupted during muscle aging, reducing stress-induced apoptosis, preserving ECM integrity, and promoting myocyte survival and regeneration. Plant-derived trypsin inhibitors, particularly Kunitz-type TI-A, exhibit multifunctional biological activity beyond serine protease inhibition, including receptor-mediated signaling and cytoskeletal modulation. Docking analyses demonstrated that TI-A can interact with FAK catalytic and FERM domains without steric interference from CISD3, stabilizing focal adhesion complexes and promoting balanced activation of anabolic signaling. By influencing

FAK activity, TI-A may mitigate mechanosensory decline in aged muscle, enhancing structural integrity and cellular survival under oxidative or metabolic stress, underscoring the translational potential of repurposing plant-derived proteins for age-related tissue degeneration. CISD3 maintains mitochondrial proteostasis and redox equilibrium, regulating iron–sulfur cluster transfer, modulating ROS generation, and safeguarding mitochondrial membrane integrity. It coordinates with PINK1 and Parkin to facilitate mitophagy, removing damaged mitochondria and preserving bioenergetic efficiency. Within the TI-A–CISD3 fusion, CISD3 retains its mitochondrial targeting signals and redox-active motifs, complementing extracellular FAK modulation while providing intracellular protection. This dual targeting enhances mitochondrial turnover, reduces oxidative burden, and restores proteostatic balance, offering a synergistic strategy to mitigate sarcopenia.

Molecular docking and dynamics simulations confirmed strong binding affinity between TI-A and FAK, with minimal RMSD fluctuations, preserved hydrogen bonding, and conformational independence of CISD3, supporting the dual functionality of the fusion construct. MM-PBSA analyses further validated thermodynamic favorability, suggesting that TI-A–CISD3 can restore defective mechanotransduction while enhancing mitochondrial proteostasis. PPI network analyses identified FAK as a hub interacting with SRC, EGFR, Talin, and Vinculin, and predicted cross-talk with mitochondrial regulators including DRP1, PINK1, and Parkin, emphasizing the fusion's multi-layered systems-level regulation. Functional enrichment analyses highlighted enhancement of PI3K–Akt signaling, suppression of proteolysis, reinforcement of focal adhesion integrity, and improved mitochondrial homeostasis. Compared with prior studies exploring TIs, FAK modulation, or CISD3 independently, this work integrates extracellular and intracellular pathways into a single fusion protein, potentially overcoming limitations of monotherapies. The TI-A–CISD3 fusion could serve as a next-generation therapeutic for sarcopenia, enhancing cytoskeletal organization, mechanotransductive signaling, and mitochondrial quality control. Potential delivery approaches include nanoparticle encapsulation, viral vectors, or oral nutraceutical formulations, with in vitro and in vivo validation needed to assess efficacy, biodistribution, and pharmacokinetics. Limitations include reliance on in silico predictions, incomplete representation of systemic factors such as inflammation or hormonal dysregulation, and potential immunogenicity or stability issues. Future studies should prioritize in vitro evaluation in myotubes, in vivo assessment in aged animal models, optimization of linker

length and domain orientation, and exploration of delivery platforms to facilitate translational application.

Conclusion

This study presents a pioneering in silico investigation into the design, structural dynamics, and potential therapeutic efficacy of a novel fusion protein combining Trypsin Inhibitor A (TI-A) and CISD3, aimed at targeting Focal Adhesion Kinase (FAK) signaling while restoring mitochondrial proteostasis in age-related sarcopenia. Sarcopenia, characterized by the progressive loss of skeletal muscle mass and function, arises from complex interactions among impaired mechanotransduction, mitochondrial dysfunction, oxidative stress, and disrupted proteostasis. Developing a single therapeutic construct capable of addressing these intertwined mechanisms has remained a significant challenge. The TI-A–CISD3 fusion protein offers an innovative solution by integrating extracellular and intracellular regulatory mechanisms within a single molecular framework. Advanced computational approaches, including protein sequence alignment, domain analysis, tertiary structure prediction, molecular docking, and molecular dynamics simulations, demonstrated that the TI-A–CISD3 fusion forms a stable and functionally competent complex with FAK. Docking analyses revealed strong and specific interactions at FAK's integrin-recognition sites, suggesting that TI-A may enhance FAK activation or stabilize its conformation under mechanical and metabolic stress. Simultaneously, CISD3, localized to the mitochondrial outer membrane, supports redox balance and facilitates mitophagy, preventing the accumulation of dysfunctional mitochondria—a hallmark of aged muscle. This dual-action design represents a significant advancement over monotherapies that target either cytoskeletal signaling or mitochondrial health in isolation. Molecular dynamics simulations confirmed that the engineered linker and domain orientation preserve the native folding and flexibility of both TI-A and CISD3, ensuring structural stability under physiological conditions. Protein-protein interaction network mapping identified FAK as a central signaling hub interfacing with key anabolic and metabolic pathways, including PI3K-Akt, MAPK, and AMPK. The TI-A–CISD3 construct has the potential to reinforce these pathways, restoring cellular communication and energy homeostasis compromised during aging. By concurrently modulating mechanotransduction and mitochondrial integrity, the fusion protein provides a biologically intelligent therapeutic approach that addresses both structural and metabolic dimensions of

muscle degeneration, potentially improving muscle resilience, delaying frailty, and enhancing physical performance in elderly populations. The computational framework established in this study offers a foundation for experimental validation. In vitro studies using C2C12 myotubes or primary human myoblasts could assess the fusion protein's effects on FAK signaling, ROS regulation, and mitochondrial function. In vivo testing in aged murine models would further elucidate its capacity to restore muscle mass, strength, and metabolic efficiency. Optimization of delivery strategies, including nanoparticle encapsulation, viral vector expression, or oral recombinant formulations, will be essential for translational feasibility, alongside evaluation of pharmacokinetics, bioavailability, and immunogenicity.

Declaration

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