In Vitro Motility Assay, Analyzing the Full-Length α-Actinin-2 Wild-Type and Phosphomimic Variants.

FSU UNDERGRADUATE RESEARCH OPPORTUNITY PROGRAM
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Abstract

Muscle contraction relies on interactions between actin filaments and myosin motors, but these interactions are regulated by additional structural proteins within the cytoskeleton. One such protein, α-actinin-2, crosslinks actin filaments and helps organize muscle architecture. Small chemical modifications such as phosphorylation may alter the structure of α-actinin-2 and influence how strongly it binds to actin. This project investigates whether phosphorylation-like mutations in the actin-binding domain of α-actinin-2 change the speed at which actin filaments move across myosin motors.

To address this question, computational modeling will predict structural changes in α-actinin-2 and estimate its binding strength to actin. Experimentally, purified actin and myosin from porcine cardiac tissue and various states of α-actinin-2 proteins will be tested using a Total Internal Reflection Fluorescence (TIRF) in vitro motility assay. By measuring actin filament sliding velocities under different conditions, we aim to determine whether phosphorylation strengthens actin binding and increases frictional resistance. These findings will provide new insight into how cytoskeletal proteins regulate muscle mechanics.

Modeling / Statistics

$$V = \frac{V_{max} \times F_d}{F_d + \frac{V_{max} \times \kappa \times \zeta \times L \times r \times k_A \times \chi \times [\alpha]^{5/2}}{k_D \times (k_A \times \chi \times [\alpha]^{3/2} + k_D)}}$$

Vmax = maximal sliding velocity
F_d = driving force of the bed of myosins
κ = system compliance associated with the ABP and the ABP linkages
L = length of a typical actin filament
r = reach of an ABP to bind to an actin filament
k_A = second order rate constant for ABP attachment to actin
k_D = ABP detachment rate
f and v are constants that define the surface geometry
ζ, χ = constants that define the surface geometry

Resources

1- AlphaFold3 Server and ChimeraX Software for protein structure predictions
2- FigureLabs.AI for protocol visualization and data graph visualization

Protein Purification

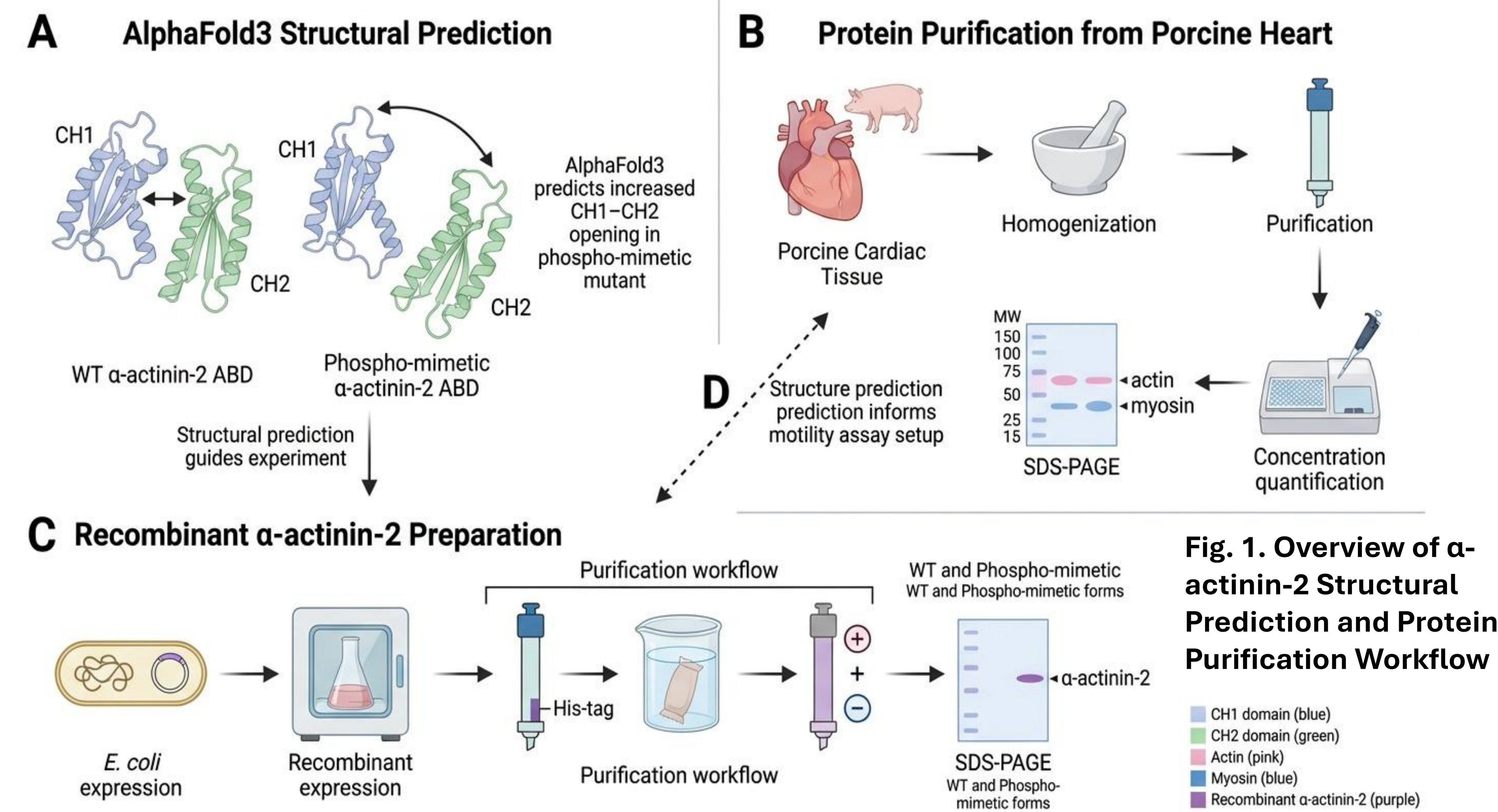


Fig. 1. Overview of α-actinin-2 Structural Prediction and Protein Purification Workflow

Predicted Results

Key idea: α-Actinin bound to actin acts like a molecular "brake." Stronger binding = more drag = slower movement.

WT condition: WT α-actinin-2 is expected to cause some slowing of actin gliding due to frictional loading.

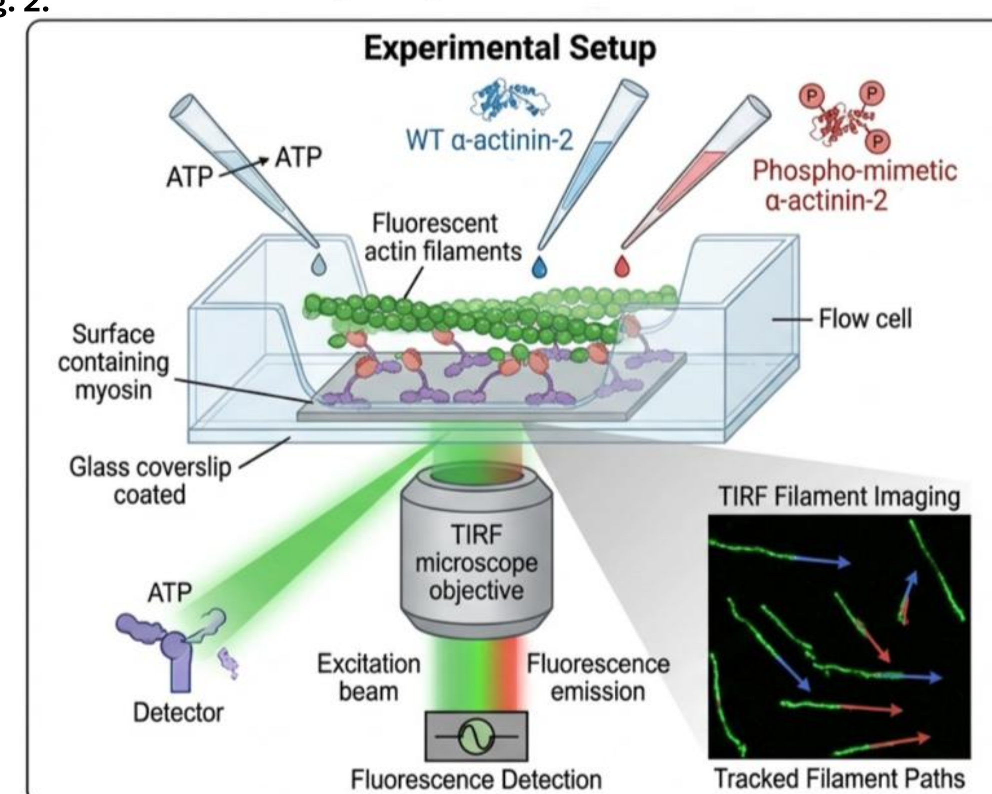
Phospho-mimetic condition: Phospho-mimetic is expected to bind actin more tightly, creating greater resistive drag, so actin filaments show a larger decrease in sliding velocity than WT.

What the workflow supports: As α-actinin "load" increases, velocity should drop; the phospho-mimetic curves should shift toward lower velocities (greater frictional effect) compared with WT.

Computational + experimental agreement: If AlphaFold predict increased binding affinity, the motility assay should show slower average filament velocity and stronger frictional loading for phospho-mimetics.

Take-home conclusion: Phosphorylation-like changes in α-actinin-2 can tune muscle cytoskeletal mechanics by increasing actin binding and resisting actomyosin-driven movement.

Fig. 2. TIRF *In Vitro* Motility Assay for α-Actinin-2 ABD Functional Test.



TIRF-based *in vitro* motility assay to measure actin sliding velocity with WT vs. phospho-mimetic α-actinin-2. Analysis compares filament velocities using curve fitting and statistical significance testing.

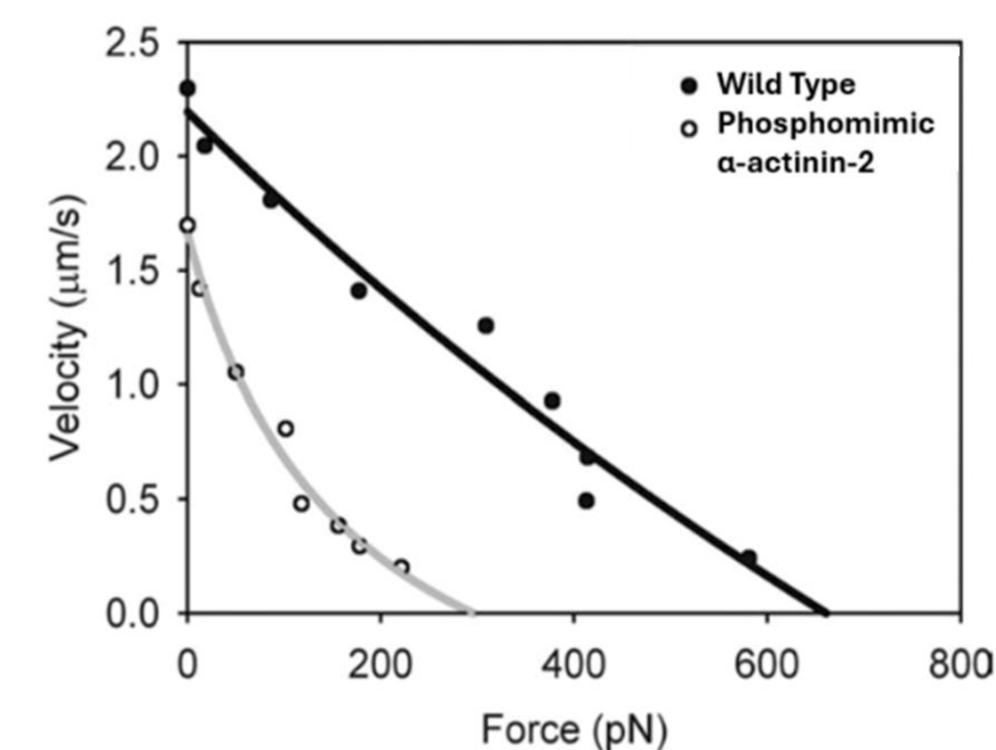


Fig. 3. Force-velocity curves fit to the Hill equation for cardiac muscle myosin WT vs Phosphomimic α-actinin-2

In silico Predictions And Statistical Analysis

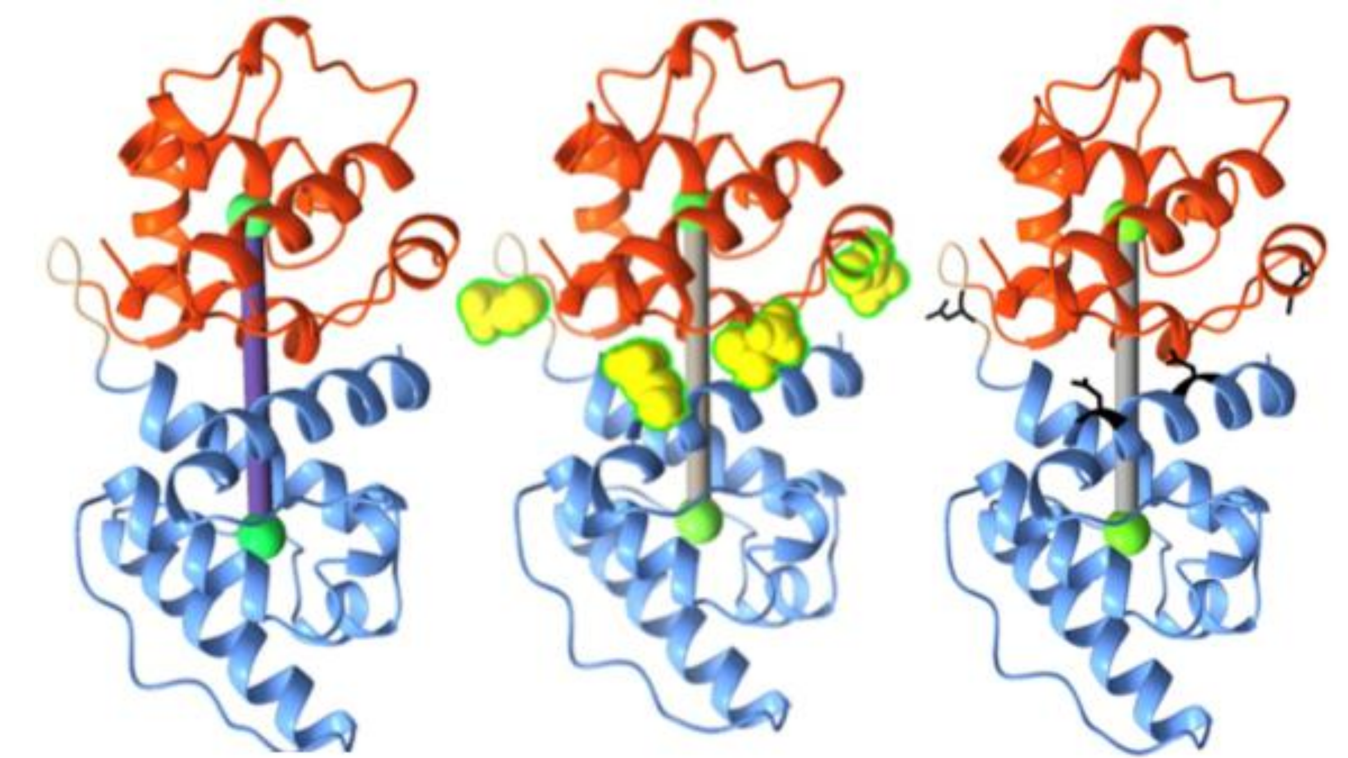


Fig. 4. 3D Polypeptide Models of Wild Type α-Actinin-2 (Left) Phosphorylated α-Actinin-2 (Middle) Pseudophosphorylated Type α-Actinin-2 (Right)

Comparison of each vs ω	Mean difference vs ω (Å)	p-value
Δ	+0.3491	1.69×10^{-5}
T43p	+0.1602	0.00357
S50p	+0.2973	5.29×10^{-5}
S147p	+0.2721	9.42×10^{-5}
T237p	+0.2952	1.37×10^{-4}
Ψ	+0.4203	8.47×10^{-6}
T43D	+0.3668	1.98×10^{-5}
S50D	+0.3995	6.50×10^{-6}
S147D	+0.3710	1.19×10^{-5}
T237D	+0.3775	1.61×10^{-5}

Fig. 5. Pairwise comparisons between WT α-actinin-2 and phosphorylated or pseudophosphorylated variants were based on modeled structural length (Å). All Phosphorylated (Δ) contains all four phosphorylation sites, and All Pseudophosphorylated (Ψ) contains all four Asp phospho-mimetic substitutions. Positive Δ values indicate greater length than WT. All variants were significantly higher than WT (α = 0.05).