Structural and Functional Characterization of the Acidic Region from the RIZ Tumor Suppressor

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BCHM 4850
March 1st, 2023

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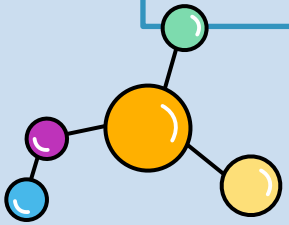
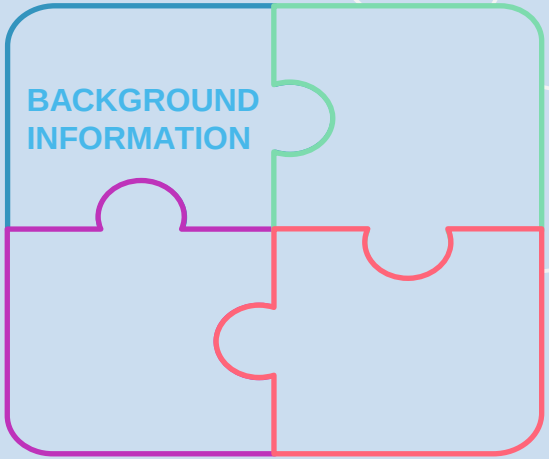
WHAT ARE WE TALKING ABOUT AND WHY DO WE CARE?

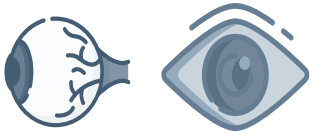
Background Information



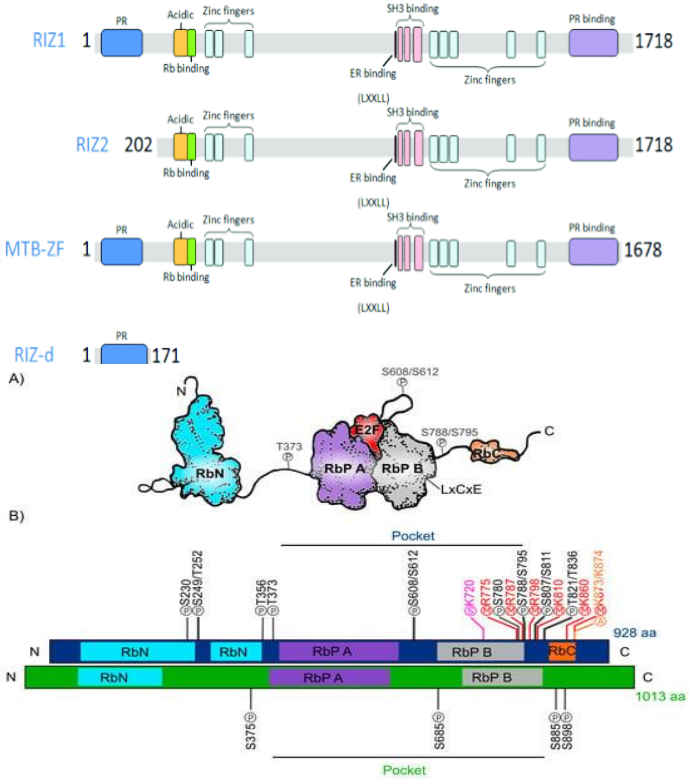


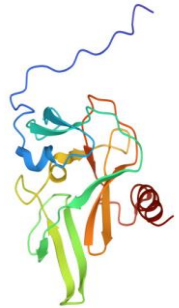
What is the focus of this presentation?

- Retinoblastoma protein-interacting zinc finger protein (RIZ)
  - -Tumor Suppressor
  - -Transcriptional Regulator
  - -Binds to retinoblastoma protein (Rb)



- Retinoblastoma protein (Rb)
  - -Tumor suppressor
  - -Regulates the cell cycle, senescence, apoptosis, differentiation, and chromosomal stability.





How does the mechanism of RIZ binding work?

- The make up of RIZ
  - RIZ1 vs RIZ2
  - The Acidic Region (AR)
  - Zinc Finger motifs
- The make up of Rb
  - Cyclin Box B
  - Similarity of the AR to the consensus RB-binding sequence
  - LXCXE Sequence

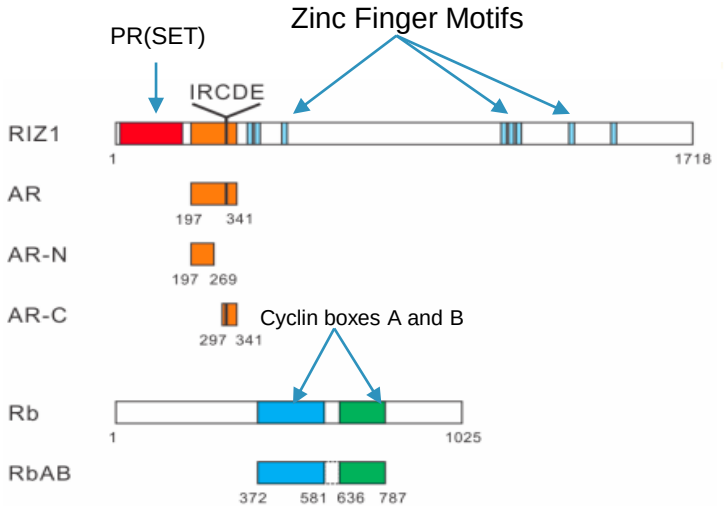


Figure 1. Schematic representation of RIZ1, Rb, and the recombinant constructs used in this study. In RIZ1, the PR(SET) domain is colored red, the acidic region (AR) orange, and the C2H2-like zinc finger domains light blue. The position of the IRCDE motif is indicated. In the Rb protein, cyclin boxes A and B that form the pocket domain are colored blue and green, respectively. The sequence between the cyclin boxes (residues 582–635; dashed) in the RbAB construct is replaced with a two-residue linker (EF).

RIZ sequence

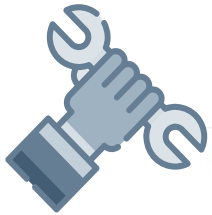
IRCDE

LXCXE

Rb binding sequence

Zooming in on RB Protein

- What will mutations to the LXCXE-binding sequence cause?
- What does the binding site cause interactions with?



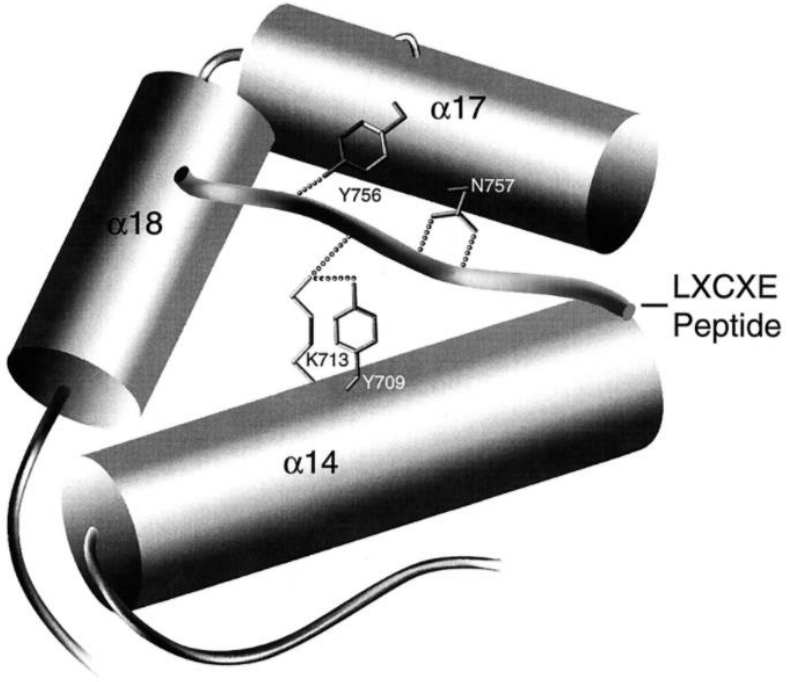


Fig. 1. Diagram of the LXCXE binding site derived from cocrystallization of the Rb pocket with an LXCXE peptide (26). Tyr 709, Lys 713, Tyr 756, and Asn 757 are conserved amino acids in the Rb pocket that appear to make important contacts with the backbone of the LXCXE peptide. Each of these amino acids was mutated to alanine either individually or in combinations.

Objectives:

- AR is intrinsically disordered
- Interaction of RIZ to Rb shows direct contact physically and not just functionally, and does not require bridging molecules.
- If the homologous region, IRCDE, is responsible for the binding of RIZ to Rb
- RIZ binding has a similar affinity that is comparable to the affinity of peptides containing the LXCXE motif of viral oncoproteins (K_d 100-200nM)

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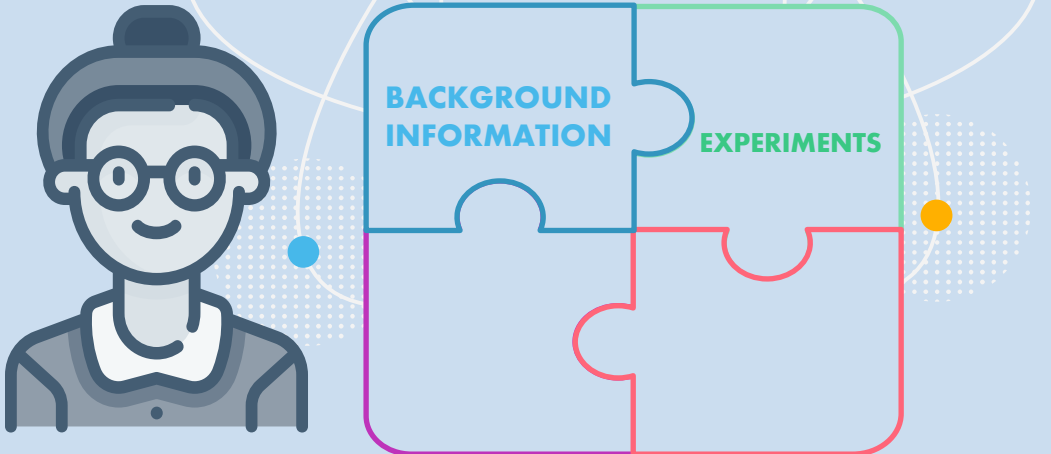
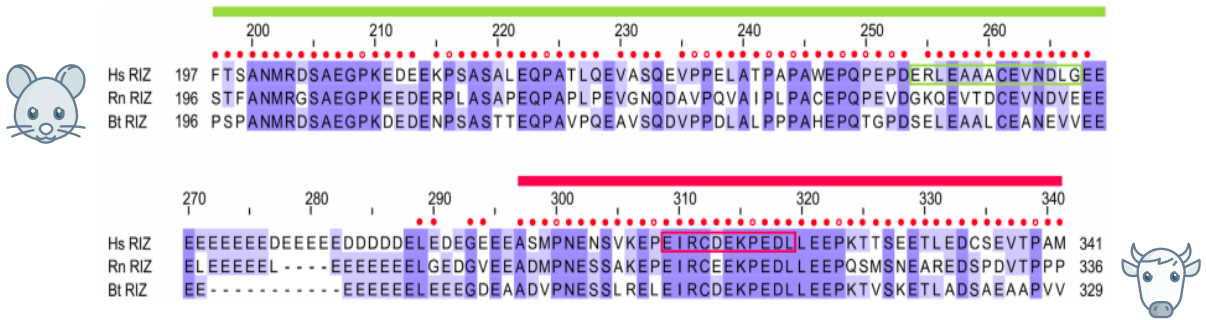
What was done?

Methods/Experiments



How were RIZ and Rb Prepared For Experimental Use?

- Recombinant Forms of the Acidic Regions of the RIZ Protein Were Used.



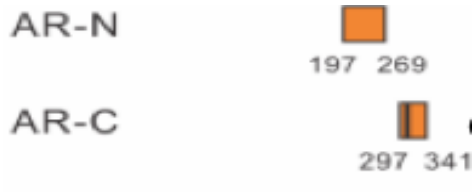


Figure 2. Amino acid sequence of AR. Sequence conservation in RIZ proteins from *Homo sapiens* (Q13029), *Rattus norvegicus* (Q63755), and *Bos taurus* (F1N790) is highlighted by blue shading. The spans of the recombinant AR-N and AR-C protein constructs are shown as green and red bars, respectively, and the peptides used in this study are marked with green and red boxes. The extent of backbone assignment in AR is also indicated. Filled red circles mark residues whose ^{1}H and ^{15}N backbone amide chemical shifts are assigned, and empty red circles denote prolines. AR contains a high proportion of acidic residues, 45 glutamates (30%) and 14 aspartates (9%), and the majority of residues with missing assignments (269−288, 291, 292, 295, and 296) are glutamates and aspartates that are located in the highly degenerate central region of the AR construct. The remaining residues with missing assignments (214, 229, 234, and 254) are glutamates in other parts of AR.

- Cyclin Boxes A and B Were Amplified Resulting In The Protein Construct **RbAB**

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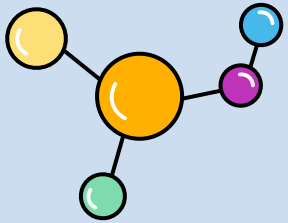
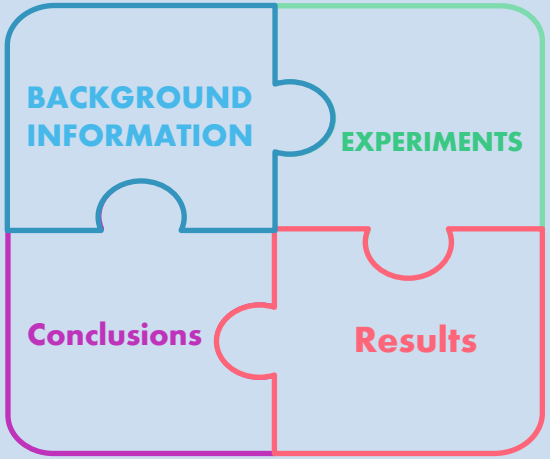
What was found

Results





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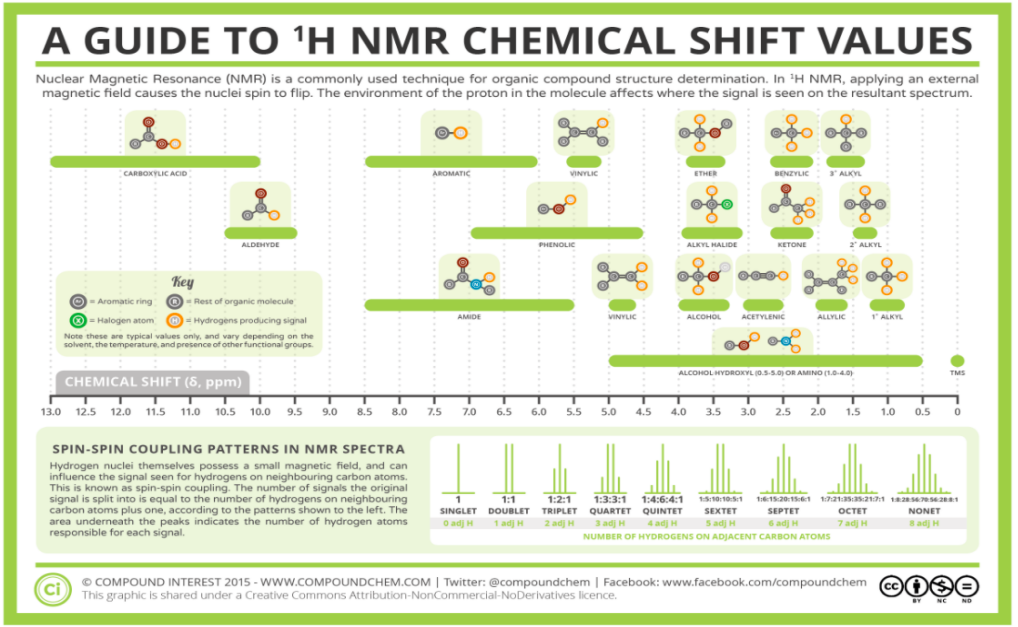
What do they mean?

Conclusions

Nuclear Magnetic Resonance (NMR) Spectroscopy

➢ What Is NMR and The Setup

- ^{13}C and ^{15}N or just ^{15}N
- Chemical shifts in AR were based of 3D ROCSY, HNCACB, CBCA(CO)NH, C(CCO)NH, HNCO, and HN(CA)CO spectra.
- Nuclear Overhauser effect (NOE)
- Heteronuclear single-quantum correlation HSQC
- SSP scores



Nuclear Magnetic Resonance (NMR) Spectroscopy

➢ Results / Conclusions

- Unfolded protein and highly flexible
  -Intrinsically Disordered

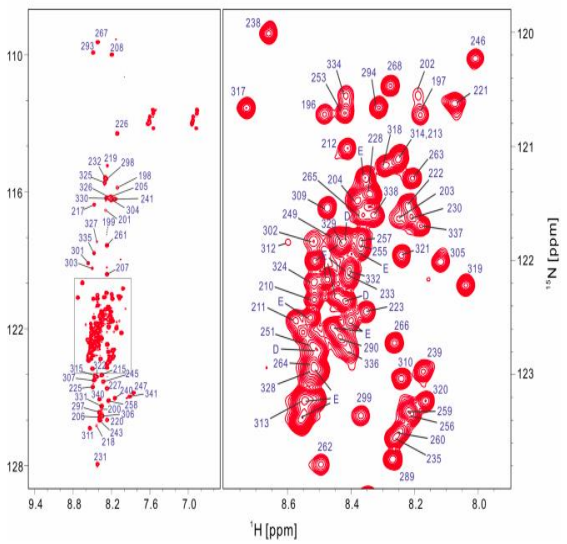


Figure 3. 2D ^{1}H–^{15}N HSQC spectrum of ^{15}N-labeled AR. The crowded central region is shown expanded in the right panel. Sequence specific assignments are indicated, and signals from several aspartates (D) and glutamates (E) that have not been assigned in a sequence specific manner are also labeled.

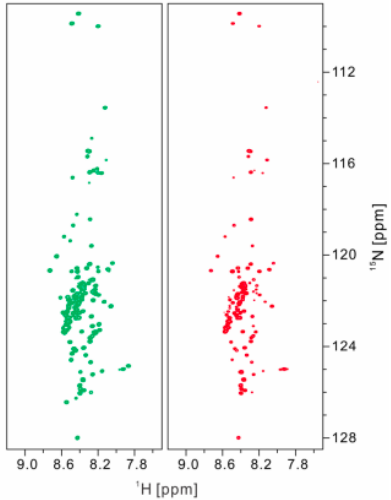


Figure 4. ^{1}H–^{15}N heteronuclear NOE in AR. A reference spectrum without ^{1}H saturation is shown in the left panel, and a ^{1}H–^{15}N NOE spectrum with ^{1}H saturation is shown in the right panel. Positive and negative contour levels are colored green and red, respectively. There are no observable positive signals in the ^{1}H–^{15}N NOE spectrum.

Nuclear Magnetic Resonance (NMR) Spectroscopy

➢ Results / Conclusions Continued

- Figure 6 (HSQC)
  - ➢ Residues 306-322 affected most by RbAB binding
  - ➢ Residues 255-267 also affected by RBAB binding. (Lower affinity)
  - ➢ AR regions are binding to RB

- Figure 9 (HSQC)
  - ➢ Black Peaks line up with Red Peaks
  - ➢ AR-N is binding to RB

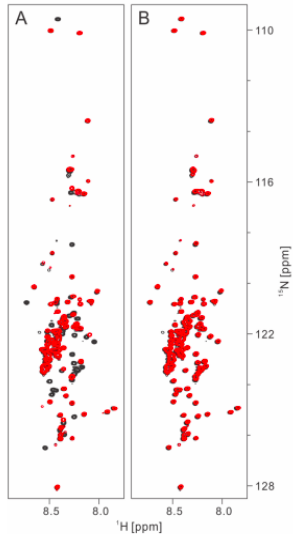


Figure 6. Effect of RbAB on NMR spectra of AR. (A) Superimposed 2D ^{1}H−^{15}N HSQC spectra of 86 μM ^{15}N-labeled AR alone (black) or in the presence of an ~2-fold molar excess of RbAB (red). (B) Same as panel A, but the sample with 86 μM ^{15}N-labeled AR and an ~2-fold molar excess of RbAB (red) also contained a 34-fold molar excess of the RIZ(309−319) peptide.

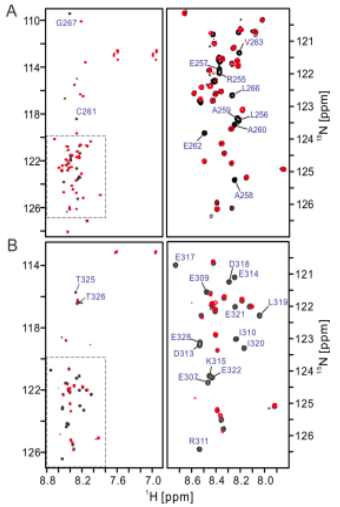


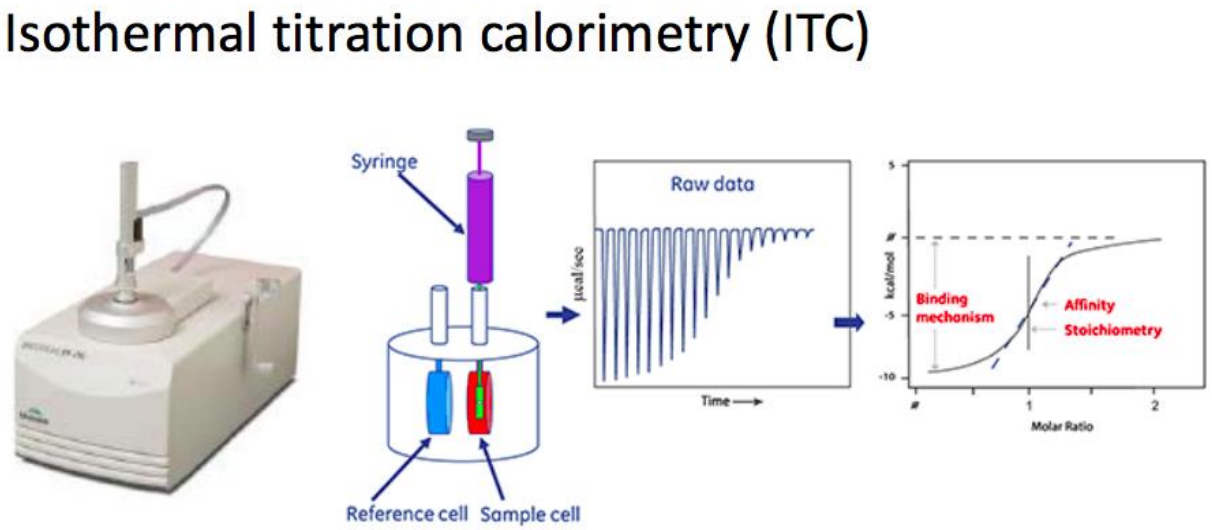
Figure 9. Effect of RbAB on NMR spectra of AR-N and AR-C. (A) Superimposed 2D ^{1}H−^{15}N HSQC spectra of 45 μM ^{15}N-labeled AR-N alone (black) or in the presence of an ~1.3-fold molar excess of RbAB (red). (B) Superimposed 2D ^{1}H−^{15}N HSQC spectra of 167 μM ^{15}N-labeled AR-C alone (black) or in the presence of an ~1.4-fold molar excess of RbAB (red). For each construct, the spectral region that is boxed in the left panel is shown expanded on the right, and assignments for the signals that are the most affected by RbAB are indicated.

Isothermal Titration Calorimetry (ITC)

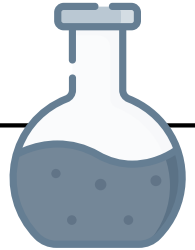
➢ What Is ITC and The Setup

- RbAb injected into the calorimeter (heats of binding)
- Affinity of RbAb for AR-N and FITZ-RIZ(254-267)

-Dissoication Constant



Isothermal Titration Calorimetry (ITC)



➢ Results / Conclusions

- AR + RbAB
- AR-N + RbAB
- AR-C +RbAB

- AR-C binds to RbAB. AR-N?

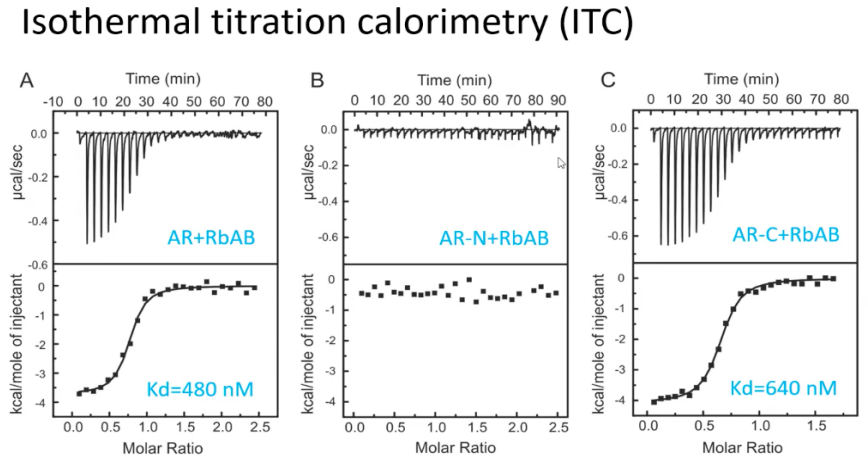
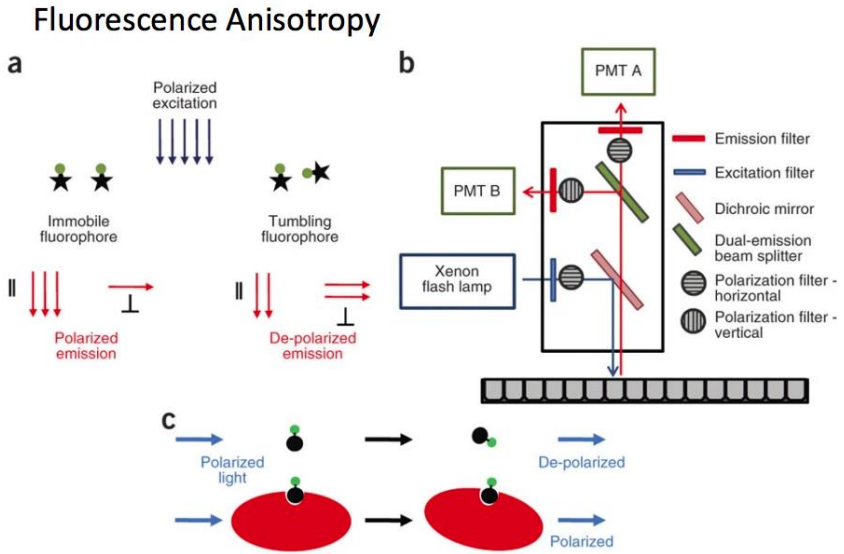


Figure 8. Calorimetric titrations of AR, AR-N, and AR-C with RbAB. Representative ITC traces and binding curves fit to the data using a single-binding site model are shown in the top and bottom panels, respectively. (A) Titration of 28 μM AR with 486 μM RbAB. (B) Titation of 39 μM AR-N with 524 μM RbAB. (C) Titration of 48 μM AR-C with 524 μM RbAB.

Fluorescence Anisotropy

➢ What Is Fluorescence Anisotropy and The Setup

- Direct Binding Assays
- Competitive Binding Assays
- Dissociation Constants



Fluorescence Anisotropy

➢ Results / Conclusions

- AR-N-FITC peptide + RbAB
- AR-C-FITC peptide + RbAB

- (AR-N-FITC peptide + RbAB) +AR-N = (AR-N + RbAB) + AR-N-FITC peptide

- (AR-C-FITC peptide + RbAB) +AR-C = (AR-C + RbAB) + AR-C-FITC peptide

- AR-C AND AR-N bind to RbAB

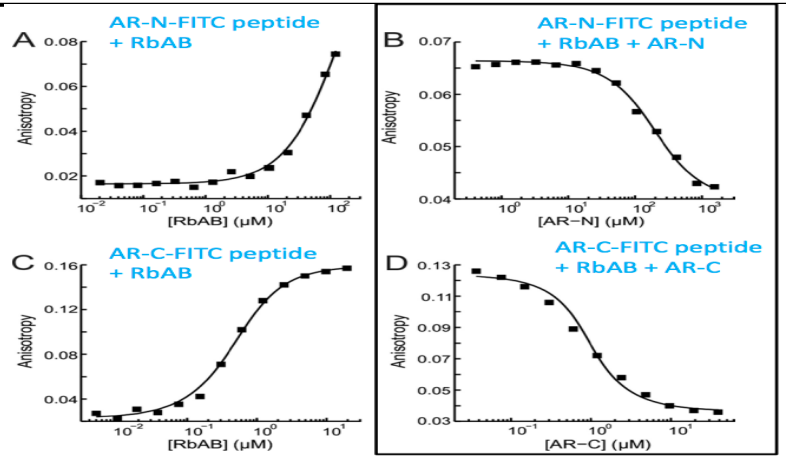
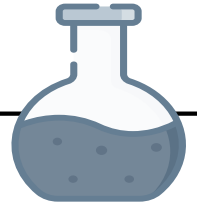


Figure 11. Fluorescence anisotropy binding assays. (A) Direct binding experiment with 0.33 μM FITC-RIZ(254—267) and the concentration of RbAB varied. (B) Competitive binding experiment with 0.33 μM FITC-RIZ(254—267), 91 μM RbAB, and the concentration of AR-N varied. (C) Direct binding experiment with 0.40 μM FITC-RIZ(309—319) and the concentration of RbAb varied. (D) Competitive binding experiment with 0.20 μM FITC-RIZ(309—319), 0.80 μM RbAB, and the concentration of AR-C varied. The binding curves fit to the data are shown as black lines. The experiments with FITC-RIZ(254—267) and FITC-RIZ(309—319) were performed at 25 and 20 °C, respectively.

➢ ITC Results / Conclusions (Continued)



- Competitive ITC Experiment

AR-N-FITC peptide + **(RbAB +AR-C)** = AR-C + **(RbAB + AR-N-FITC peptide)**

AR-N + **(RbAB +AR-C)** = AR-C + **(RbAB + AR-N)**

- AR-N does bind to RbAB
- ΔH° = 0 for prior ITC Experiment

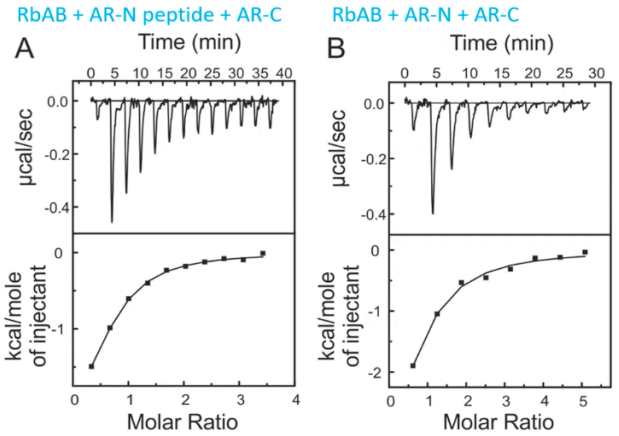


Figure 12. Calorimetric titrations of RbAB with AR-C in the presence of FITC-RIZ(254–267) or AR-N. (A) Titration of 20 μM RbAB with 1154 μM AR-C in the presence of 650 μM FITC-RIZ(254–267). (B) Titration of 9 μM RbAB with 984 μM AR-C in the presence of 459 μM AR-N. Representative ITC traces and binding curves fit to the data are shown in the top and bottom panels, respectively.

SUMMARY

- AR is intrinsically disordered
- The AR region of the RIZ protein does bind RB
- The homologous region, in the AR, region, IRCDE is responsible of binding to RB
- RIZ has similar affinity when compared to other peptides containing the LXCXE motifs

THANKS

Do you have any questions?

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Sources:

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Image Sources:

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