Structural insights into PROTAC®-induced proximity

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Overview of today’s presentation

• Brief background on PROTAC® degraders

• CryoEM success stories enabled by our optimized cryoEM workflow
  
  • Structural insights into ARV-471-induced proximity between the estrogen receptor (ER) and the CRBN E3 ligase

  • Mechanistic & structural basis of substrate-recruitment by a novel, PROTACable E3 ligase, KLHDC2
PROTAC® protein degraders harness the ubiquitin-proteasome system to induce the degradation of disease-causing proteins.
Structural insights into ARV-471-induced proximity between the estrogen receptor (ER) and the CRBN E3 ligase
ARV-471: Induces proximity between CRBN E3 ligase & the estrogen receptor

- ER-LBD is pulled into a higher mw complex with CRBN:DDB1 by the presence of ARV-471
- The ER:ARV-471:CRBN ternary complex can be separated by size-exclusion chromatography

presented at the NESBA symposium on Jan 24, 2023
Although robust ternary complex formation occurs in solution, this is not the case once frozen.

- 2D classification yields apo-DDB1
Optimization of crosslinking of ternary complex

- **ER:ARV-471:CRBN complex can be crosslinked** for cryoEM studies

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Crosslinked ternary complex also does not show robust ER density.

- 2D classification of crosslinked complex yields DDB1/CRBN and little hint of ER.

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Ultra-fast vitrification yields first evidence of ER in ternary complex by cryoEM

- 2D classification yields ~356k good particles
- Non-uniform refinement
- 3D variability analysis and cluster display

* single particle cryoEM on Krios™ G4 (E-CFEG) / Selectris Falcon 4 / CryoSPARC on AWS / ThermoFisherScientific cryoEM collaboration

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Mechanistic insights into a clinical stage PROTAC
ARV-471: Induces proximity between CRBN E3 ligase & the estrogen receptor, leading to ER degradation

- Highly dynamic ternary complex as imaged by cryoEM
- ER is flexible, and it is not possible to define a single ER binding pose
- CRBN in "closed” conformation
- DDB1 resolved to 2.7Å

*Chameleon™ grid prep / single particle cryoEM on Krios™ G4 (E-CFEG) / Selectiris Falcon 4 / CryoSPARC on AWS / ThermoFisherScientific cryoEM collaboration

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Mechanistic insights into a clinical stage PROTAC

**ARV-471:** Induces proximity between CRBN E3 ligase & the estrogen receptor, leading to ER degradation

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- **CRBN** in “closed” conformation
- **DDB1** resolved to 2.7Å
- **ARV-471** not resolved

* ER-LBD + CRBN + DDB1 colored independently, image prepared by ChimeraX / MD fits of ternary complex not shown
Mechanistic & structural basis of substrate-recruitment by KLHDC2
PROTAC discovery – one case study from the Arvinas E3 repertoire

The next frontier is discovering new E3 ligases for TPD – how do we discover them?

E3 ligase with degrader potential → ligand ID → validated E3 ligand → lead-to-PROTAC → active PROTAC → orally bioavailable degrader

E3 ligand-to-PROTAC discovery → novel CRL2^KLHDC2 PROTAC degraders

Discovery & characterization of KLHDC2 ligands for PROTAC applications:

1) Rapid de novo ligand design by CADD & ligand evolution
2) Ligand-to-PROTAC conversion & on-mechanism activity validation
3) Mechanistic & structural understanding of E3 assembly

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KLHDC2 is an active E3 ligase that can be exploited for PROTAC discovery

- KLHDC2 is a CRL2-associated substrate receptor
- KLHDC2 has been shown to recognize C-terminal glycine residues as a high affinity degron
- C-term Gly recognition has been structurally elucidated

In-house validation of KLHDC2 as a C-terminal degron targeting CRL2 E3 ligase using NanoLuc-degron (NLD) fusions

- NanoLuc-substrate [raw luminescence]
- WB:
  - siRNA: scrambled
  - siRNA: KLHDC2
- NanoLuc-SelK-degron
- loading control

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Structure-based, de novo ligand design by CADD & rapid ligand evolution yielded potent and novel KLHDC2 ligands

- Multiple co-crystal structures solved with our CADD-based KLHDC2 ligands
- KLHDC2 ligands extensively occupy and fill the substrate-binding pocket
- Crystal structures allow rational design of an E3-dead analogue; and illuminate multiple exit vectors for PROTAC development
The full-length KLHDC2/EloB/EloC ligase complex is a dynamic oligomer

- apo KLHDC2/EloB/EloC ligase complex is oligomeric
- SelK-peptide-bound KBC complex shifts to a smaller size (as by measured by SEC)

SEC trace of apo & substrate-bound ligase complex

Expanded gels showing all components of the ligase complex
The KLHDC2/EloB/EloC complex is self-regulated.

- The C-terminus of KLHDC2 ends in -GlySer
- The substrate (SelK) peptide ends in -GlyGly
- A possible scenario: loosely held together complex via KLHDC2 C-term is outcompeted by a substrate

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KLHDC2 can bind itself in trans

**KLHDC2 C-term peptides display low affinity to KLHDC2**

**Low affinity C-term KLHDC2 peptides look to partially dissociate the oligomeric KBC complex**

**KLHDC2 C-term co-crystallized with KLHDC2_{\text{KD}}, adopting the conformation of the SelK peptide**
Oligomeric KLHDC2 complex organization is dynamic upon substrate binding, which can be recapitulated by small molecule ligand binding.

KLHDC2 oligomerization altered by high affinity substrate binding

KLHDC2 oligomerization altered by a C-terminal mutant

KLHDC2 oligomerization can also be altered by small molecule ligands

KLHDC2/EloB/EloC complex is a dynamic oligomer

C-terminal KLHDC2 mutant purifies as a monomer

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CryoEM structure of the apo KLHDC2/EloB/EloC complex reveals a tetrameric arrangement, consistent with the model.
CryoEM structure of the complex supports oligomerization mediated by C-terminus

- 4 individual KLHDC2/EloB/EloC complexes have good density & can be visualized in the final complex
- Focusing on one KBC reveals an extended C-terminus of KLHDC2
KLHDC2 targeting small molecules alter oligomeric assembly of KBC

KLHDC2 oligomerization can also be altered by high affinity small molecule ligands

Continuing to look at assembly of:
- KBC bound to substrate-peptides
- KBC bound to small molecules
- KBC bound to PROTACs & PROTAC-POI complexes
- KBC bound to full CRL2 complex -/+ substrates/compds

→ understanding these offers insight into PROTACs
PROTACs based on KLHDC2 ligands ubiquitylate target proteins

Using purified, full-length KLHDC2/EloB/EloC complex in cell-free, biochemical ubiquitination assays, PROTACs ubiquitylate a target in an KLHDC2-recruitment-dependent manner.

**PROTAC 1**
- [active]

**PROTAC 1**
- [E3-dead]

**PROTAC 4**
- [active]

- + ATP
- + Ub
- + E1
- + E2
- + CRL2
- + KLHDC2/EloB/EloC
- + PROTACs

Poly-ubiquitylated \((\text{Ub}^n)\)

POI target

POI target

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KLHDC2-based PROTAC optimization using JQ1 yields potent pan-BET degraders

- Our novel KLHDC2-based BET-family PROTACs are:
  - robust → greater than 90% $D_{\text{max}}$
  - potent → DC$_{50}$ in the low nM range
  - on-mechanism → sensitive to KLHDC2 siRNA

HiBiT degradation assay for BRD4

WB for endogenous BRD2

Immunoblot assay for BRD2

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PROTAC-able E3 ligase is now structurally and functionally enabled for TPD

- This E3 ligase can degrade target proteins using our PROTAC technology.
- PROTAC design is enabled by the quaternary structure of this E3 in its full-length, wild-type form.
- Extensive optimization of the protein complex and freezing conditions on the Vitrobot did not permit high-resolution structural determination.
- Freezing on the chameleon with optimized protein complex allowed high-resolution structural determination.
- We are excited to pursue more high-throughput, streamlined, cryoEM structural determination with the in-house chameleon instrument.

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Acknowledgements – the entire Arvinas Team (now 400+!)

Thank you!