



Rational Discovery of a Small Molecule Intramolecular Glue Inhibitor of CBL-B that Enhances T-cell Function

Stefan Gajewski

Discovery on Target 2024

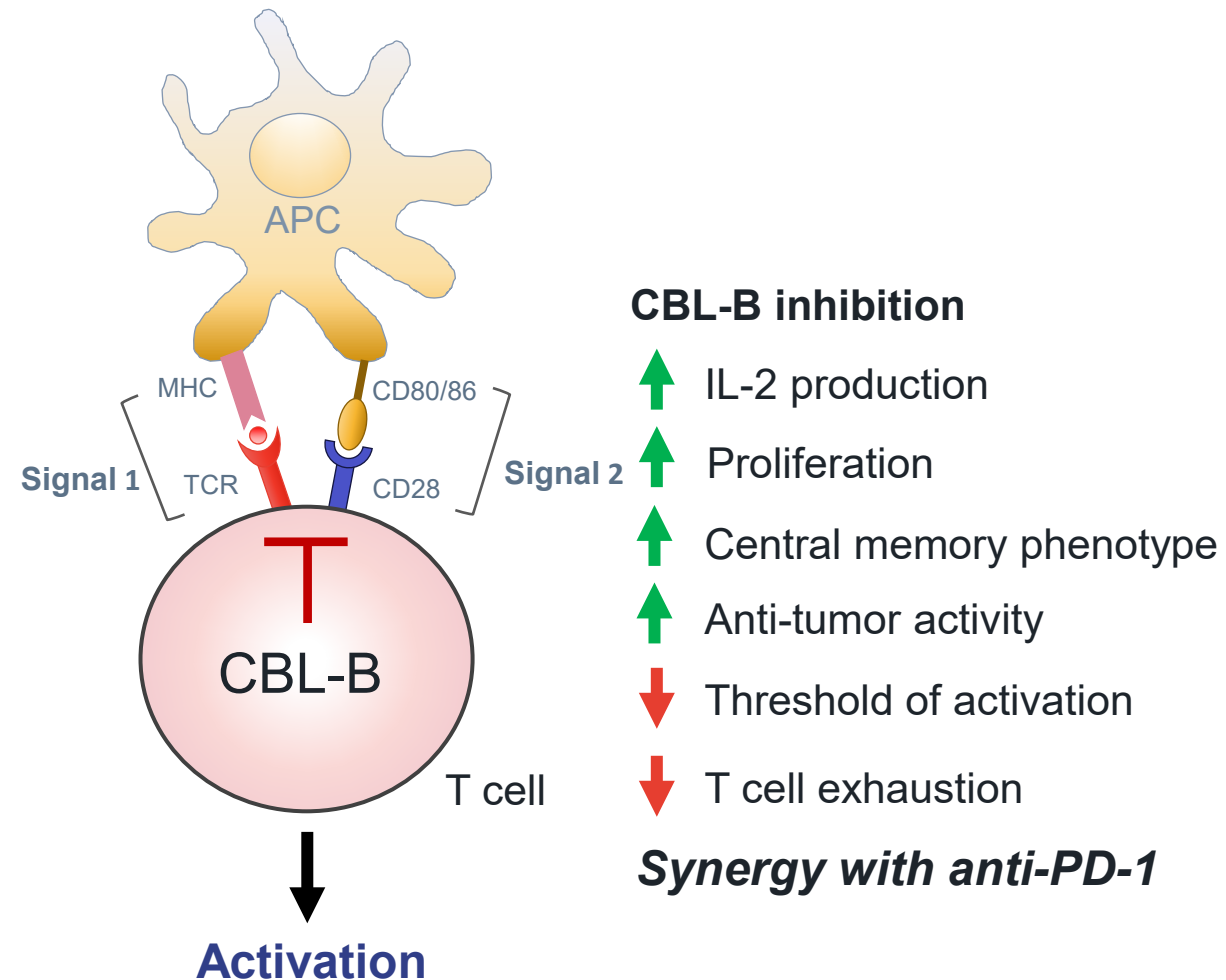
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CBL-B Is a Modulator of Immune Cell Activation

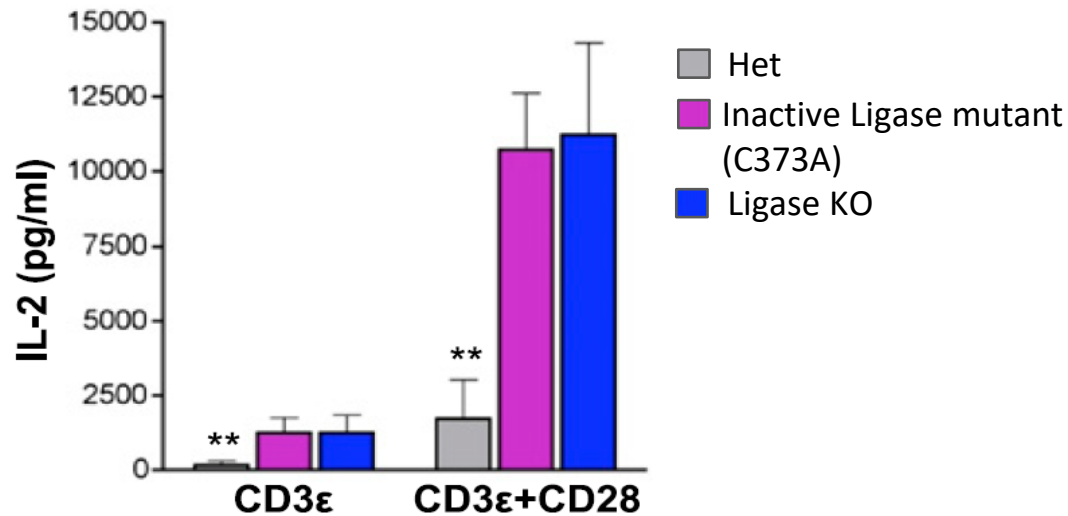
- CBL-B is an E3 ubiquitin ligase highly expressed in cells of the immune system
- CBL-B regulates T, B, and NK cell activation
- Blocking CBL-B removes a brake on the immune system
- CBL-B deficient mice demonstrate robust T cell and NK cell-mediated antitumor immunity



CBL-B Is a Modulator of Immune Cell Activation

Inactivation or deletion of CBL-B results in hyperactive T cells and inhibition of tumor growth.

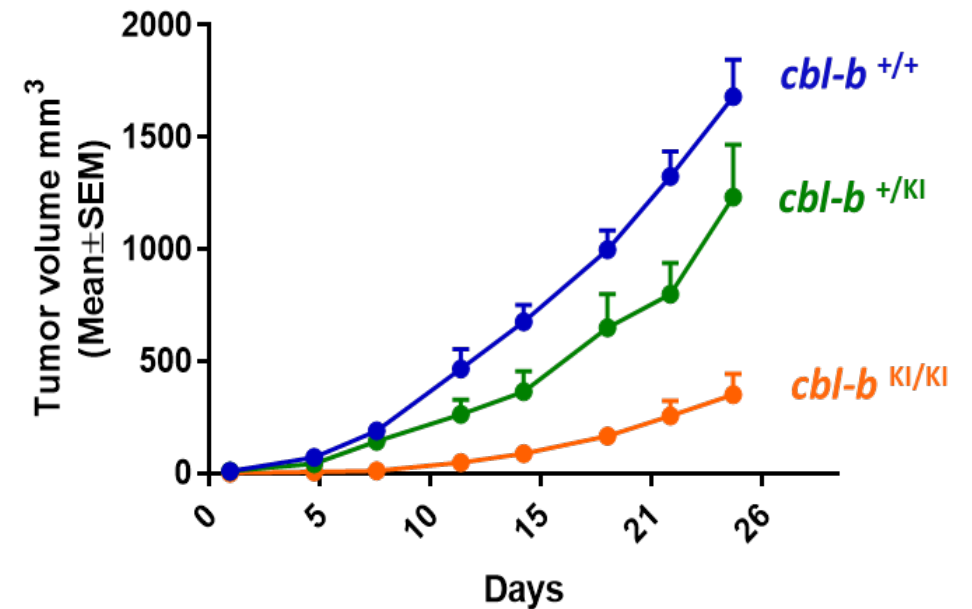
IL-2 secretion in KO and ligase inactive T cells *ex vivo*



Paolino et. al. *J. Immunology*, 2011

Ligase-inactive or KO exhibit enhanced and equivalent response to either single- or double stimulation

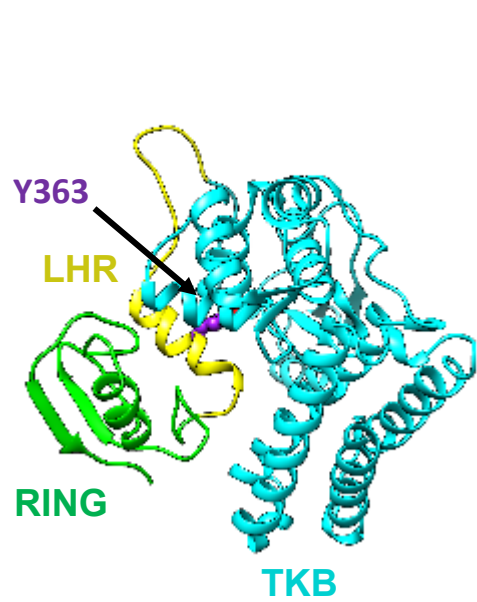
Ligase-inactive CBL-B knock-in mice inhibit tumor growth (TC-1 syngeneic model).



Unpublished Nurix Data

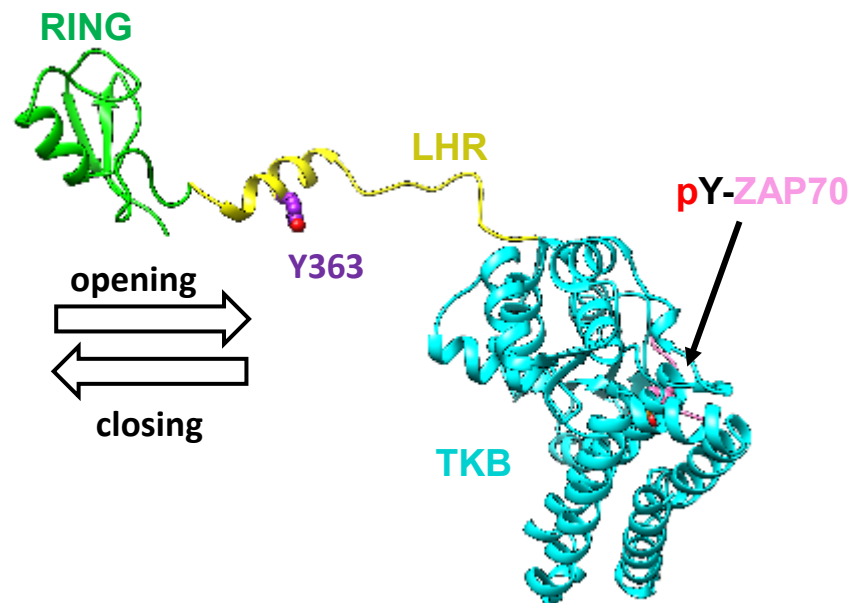
Active CBL-B Binds Ub-loaded E2 Ligases

“closed-state”
(inactive, autoinhibited)



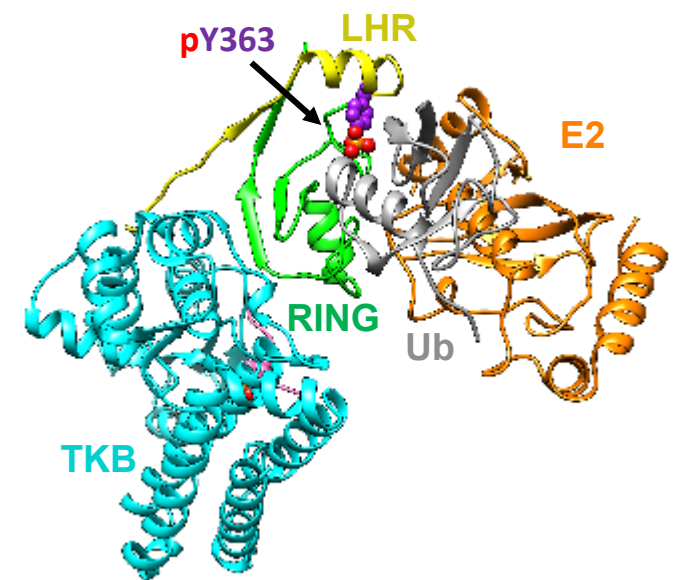
(CBL-B – Nurix,
unpublished)

“open-state”
(inactive, Y363 accessible)

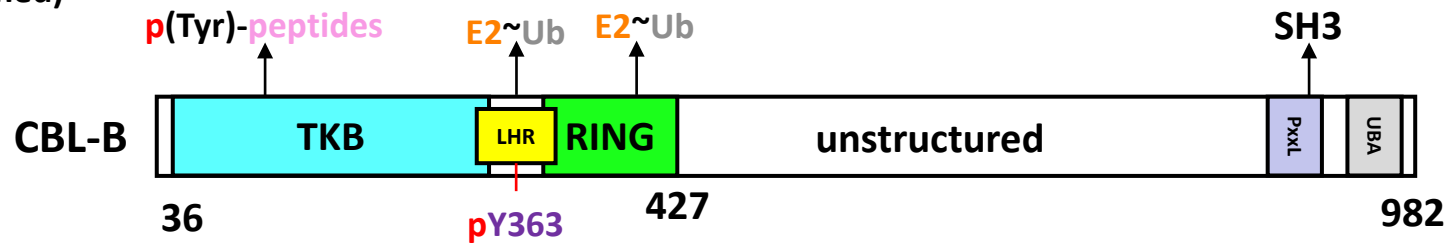


(Model)

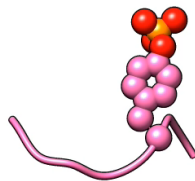
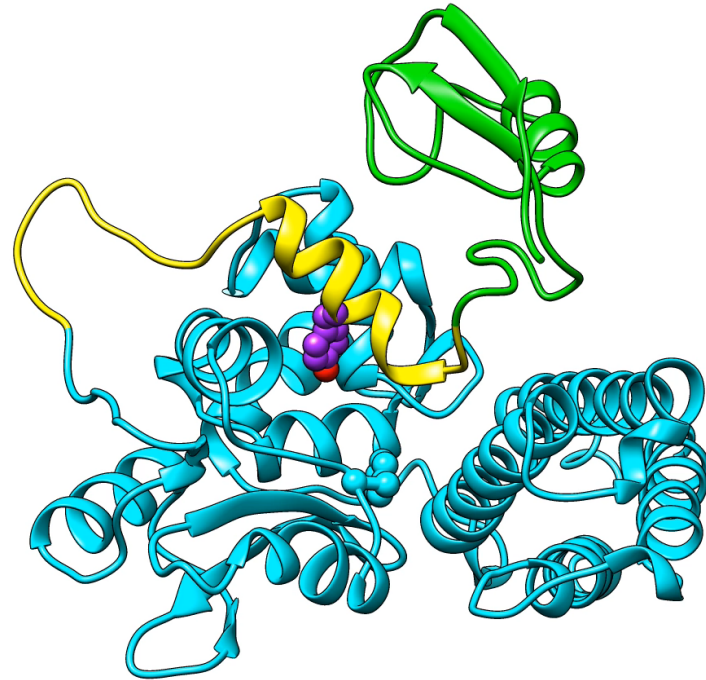
“active-state”
(active, pY363 binds E2-Ub)



(PDB: 3ZNI)



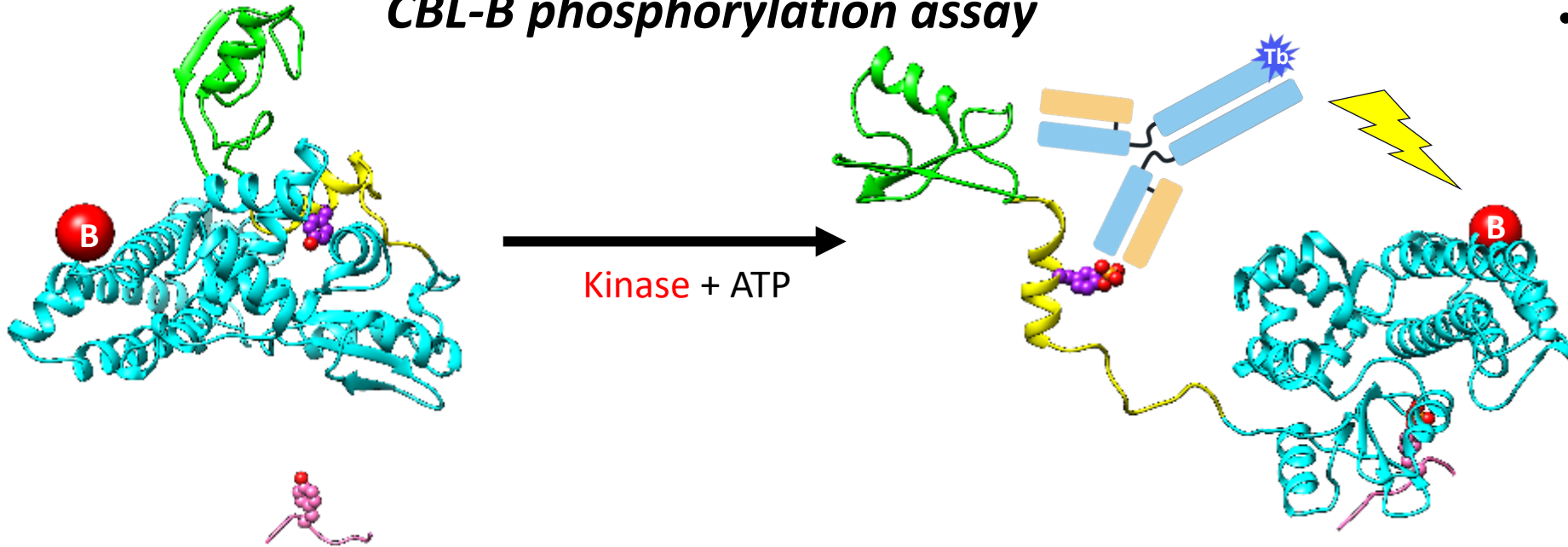
- Shahab U *et.al.*, Biochem. Biophys Res Commun (1996)
- Schmidt M and Dikic I, Nat Rev Mol Cell Biol (2005)
- Kobashigawa Y *et.al.*, Proc Natl Acad Sci U S A (2011)
- Dou H *et.al.*, Nat Struct Mol Biol (2013)



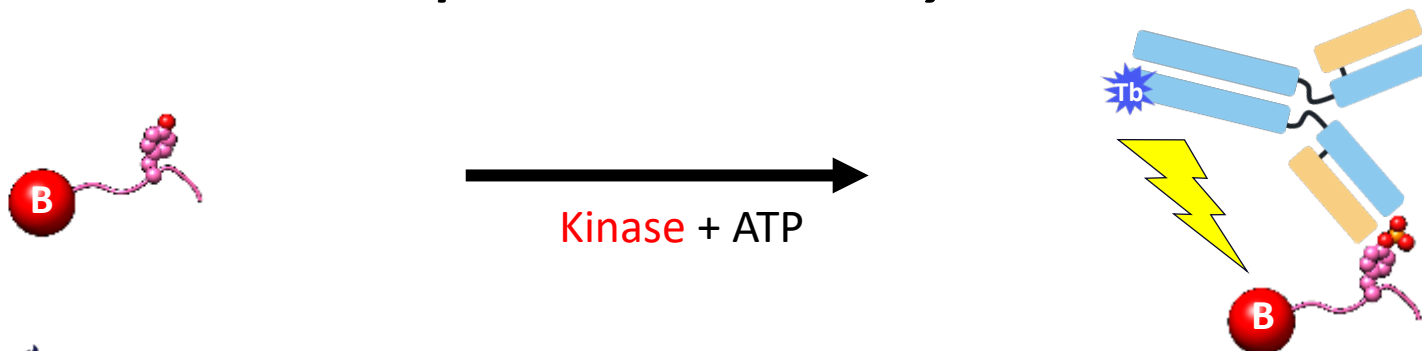
Dynamics of CBL-B activation by a Tyrosine phosphorylated ZAP70 peptide

CBL-B Phosphorylation Assay (“Closed State”)

CBL-B phosphorylation assay

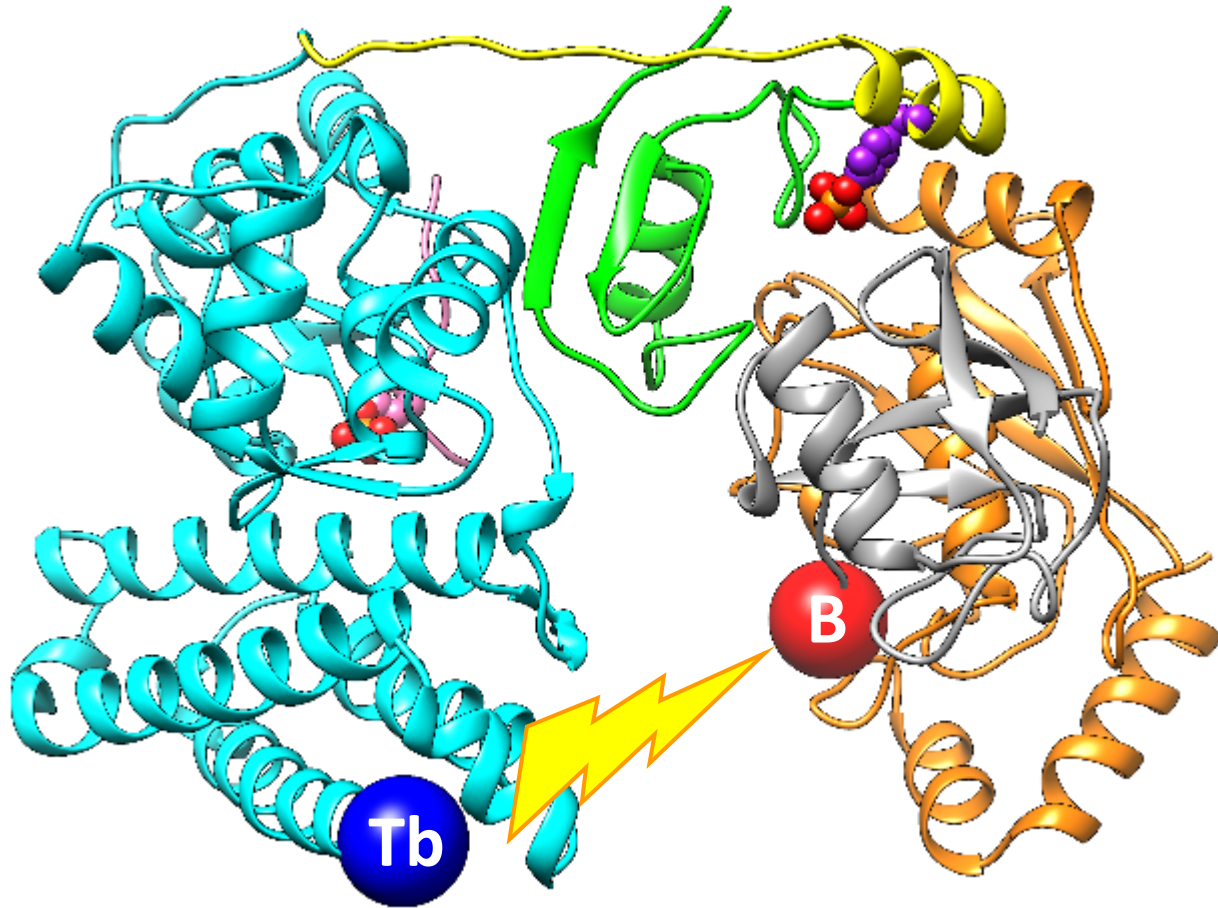


Peptide counter assay



- FRET based pY(363)-CBL-B phosphorylation assay:
 - CBL-B N-terminal labeled with Bodipy
 - Terbium labeled α pY mAb
 - ZAP70 peptide for CBL-B open state induction
 - Src kinase and ATP to start the reaction
- Counter screen with Bodipy labeled ZAP70 peptide

E2~Ub Binding Assay (“Open State”)

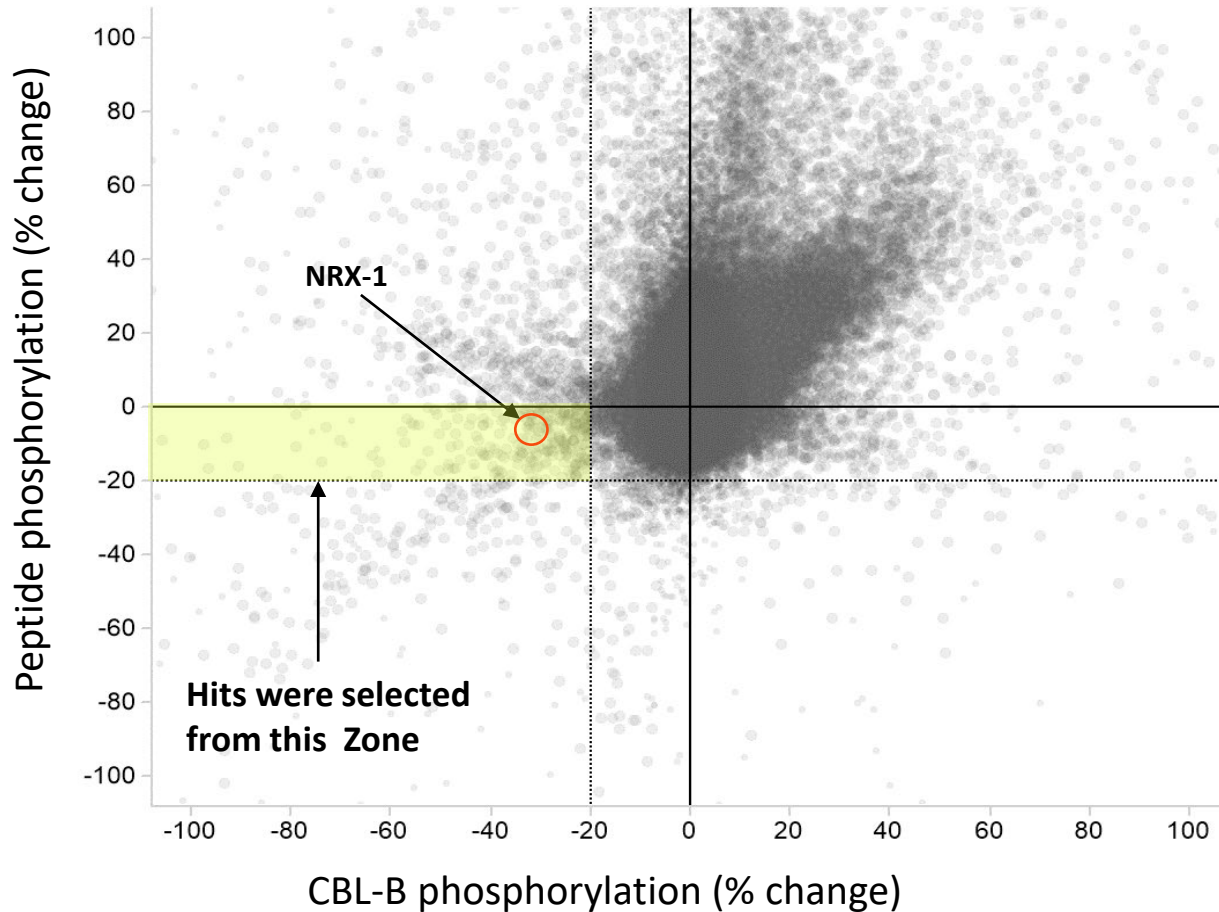


- FRET based E2~Ub binding assay to **pY(363)-CBL-B**:
 - CBL-B N-terminal labeled with biotin and streptavidin-terbium
 - E2~Ub conjugate C-terminal labeled with Bodipy

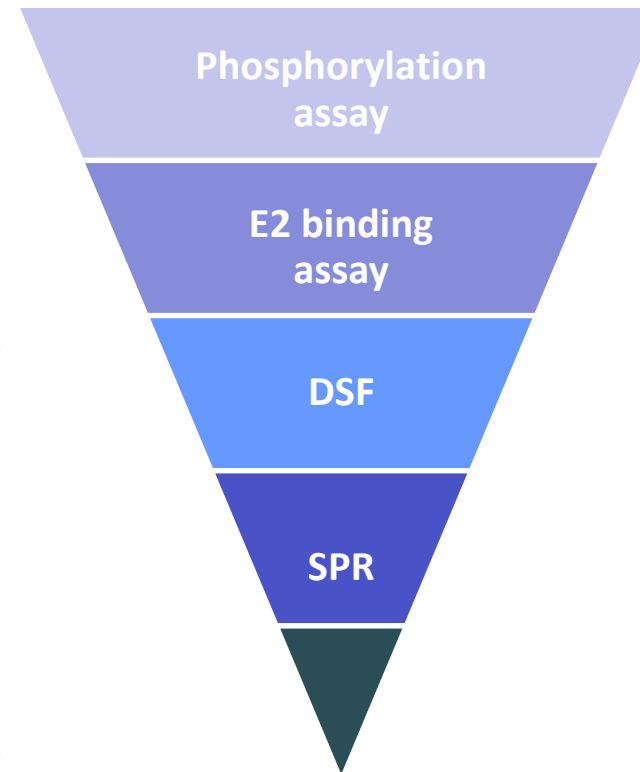
Loss of FRET signal indicates:

- 1) PPI inhibition or
- 2) closed state glue

Discovery of CBL-B Inhibitors via Open State HTS



Test Funnel



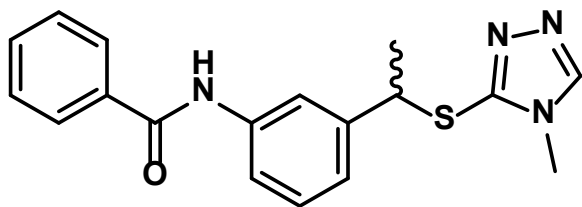
104 hits selected from phosphorylation HTS

~90% "confirmed" from crude material

14 positive in titration assays after re-purification

1 compound validated in SPR and crystallography

HTS Reveals a Singleton Hit

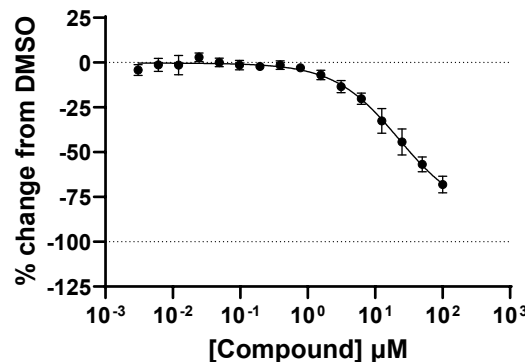


NRX-1
racemic

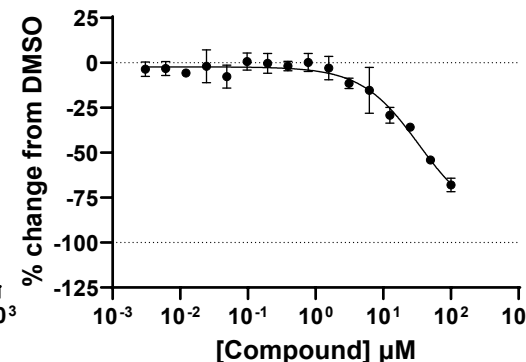
mwt = 338
K_{sol} 280 μM
cLogP 3.46
PSA 60

Phospho: IC₅₀ = 39 μM
E2~Ub: IC₅₀ = 60 μM
SPR: K_D = 37 μM

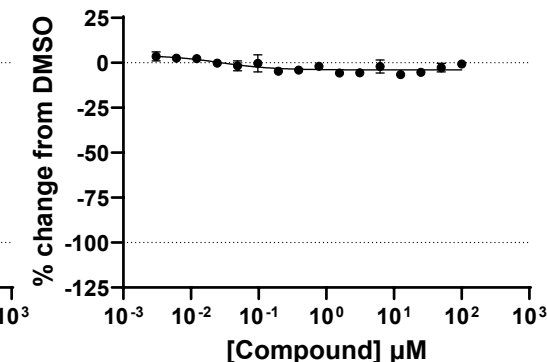
CBL-B phosphorylation



E2~Ub binding

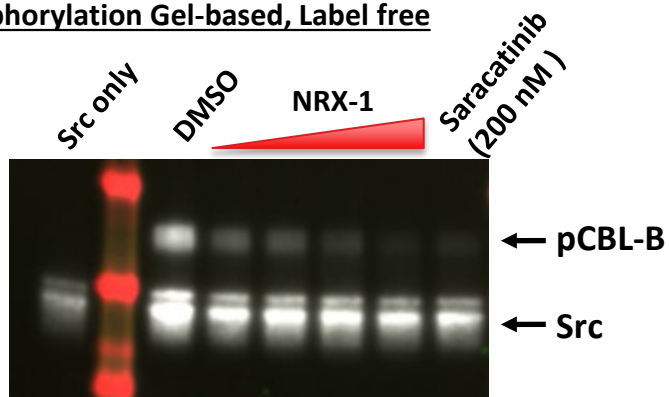


Peptide counter screen



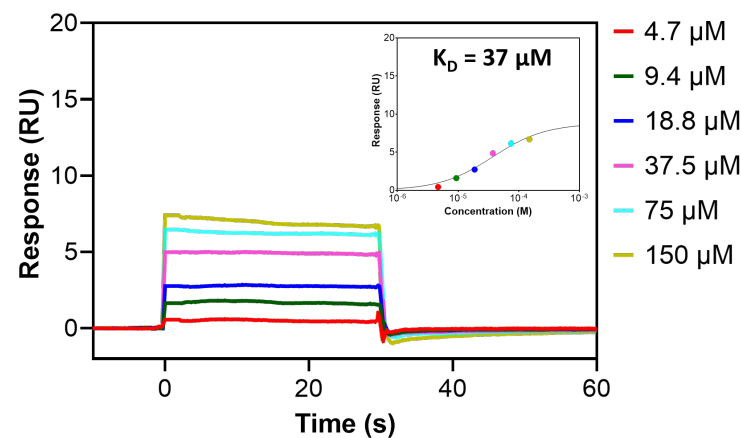
E2 binding assay and peptide counter assay to examine Src activity indicate that **NRX-1** is a CBL-B inhibitor

CBL-B Phosphorylation Gel-based, Label free

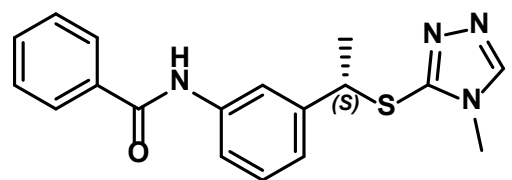


Compound titration (μM): 6.25 12.5, 25, 50, 100

Compound Binding to CBL-B by SPR



NRX-2 Is a Specific Inhibitor of CBL-B



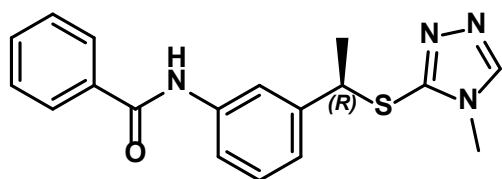
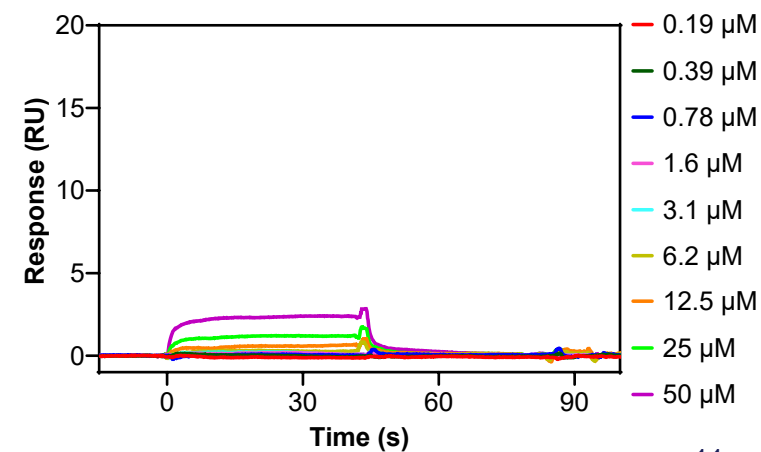
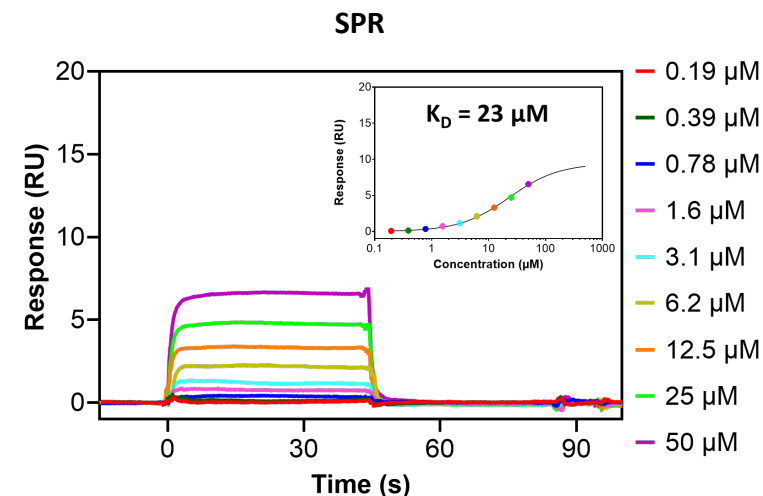
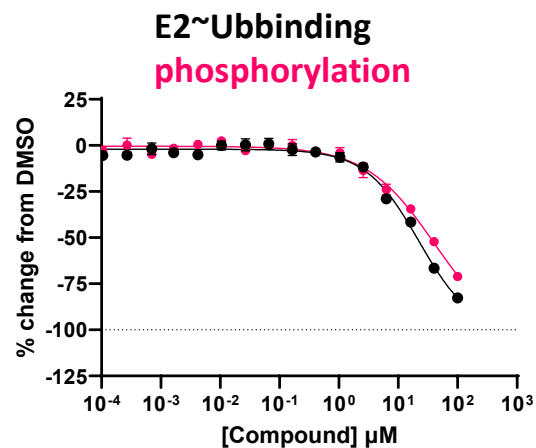
NRX-2

eutomer

Phospho: $IC_{50} = 34 \mu M$

E2~Ub: $IC_{50} = 21 \mu M$

SPR: $K_D = 23 \mu M$



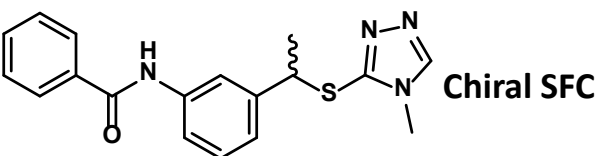
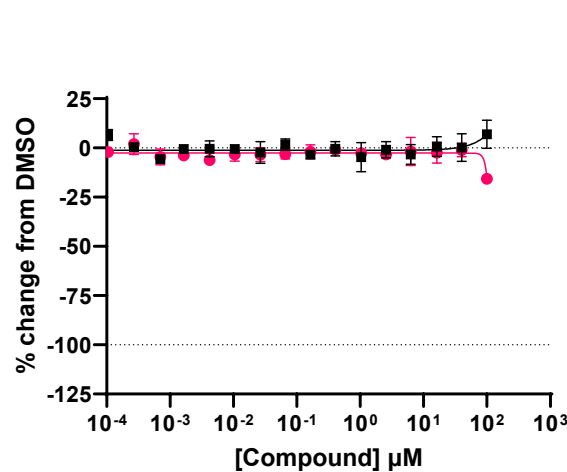
NRX-3

distomer

Phospho: $IC_{50} = n/a$

E2~Ub: $IC_{50} = n/a$

SPR: $K_D = n/a$



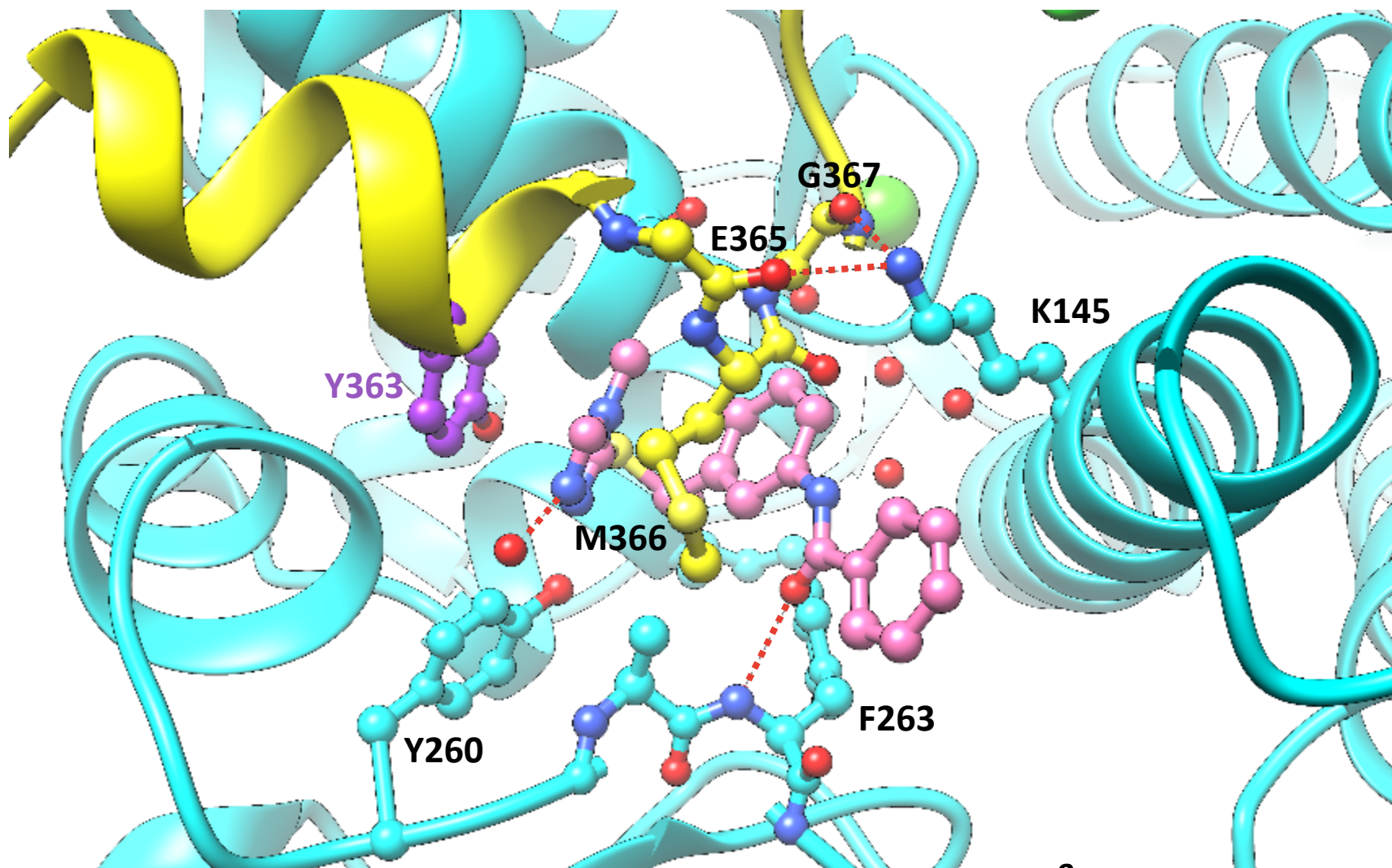
NRX-1

HTS Screening hit
(racemic)

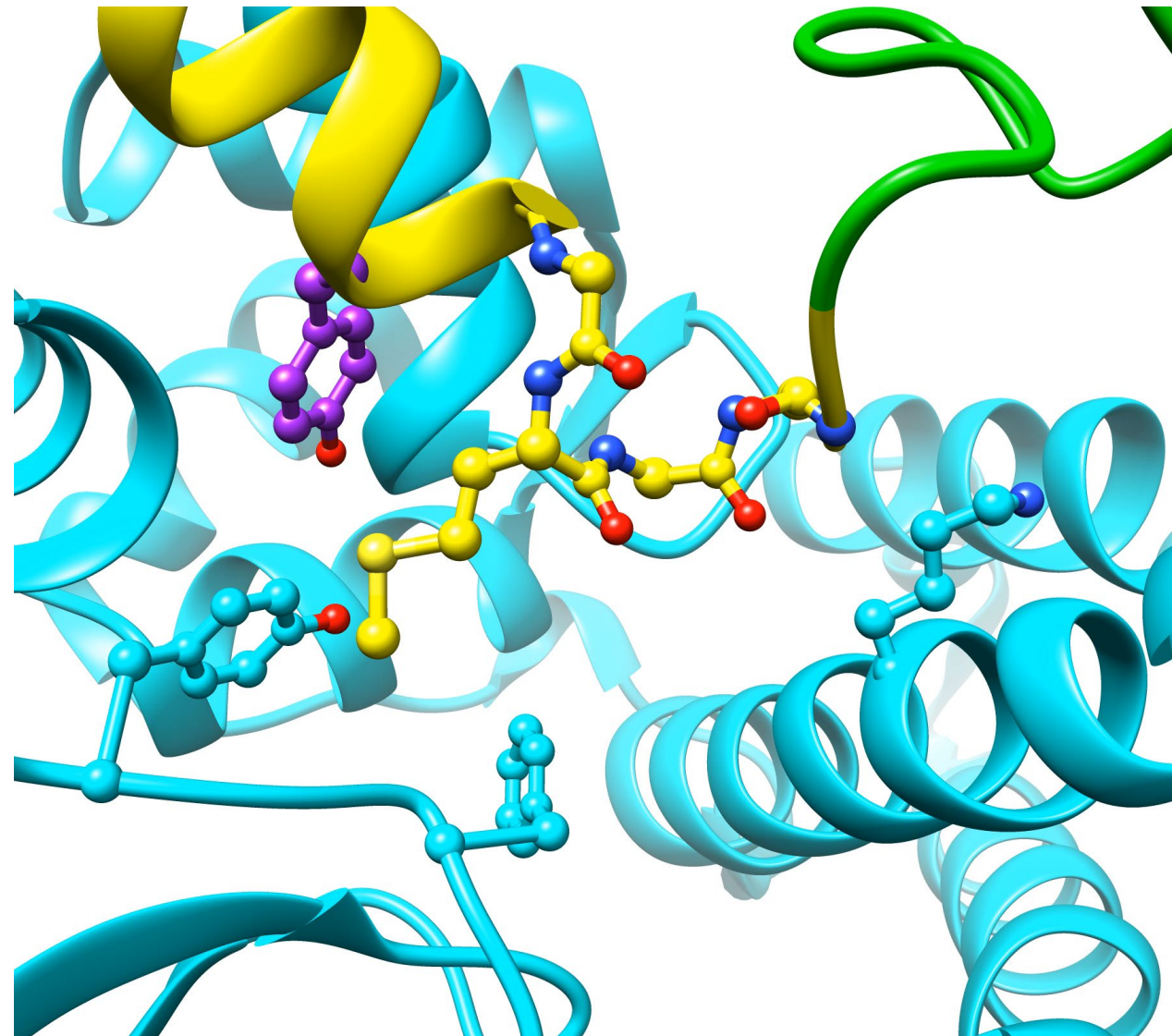
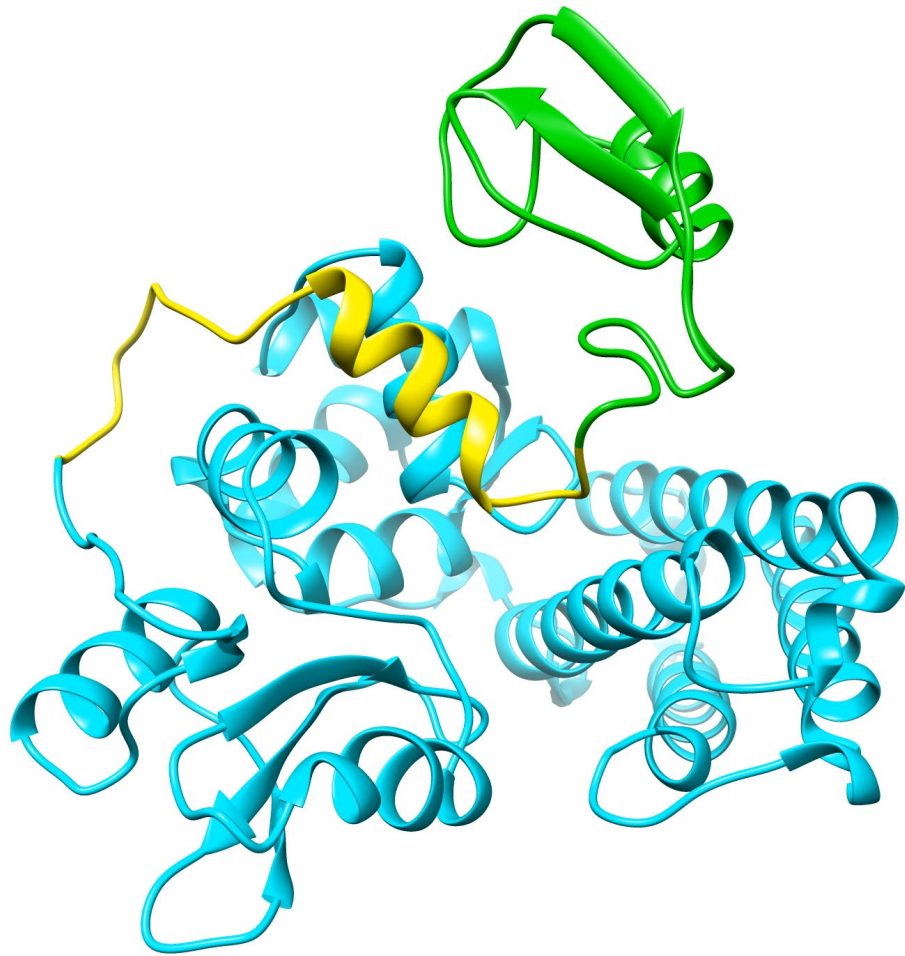


Chiral SFC

Binding Pose of NRX-2 CBL-B Inhibitor in X-ray Crystal Structure

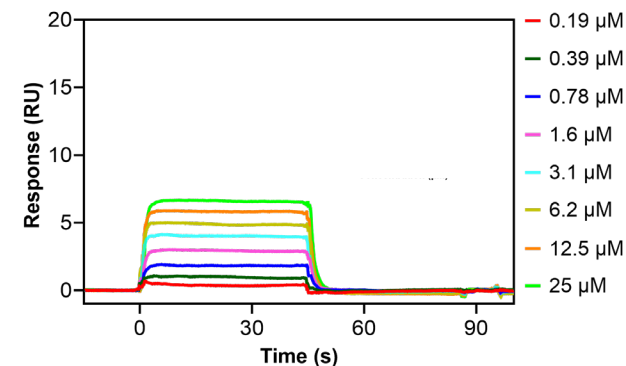
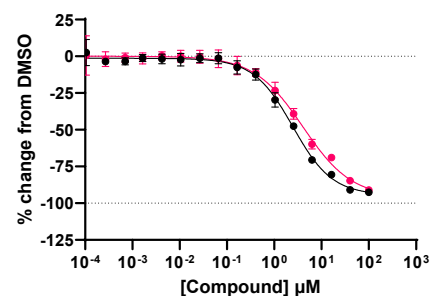
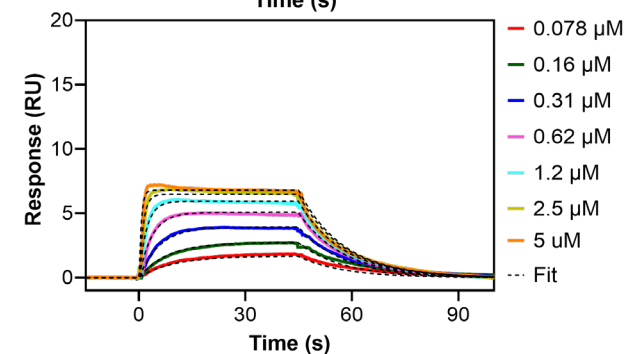
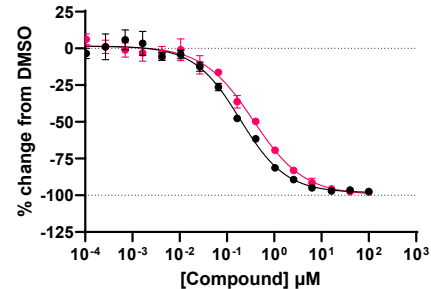
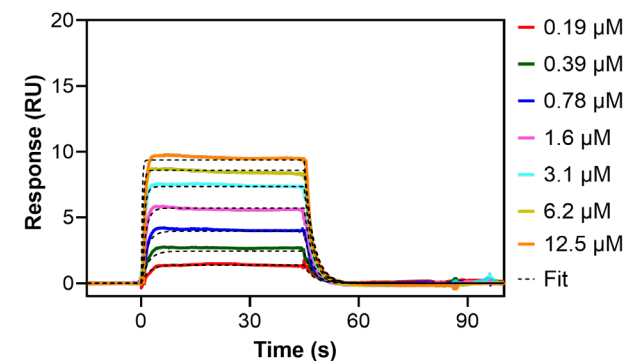
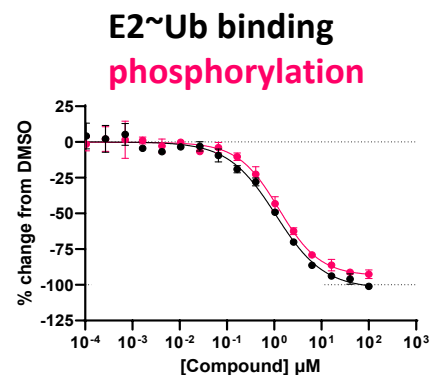
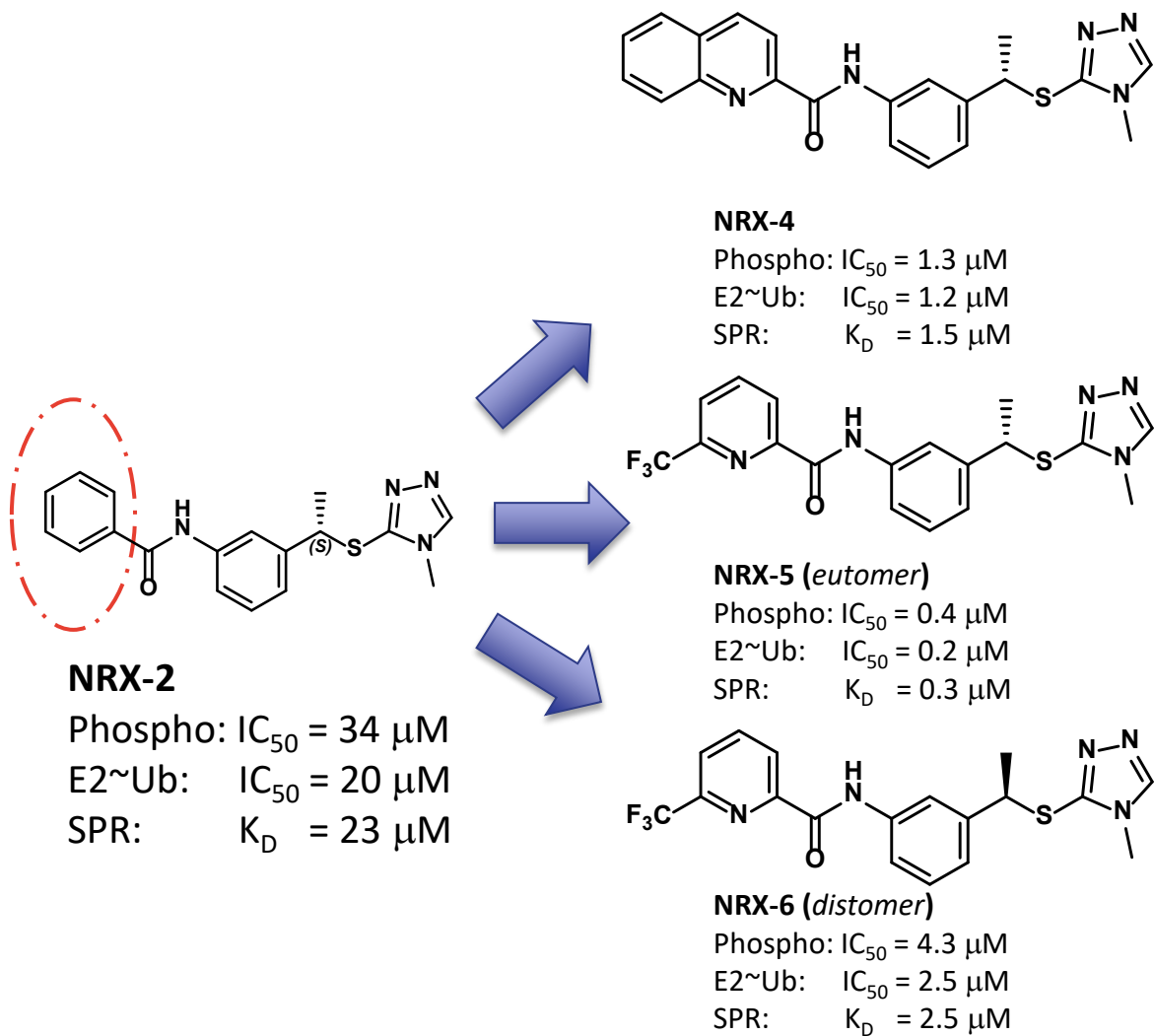


2Å resolution

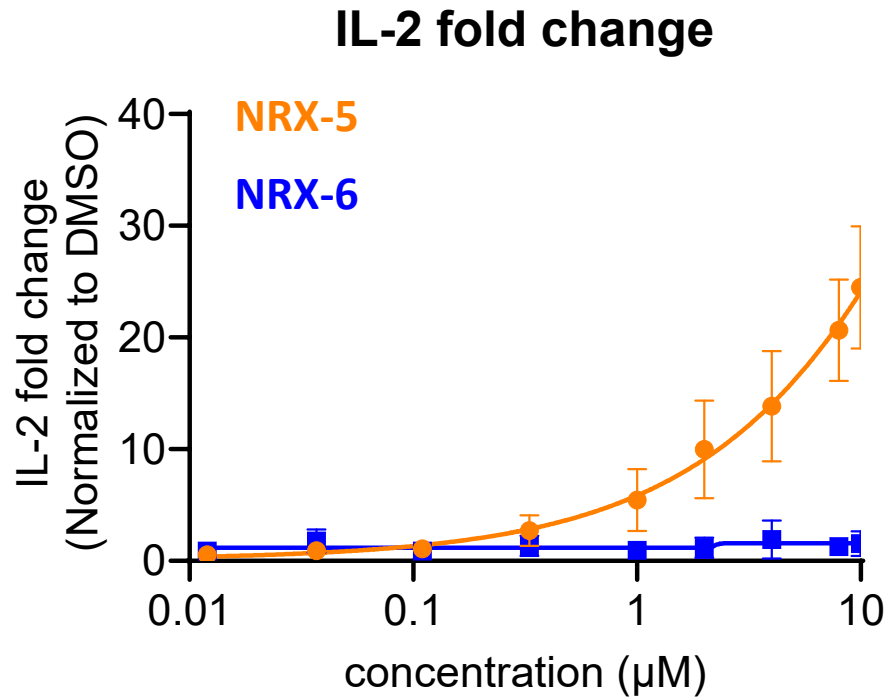


- SH3 moves away from LHR upon NRX-2 binding
- RING domain swings into a new position without perturbing LHR
- K145 stabilizes LHR conformation with a niche-3 contact

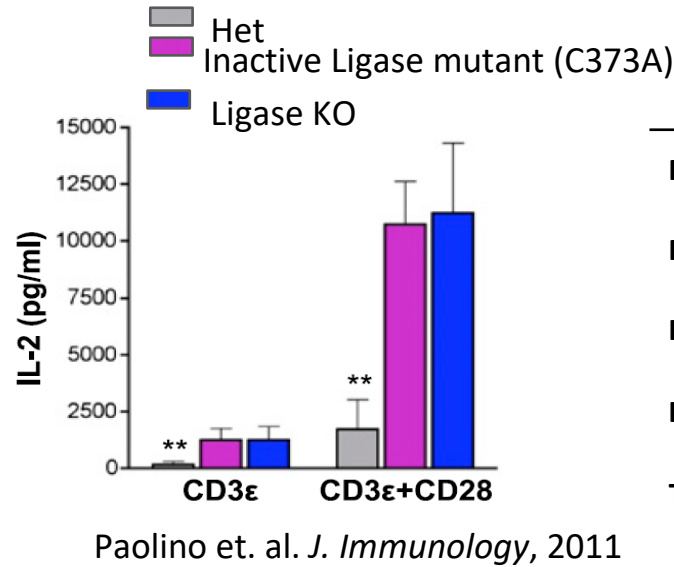
Early SAR Evaluation Led to Identification of Tool Compound NRX-5



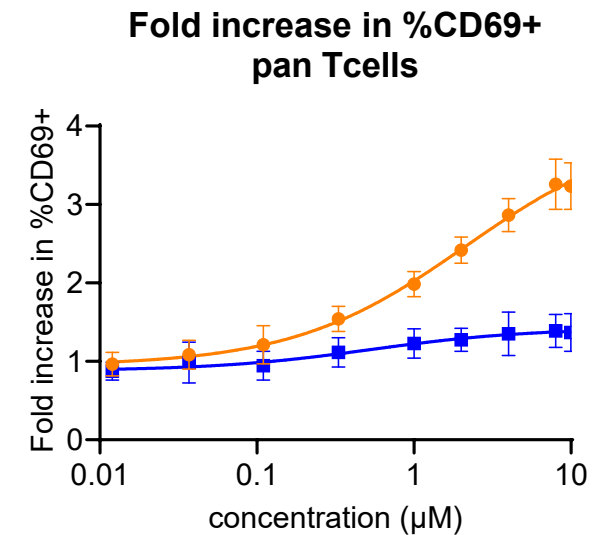
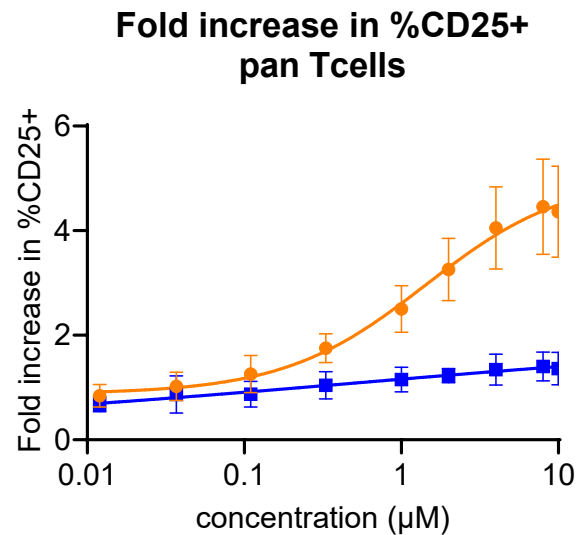
NRX-5 Stimulates Immunogenicity in PBMCs



PBMCs were incubated with compounds 1h before stimulation via CD3 and CD28



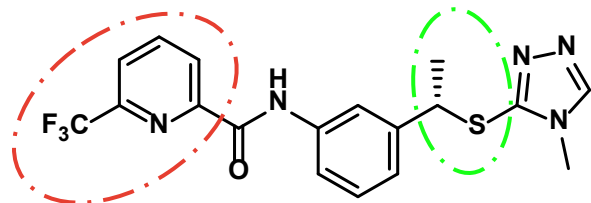
	EC50 (µM)	Emax
Fold increase in %CD69+	2.1	≥ 4.0
Fold increase in %CD25+	1.4	≥ 5.1
IFN-γ fold change	0.3	≥ 4.7
IL-2 fold change	N/D	≥ 24.5
TNF-α fold change	0.2	≥ 3.1



Further Optimization of NRX-5

Headgroup modifications

Spacer modifications

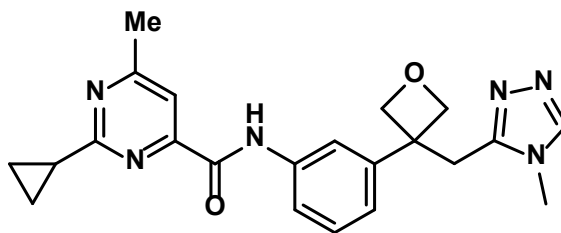


NRX-5

Phospho: $IC_{50} = 0.4 \mu M$

E2~Ub: $IC_{50} = 0.2 \mu M$

SPR: $K_D = 0.3 \mu M$

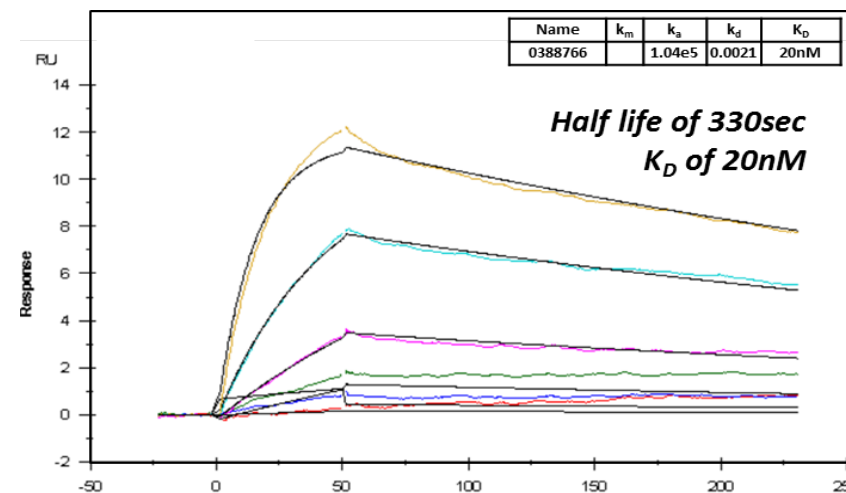
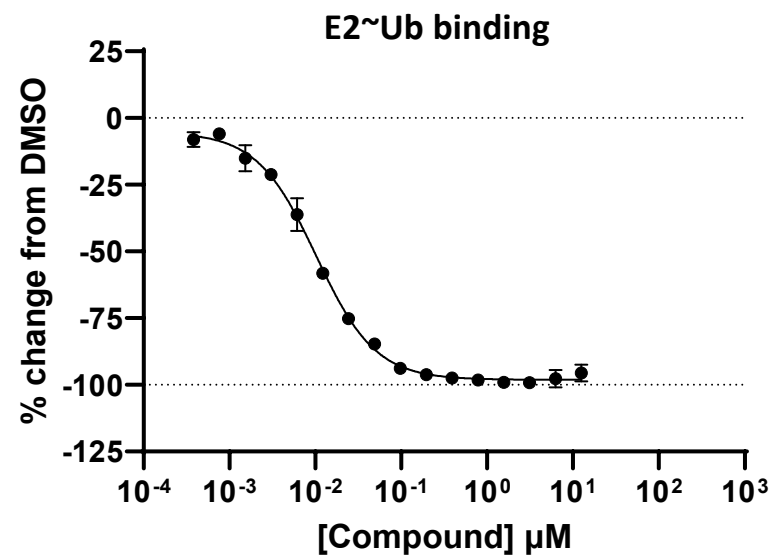


NRX-7

Phospho: $IC_{50} = n/a$

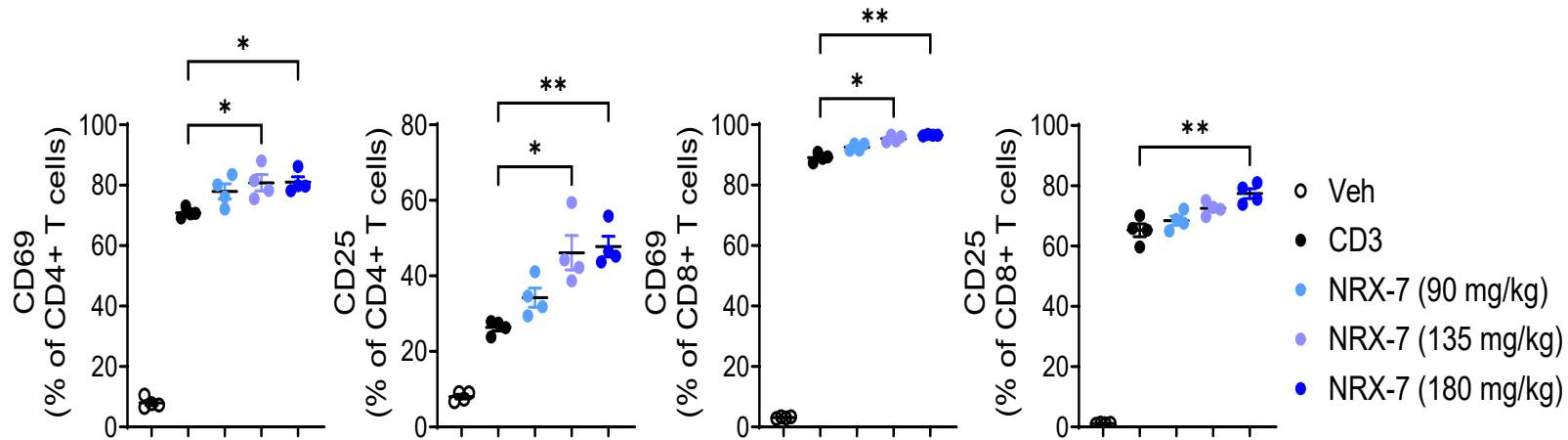
E2~Ub: $IC_{50} = 20 nM$

SPR: $K_D = 20 nM$

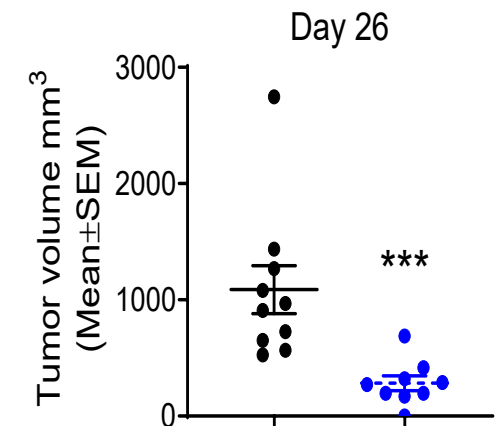
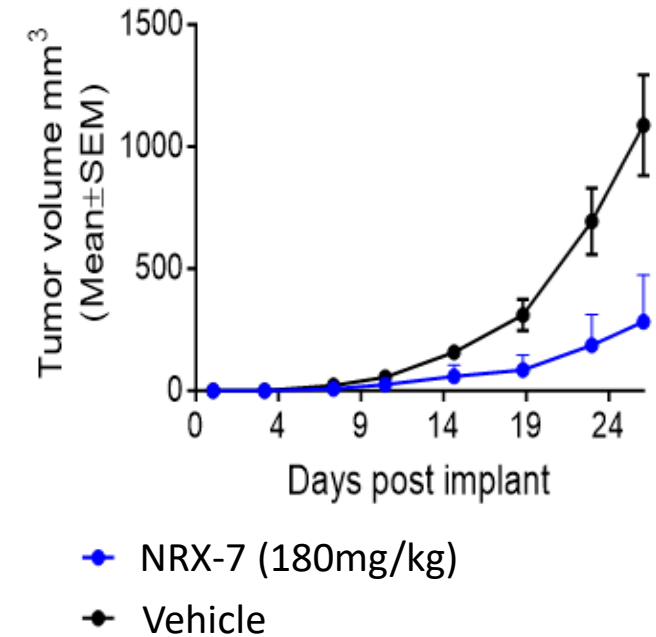


NRX-7 Enhances in vivo T-Cell Stimulation and Anti-Tumor Efficacy

Increase of CD69 and CD25 in CD4+ / CD8+ T cells 24 after CD3 stimulation – NRX-7 was administered orally before CD3 injection



Immunocompetent mice bearing CT26 tumors were treated orally with NRX-7 starting day 3



Conclusions

- CBL-B is a well-characterized E3 ligase that functions to modulate immune cell activity
- We identified the autoinhibited state of CBL-B as the relevant target form for drug discovery and developed two orthogonal assays monitoring the sequential activation of this target protein
- A singleton hit with low potency was discovered and validated with orthogonal assays, SPR and crystallography
- The hit compound was confirmed to be an intramolecular glue, stabilizing the closed, inactive state of the ligase
- Basic modifications of the hit compound allowed for validation of CBL-B inhibition in cell culture, and further optimization of the series led to identification of an early tool compound with in vivo efficacy
- Nurix's clinical compound NX-1607 is currently in Phase 1 (NCT05107674) for immune oncology indications

Thank You !

