# Catalytic *in vivo* protein knockdown

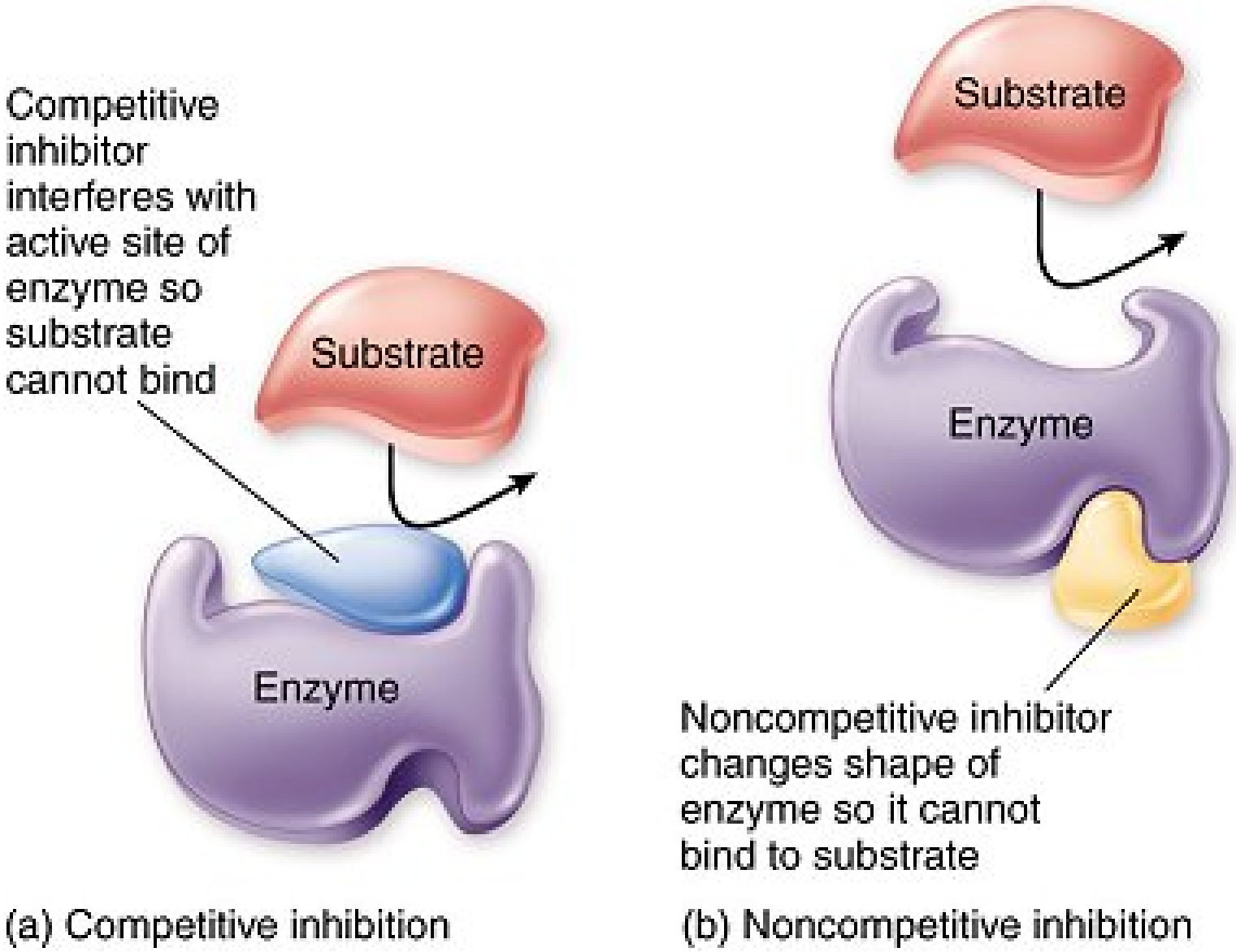
**Technical Journal Club**

Mario Nuvolone

November 15th 2016

University Hospital Zurich
Institute of Neuropathology

# Current inhibitory-based pharmacologic approach



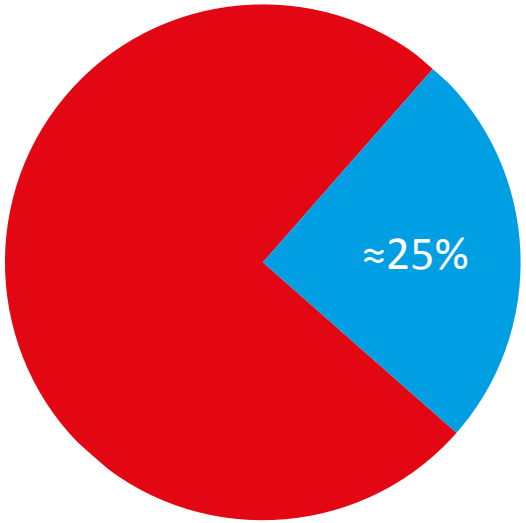

www.tokresource.com

# Limitations of inhibitory-based pharmacologic approach

1) Limited to proteins with tractable ligand binding site

→ Limited drug space (≈25% of human proteome)

**UNDRUGGABLE:**
Transcription factors
Scaffolding proteins
Other non-enzymatic proteins




**DRUGGABLE:**
Receptors
Enzymes

2) Need to maintain high systemic exposure for high *in vivo* inhibition

3) Potential off-target binding and unwanted side effects

Toure & Crews. Angew Chem Int Ed 2016
Ohoka et al. Curr Cancer Drug Targets 2016

**Alternatives approaches**

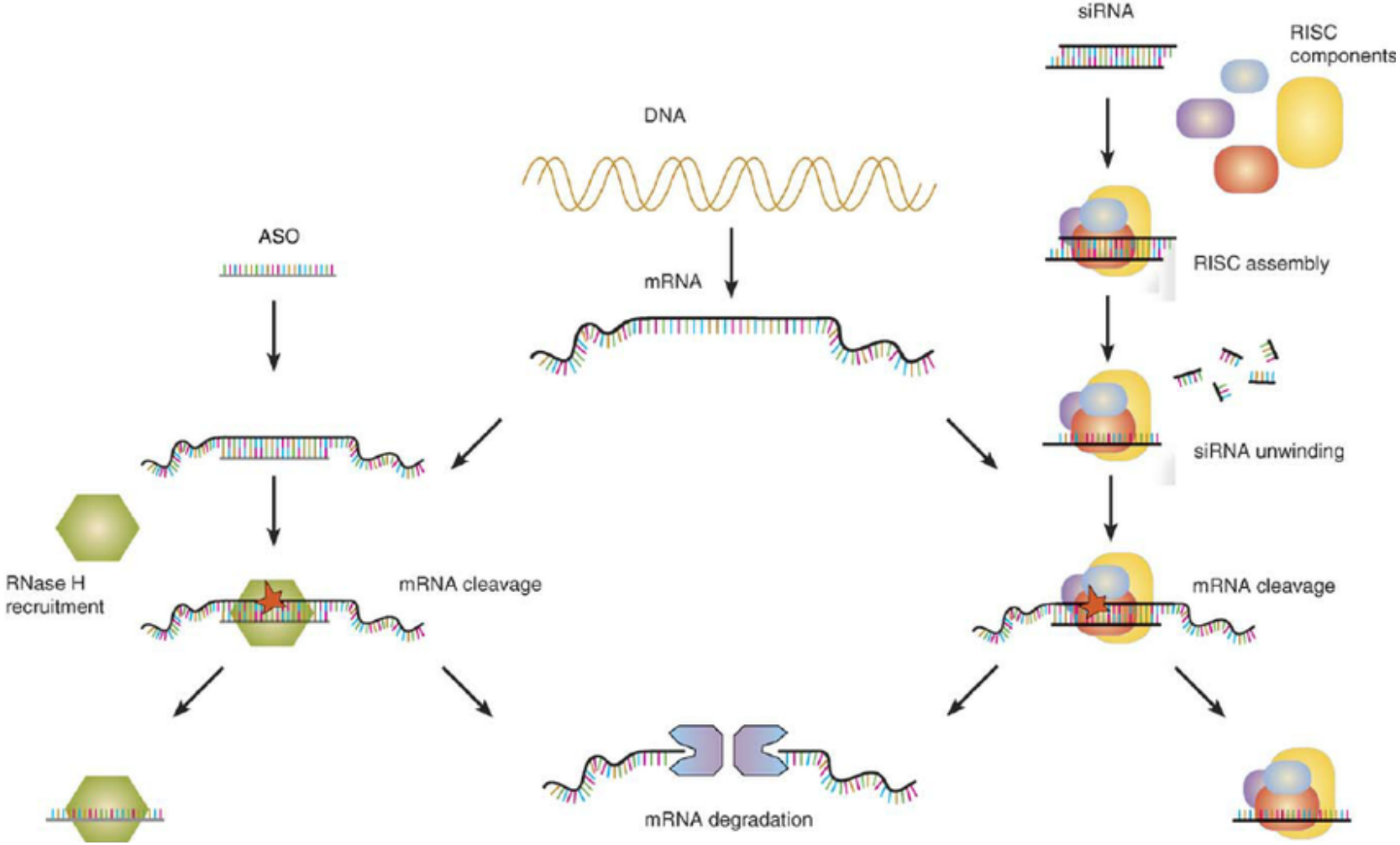
1) **Gene knockdown**

2) **Protein knockdown**

# Alternatives approaches I: Gene knockdown

1) Based on anti-sense oligonucleotides (ASOs) or RNA interference (RNAi)

2) Aims at decreasing translation of transcripts from disease-causing genes




Wacheck & Zangemeister-Wittke. Crit Rev Hematol Oncol 2006

# Limitations of Gene knockdown

1) Pharmacokinetics profile:

   - Limited stability of double- stranded RNA for RNAi

   - ASOs preferentially accumulate in the liver

2) Off-target issues

3) Requires time to achieve efficient knockdown
   (especially for target proteins with long half-life)

→ Limited therapeutic applications

Toure & Crews. Angew Chem Int Ed 2016
Ohoka et al. Curr Cancer Drug Targets 2016

Protein knockdown

1) PROTACs

2) Phtalimide conjugation

# Alternatives approaches II: PROTAC-based Protein knockdown

**PROTAC: PRO**teolysis **TA**rgeting **C**himeras

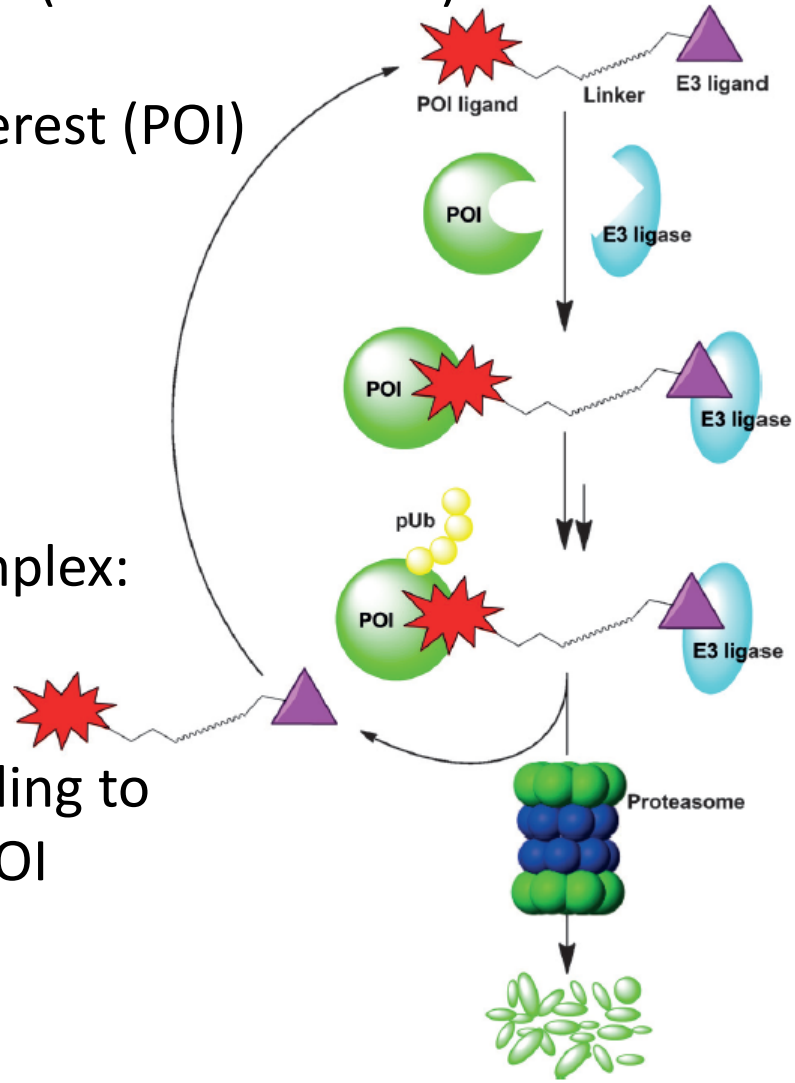
1) Based on heterobifunctional compounds (named PROTAC) with:

- Ligand specific to the Protein Of Interest (POI)
- Linker
- Ligand specific to an E3 ligase

2) Favours the formation of a ternary complex:

**POI – PROTAC – E3**

3) E3 ligase poly-ubiquitilates the POI, leading to proteasome-mediated degradation of POI



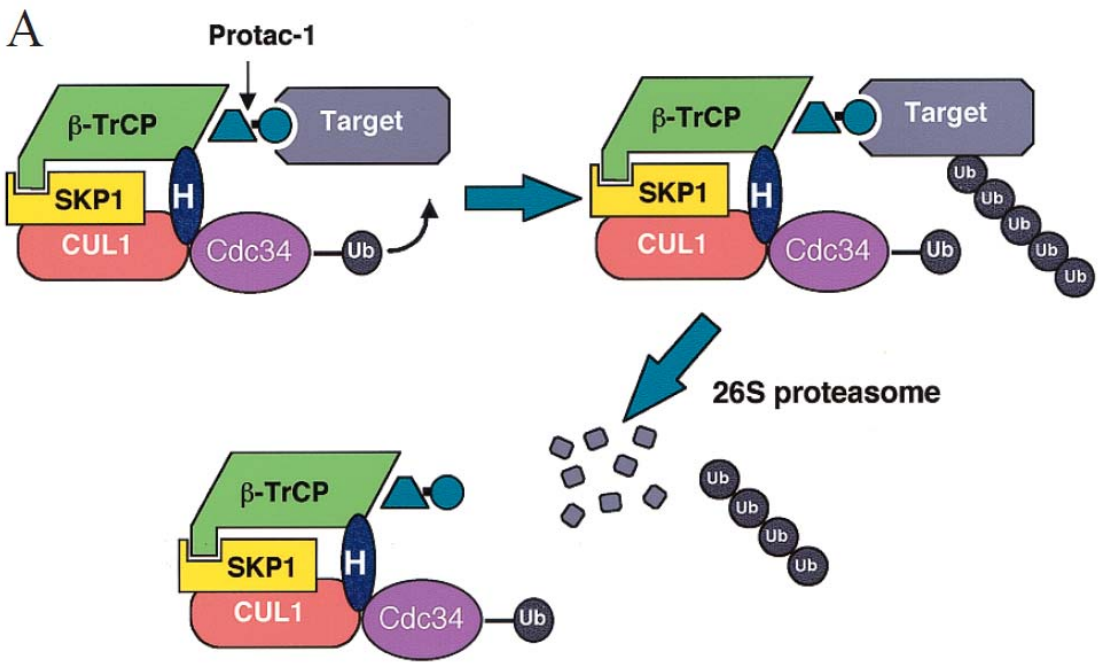

Toure & Crews. Angew Chem Int Ed 2016
Ohoka et al. Curr Cancer Drug Targets 2016

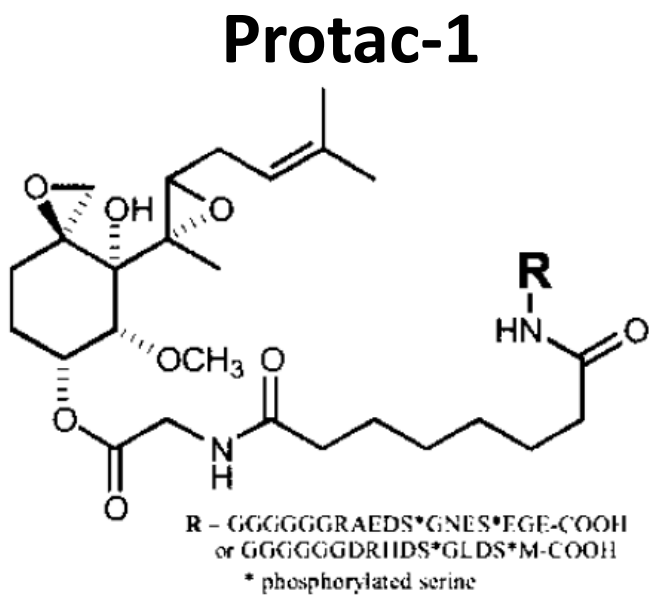
8554–8559 | PNAS | July 17, 2001 | vol. 98 | no. 15

# Protacs: Chimeric molecules that target proteins to the Skp1–Cullin–F box complex for ubiquitination and degradation

Kathleen M. Sakamoto*[†‡], Kyung B. Kim[§], Akiko Kumagai[†], Frank Mercurio[¶], Craig M. Crews[§], and Raymond J. Deshaies[†‡‖]

*Department of Pediatrics and Pathology, Mattel Children's Hospital at University of California Los Angeles, University of California Los Angeles School of Medicine, Gwynn Hazen Cherry Memorial Laboratories, and Jonsson Comprehensive Cancer Center, Los Angeles, CA 90095-1752; [†]Division of Biology, and [‖]Howard Hughes Medical Institute, California Institute of Technology, Pasadena, CA 91125; [§]Department of Molecular, Cellular, and Developmental Biology, Yale University, New Haven, CT 06520; and [¶]Signal Division, Celgene Pharmaceuticals, La Jolla, CA 92121



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**Main result:**

First proof-of-concept for the PROTAC technology

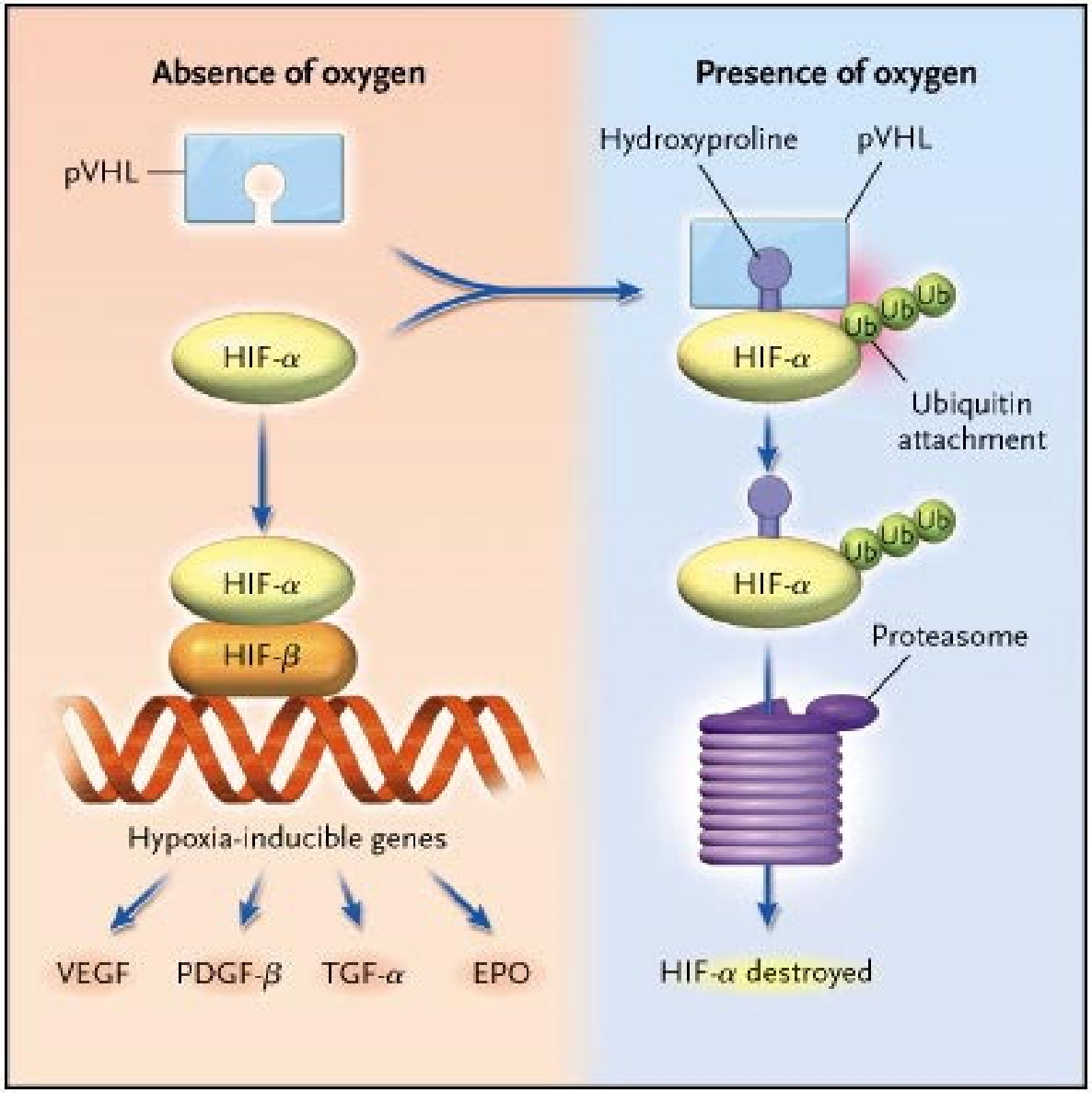
***Caveat:***

No cell permerability due to peptidic E3-recruiting moiety of PROTAC-1

| Category | Specific Name | Target (Ligand) | E3 Ligase (Ligand) | Supplement | Proposed Mechanism | Refs. |
|---|---|---|---|---|---|---|
| PROTAC | PROTAC-1 | MetAP-2 (ovalicin) | $SCF^{\beta\text{-}TRCP}$ (IκBα-phos-peptide) | | $SCF^{\beta\text{-}TRCP}$-mediated ubiquitylation and proteasomal degradation of MetAP-2 *in vitro*. | [50] |
| | PROTAC-2 | ERα (estradiol) | $SCF^{\beta\text{-}TRCP}$ (IκBα-phos-peptide) | | $SCF^{\beta\text{-}TRCP}$-mediated ubiquitylation and proteasomal degradation of ERα *in vitro*. | [51] |
| | PROTAC-3 | AR (DHT) | $SCF^{\beta\text{-}TRCP}$ (IκBα-phos-peptide) | | Proteasomal degradation of the GFP-AR protein in the microinjected cells. | [51] |
| | PROTAC-4 | FKBP12(F36V) (AP21998) | VHL E3 complex (HIF-1α-peptide) | Poly-D-Arg (tag) | VHL-mediated degradation of the EGFP-FKBP12 protein. | [52] |
| | PROTAC-5 | AR (DHT) | VHL E3 complex (HIF-1α-peptide) | Poly-D-Arg (tag) | Proteasomal degradation of the GFP-AR protein. | [52] |
| | Apigenin-PROTAC | AhR (apigenin) | VHL E3 complex (HIF-1α-peptide) | | Proteasomal degradation of the AhR protein. Repression of AhR-dependent transcriptional activation. | [53] |
| | PROTAC-A PROTAC-AA | AR (DHT) | VHL E3 complex (HIF-1α-peptide) | Poly-D-Arg (tag)(-AA) | Ubiquitylation and proteasomal degradation of AR. Cell cycle arrest of AR- dependent prostate cancer cells. | [54] |
| | PROTAC-B | ERα (estradiol) | VHL E3 complex (HIF-1α-peptide) | | Ubiquitylation and proteasomal degradation of ERα. Cell cycle arrest of ERα-dependent breast cancer cells. | [54] |
| | SARM-nutlin PROTAC | AR (SARM) | MDM2 (nutlin) | | Proteasomal degradation of the AR protein. | [56] |
| | Phospho-PROTAC ($^{TrkA}PP_{FRS2\alpha}$) | FRS2α (TrkA-peptide) | VHL E3 complex (HIF-1α-peptide) | Poly-D-Arg (tag) | NGF-dependent ubiquitylation and proteasomal degradation of FRS2α. Inhibition of NGF-dependent neuronal differentiation. | [59] |
| | Phospho-PROTAC ($^{ErbB3}PP_{PI3K}$) | PI3K (ErbB3-peptide) | VHL E3 complex (HIF-1α-peptide) | Poly-D-Arg (tag) | NRG-dependent ubiquitylation and proteasomal degradation of PI3K. Inhibition of ovarian tumor growth in xenograft model. | [59] |

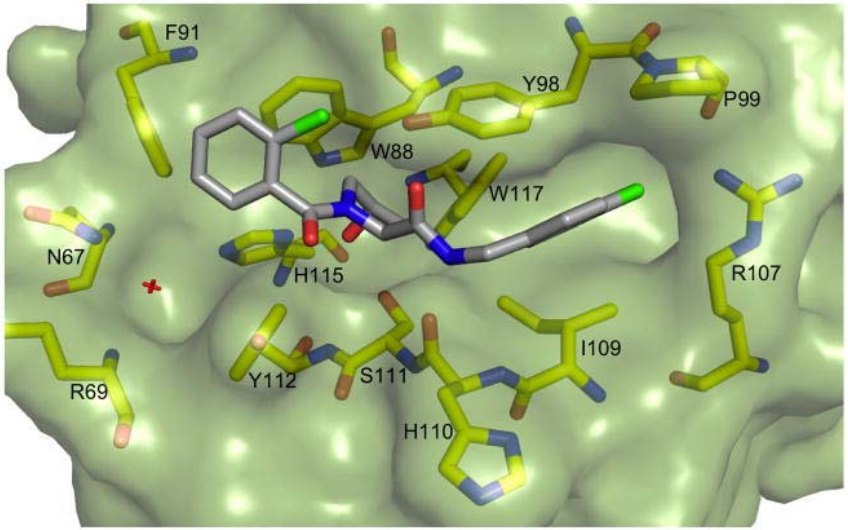
Ohoka et al. Curr Cancer Drug Targets 2016

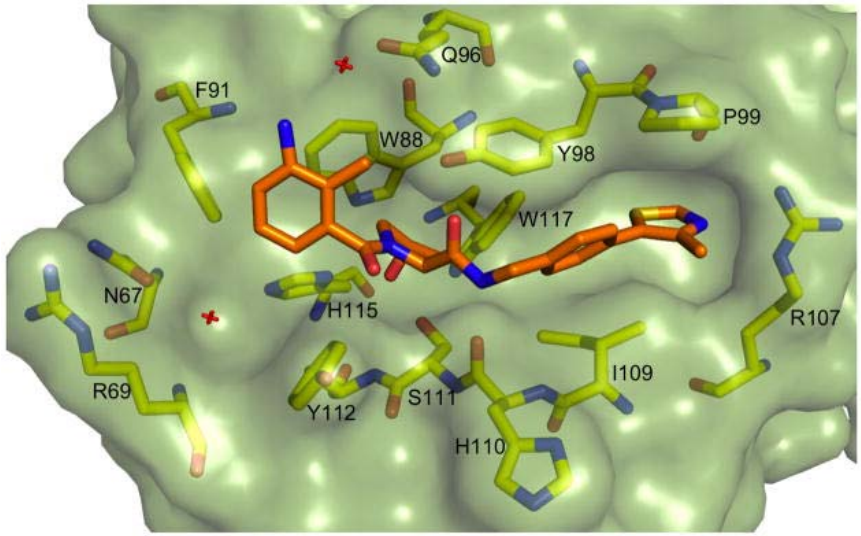
# Von Hippel Lindau (VHL) – Hypoxia inducible factor 1 α (HIF1α)

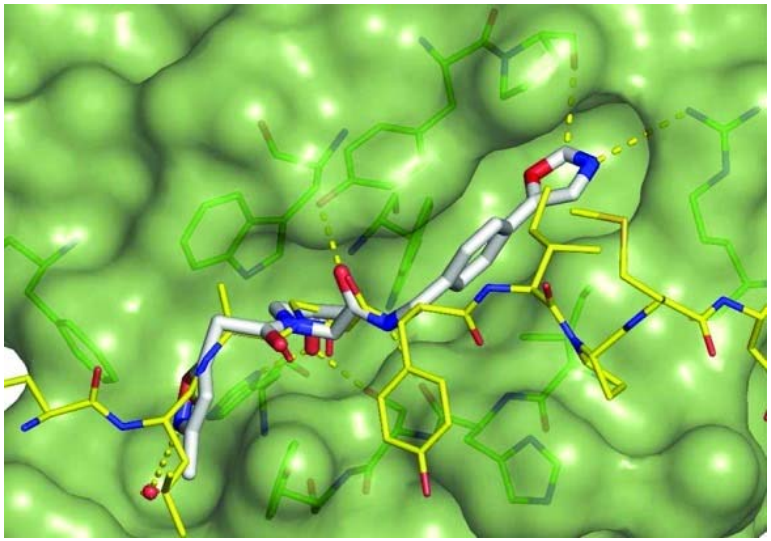



George & Kaelin New Engl J Med 2003

# Small molecule inhibitors of VHL– HIF1α interaction



Development of potent small-molecule inhibitors of the interaction between VHL and HIF1α

Buckley et al. J Am Chem Soc 2012
Buckley et al. Angew Chem Int Ed Engl 2012

nature chemical biology

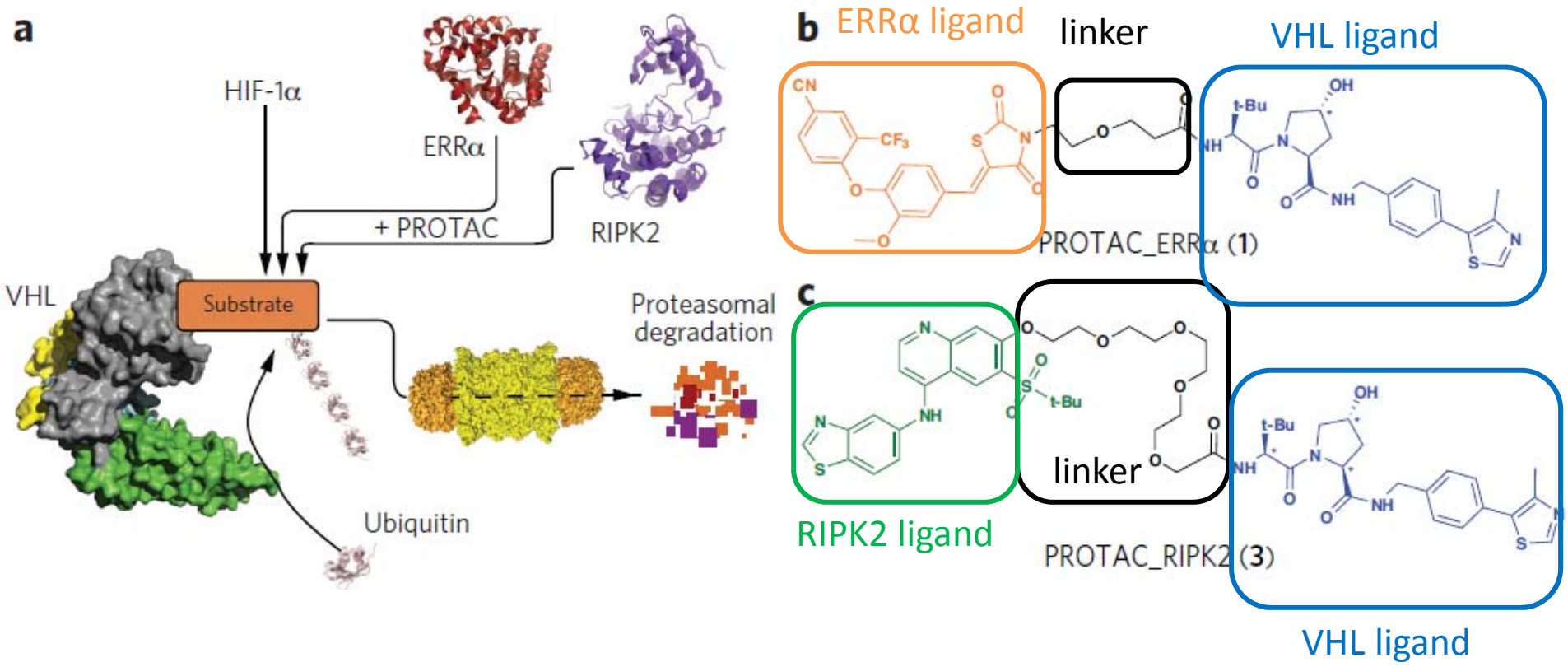
ARTICLE

PUBLISHED ONLINE: 10 JUNE 2015 | DOI: 10.1038/NCHEMBIO.1858

# Catalytic *in vivo* protein knockdown by small-molecule PROTACs

Daniel P Bondeson[1,9], Alina Mares[2,9], Ian E D Smith[2,9], Eunhwa Ko[1], Sebastien Campos[2], Afjal H Miah[2], Katie E Mulholland[2], Natasha Routly[2], Dennis L Buckley[1], Jeffrey L Gustafson[1], Nico Zinn[3], Paola Grandi[3], Satoko Shimamura[3], Giovanna Bergamini[3], Maria Faelth-Savitski[3], Marcus Bantscheff[3], Carly Cox[1], Deborah A Gordon[4], Ryan R Willard[4], John J Flanagan[4], Linda N Casillas[5], Bartholomew J Votta[5], Willem den Besten[6], Kristoffer Famm[2], Laurens Kruidenier[2], Paul S Carter[2], John D Harling[2], Ian Churcher[2*] & Craig M Crews[1,7,8*]

# Small molecule PROTACs: overview



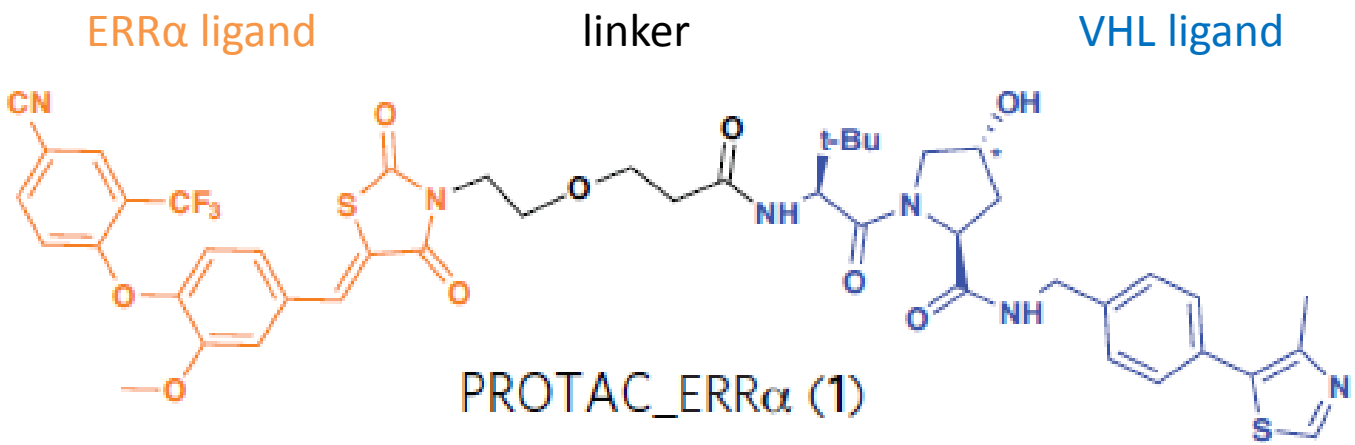

VHL ligand designed based on previous structural studies

$K_d$: 320 nM

# PROTAC_ERRα: design

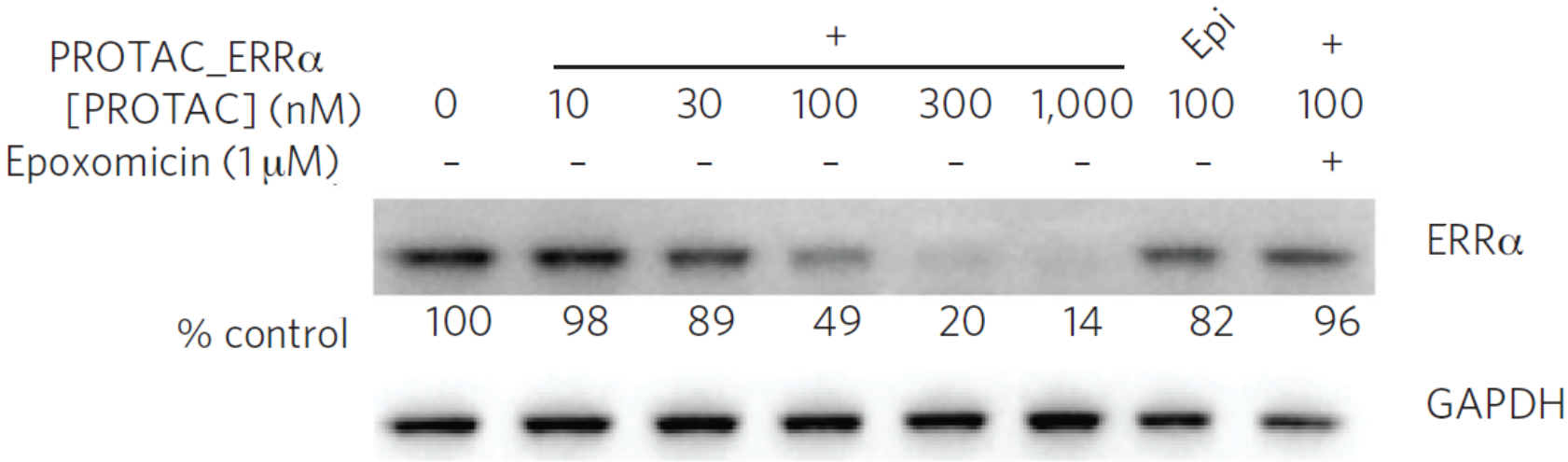
ERRα:
(Estrogen-related receptor α)
- Orphan nuclear hormone receptor
- Master regulator of cellular energy homeostasis

ERRα ligand:
- Thiazolinedione-based
- Selective for binding to ERRα over other ERR isoforms
- Not able to degrade ERRα alone



# PROTAC_ERRα: functionality

In MCF-7 breast cancer cell line

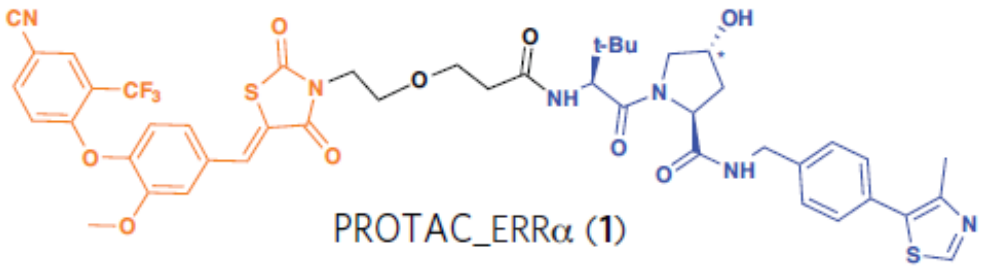



PROTAC and proteasome required

$DC_{50}$ of PROTAC_ERR : ≈ 100 nM

Epi: PROTAC_ERRα epi (inverted stereochemistry at *)

Epoxomicin: Proteosome inhibitor



# PROTAC_RIPK2: design

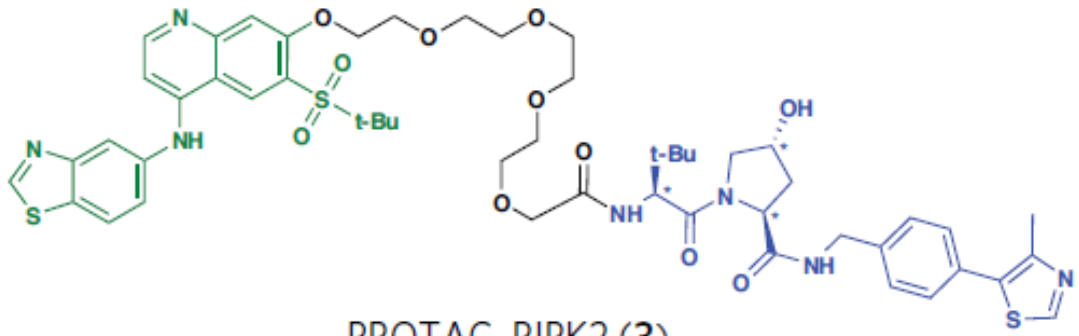
RIPK2:

- Serine-threonine kinase
- Important mediator of innate immune signaling
- Involved in NF-κB and MAPK activation
- Implicated in Blau syndrome and sarcoidosis

RIPK2 ligand:

- Modification of a previously identified inhibitor
- Optimized through study of structure-activity relationship

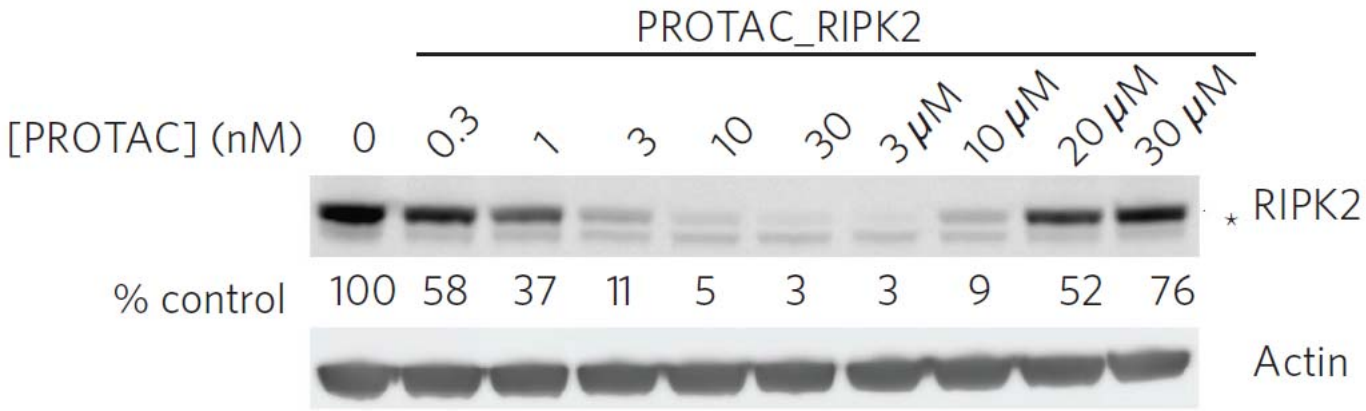
RIPK2ligand linker VHL ligand




PROTAC_RIPK2 (**3**)

# PROTAC_RIPK2: functionality I

In THP-1 human monocyte cell line

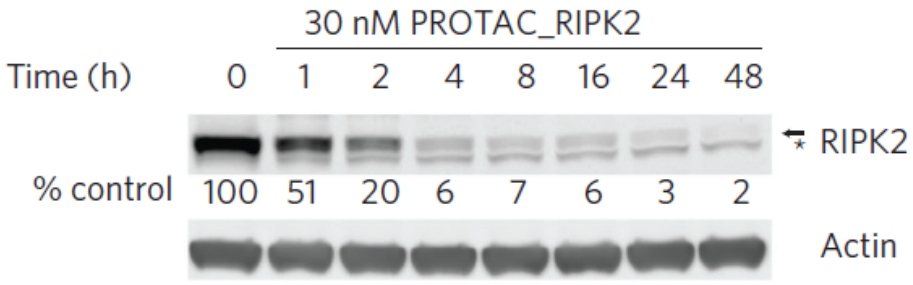


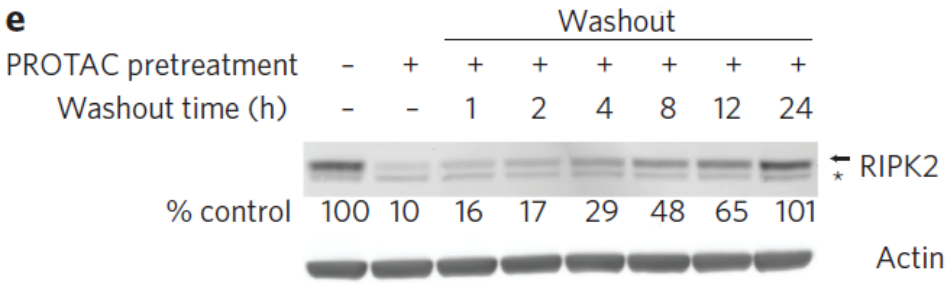

→ $DC_{50}$ of PROTAC_RIPK2 : ≈ 1.4 nM

→ Biphasic response

# PROTAC_RIPK2: functionality II

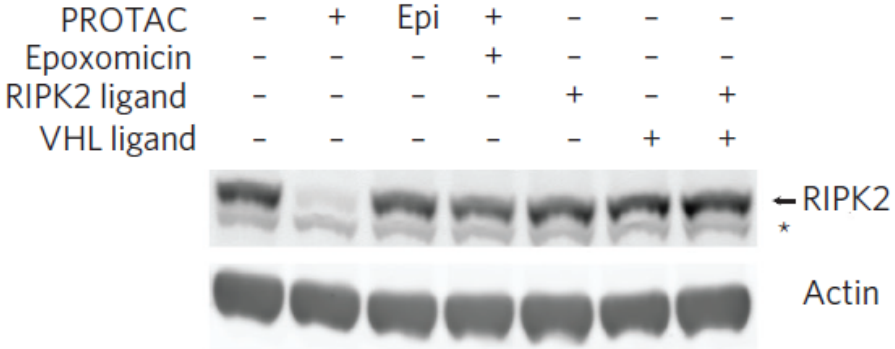
In THP-1 human monocyte cell line




Early degradation
(RIPK2 half life is ≈60h)

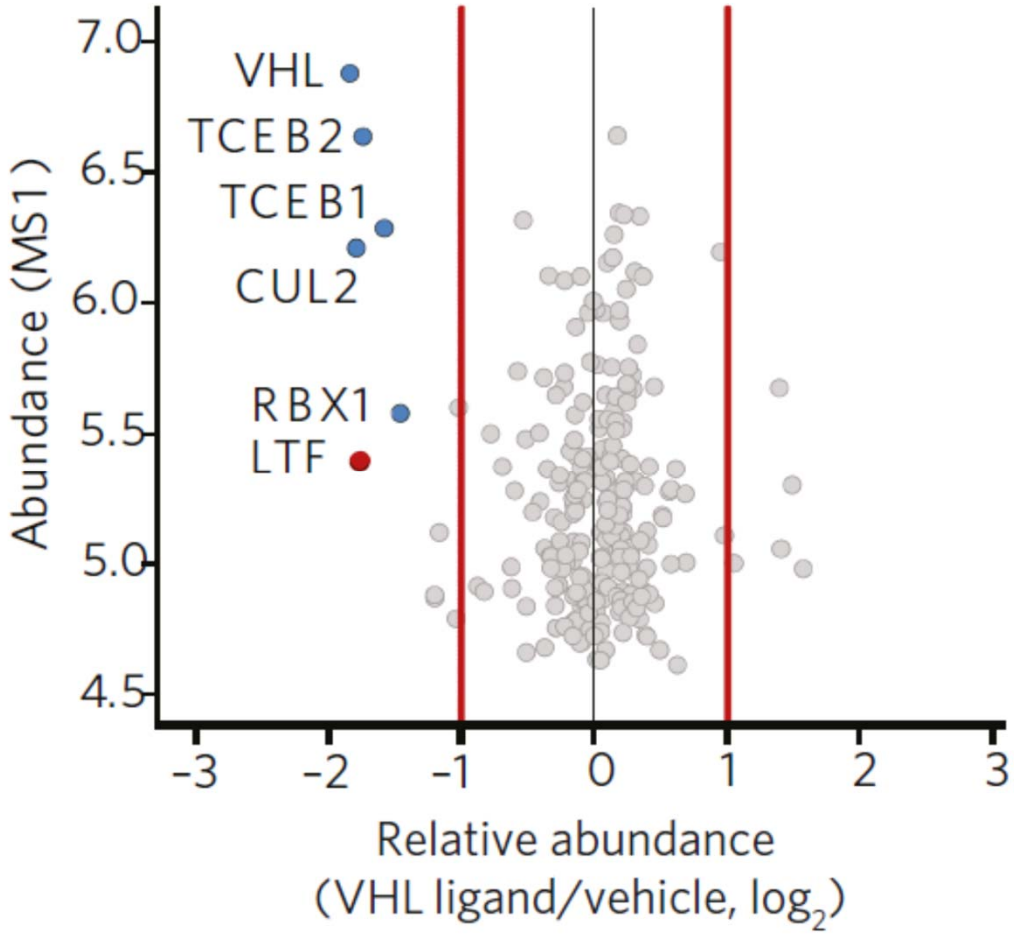
RIPK2 levels recover after PROTAC washout

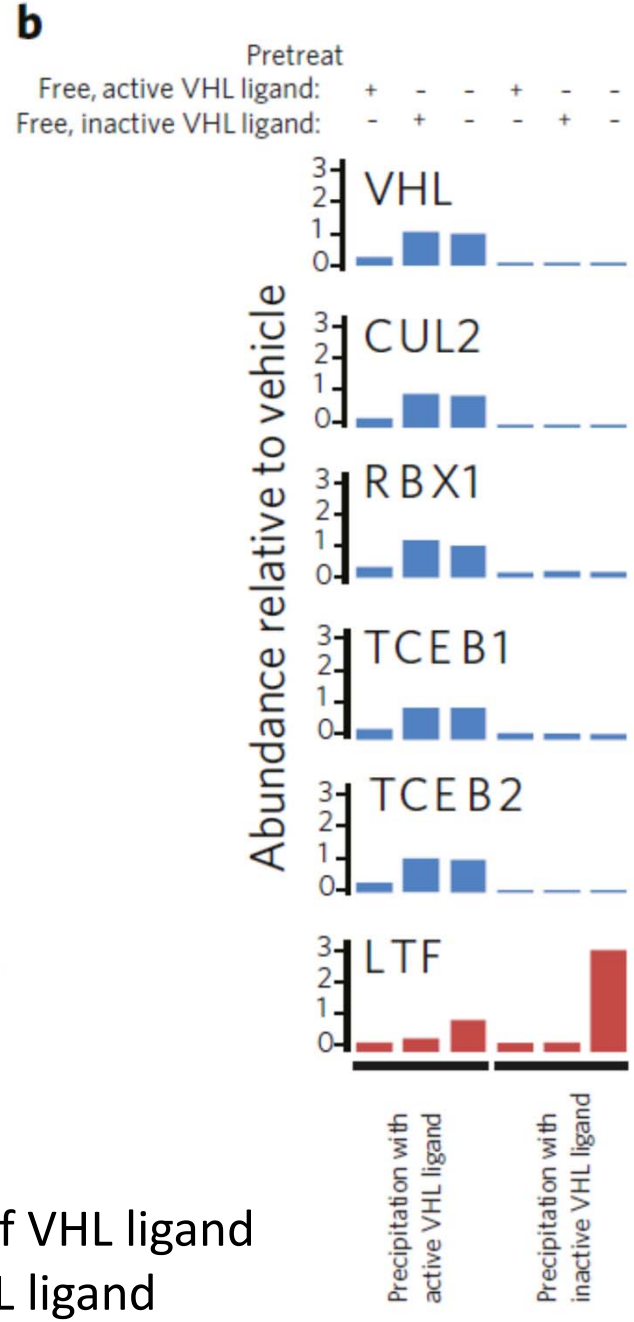



PROTAC and proteasome required
RIPK2 ligand and VHL ligand alone insufficient

# PROTAC: specificity of VHL ligand

Chemoproteomic pulldown with VHL ligand



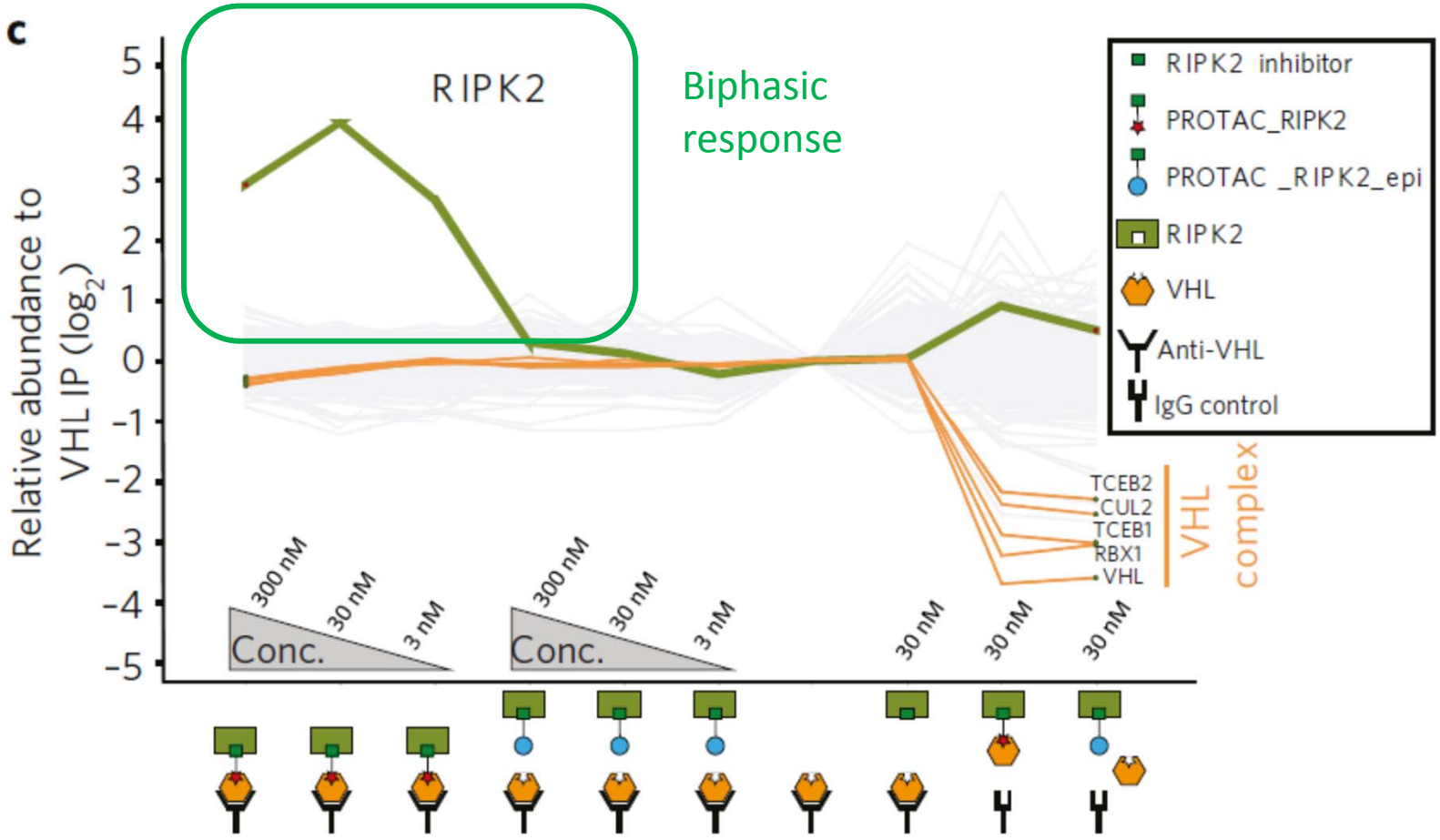

Member of the VHL complex are the only major targets of VHL ligand

LTF: unspecific binding, also with the control, inactive VHL ligand

# PROTAC: ternary complex I

Immunoprecipitation of VHL from THP-1 lysates

Multiplex quantitative MS analysis with isobaric mass tags (TMT10 labels)

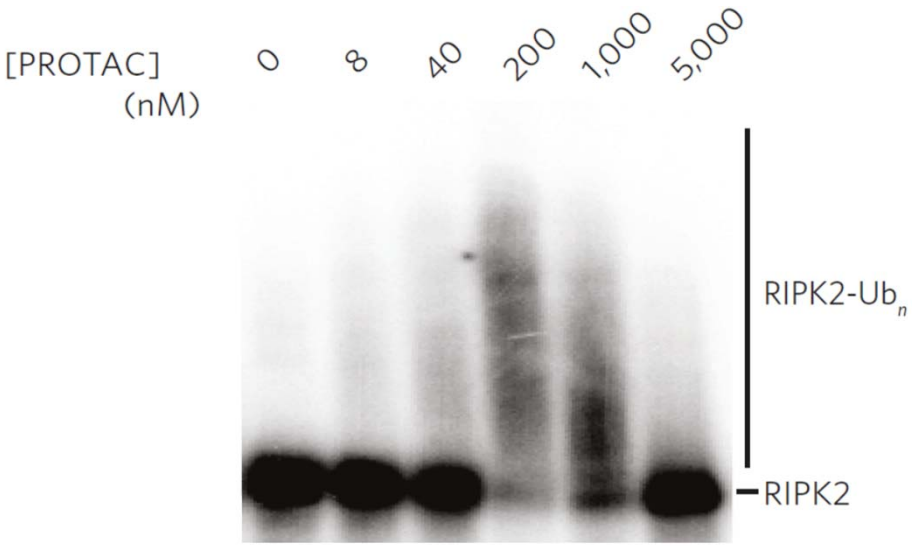


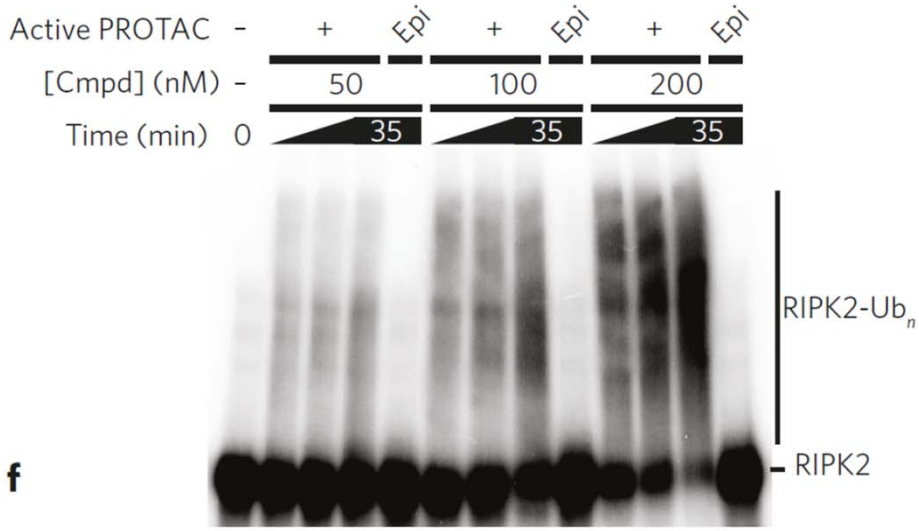

In the presence of PROTAC_RIPK2, not PROTAC_RIPK2_epi, there is co-immunoprecipitation of VHL and RIPK2

# PROTAC: ternary complex II

*In vitro* ubiquitination of radiolabelled RIPK2

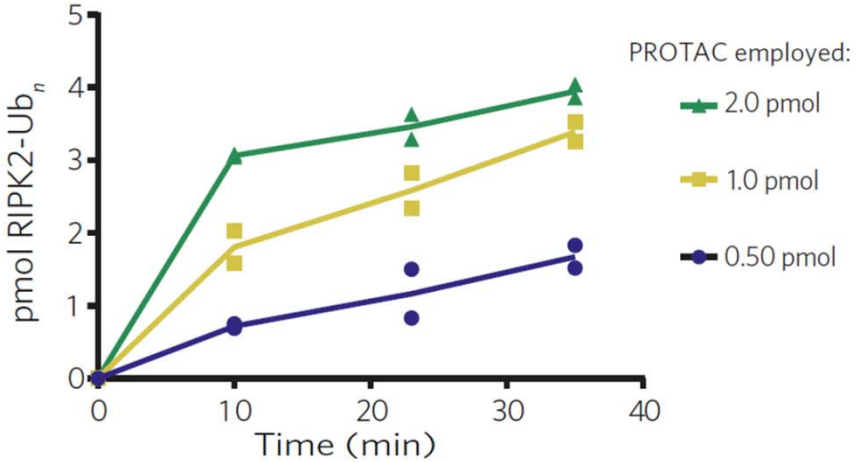
Quantification through PAGE/autoradiography and liquid scintillation of excised bands




PROTAC required for ubiquitination

Biphasic response

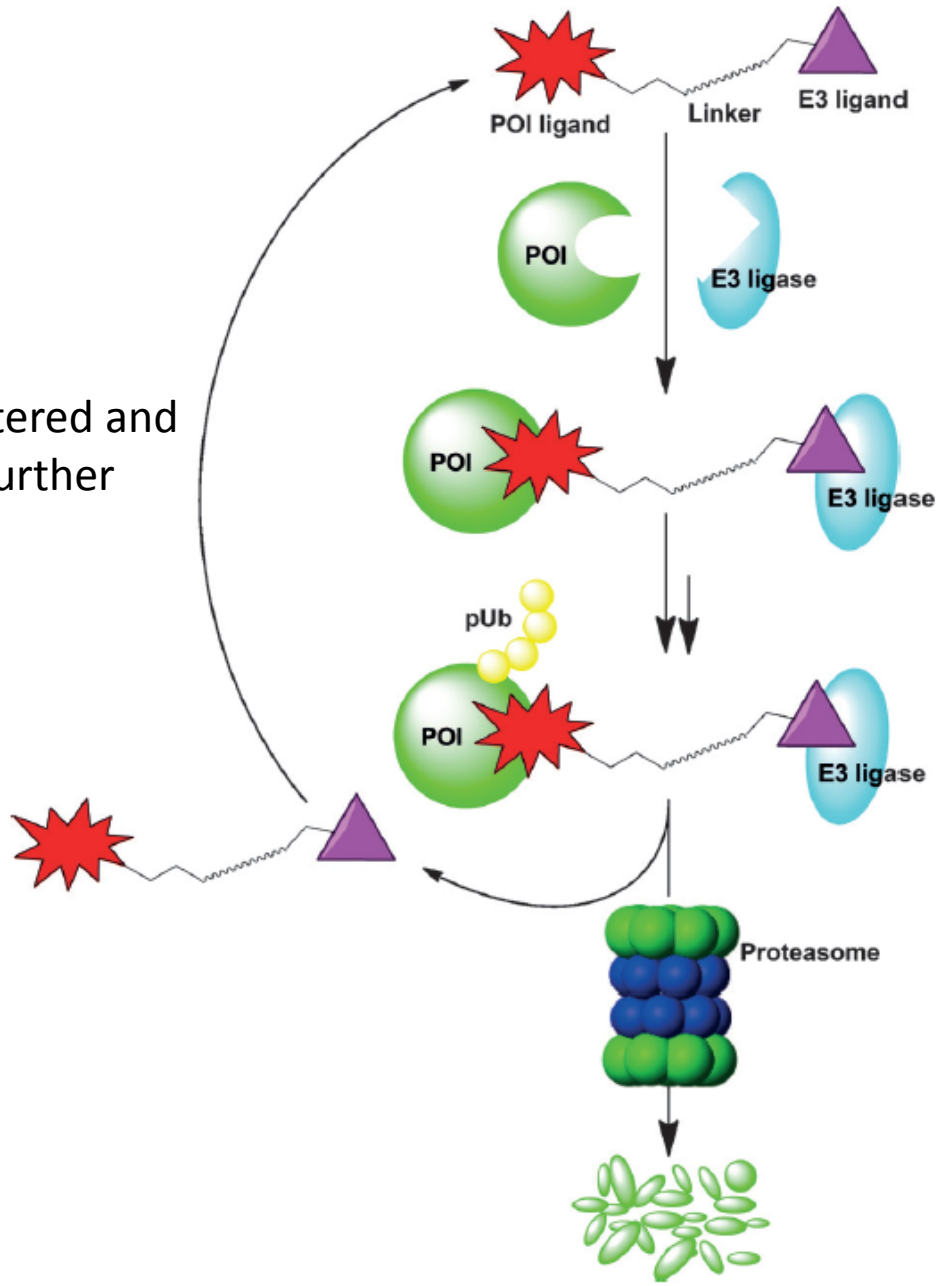



Sub-stoichiometric catalysis

| PROTAC (pmol): | 0.5 | 1.0 | 2.0 |
|---|---|---|---|
| RIPK2-Ub (pmol): | 1.7 | 3.4 | 4.0 |
| Stoichiometry: | 3.3 | 3.4 | 2.0 |

# PROTAC: catalytically active

PROTAC is unaltered and can «recycle» further

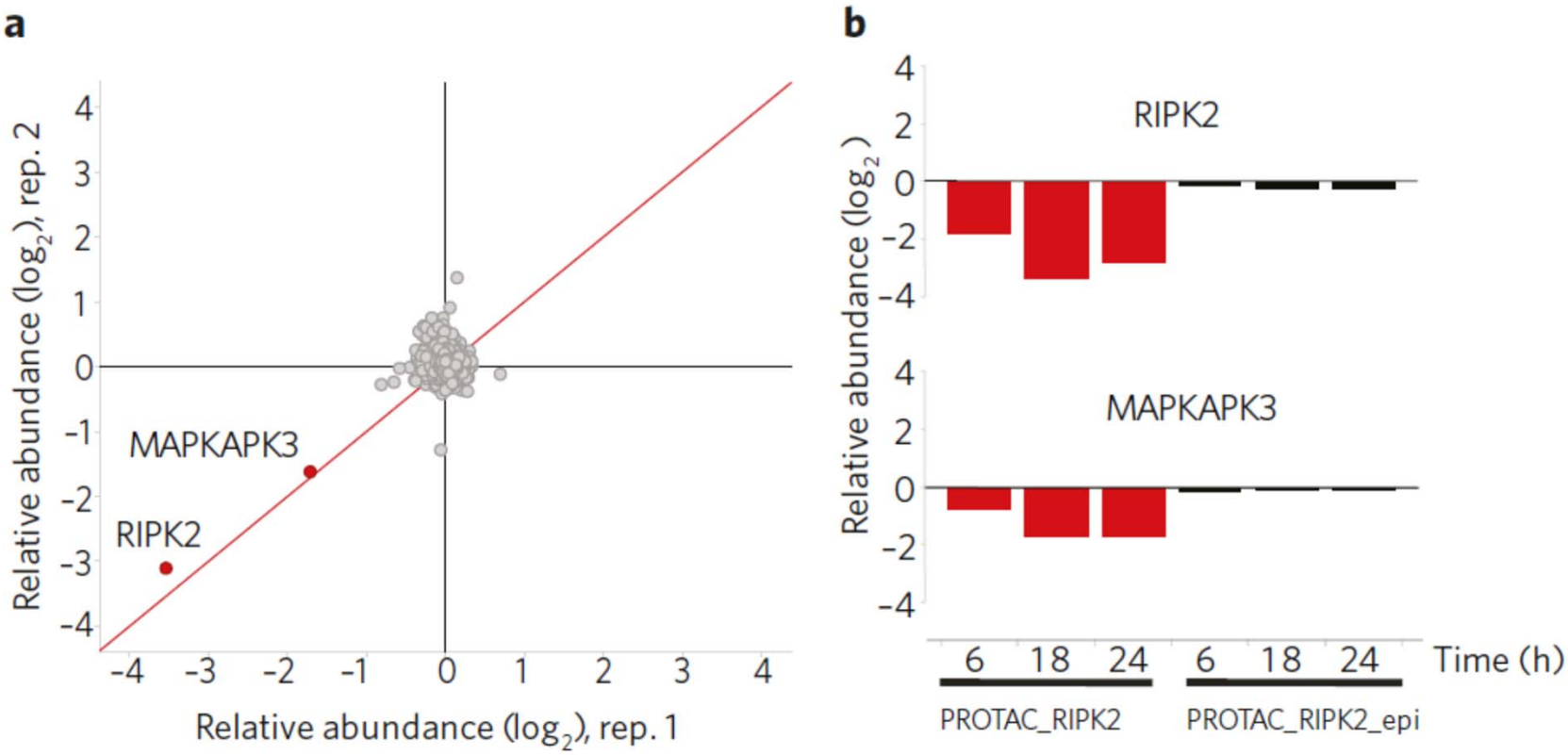


# PROTAC_RIPK2: specificity for RIPK2 degradation

Cellular expression proteomics

THP-1 treated with PROTAC_RIPK2, PROTAC_RIPK2_epi or RIPK2 alone

≈7000 quantified proteins



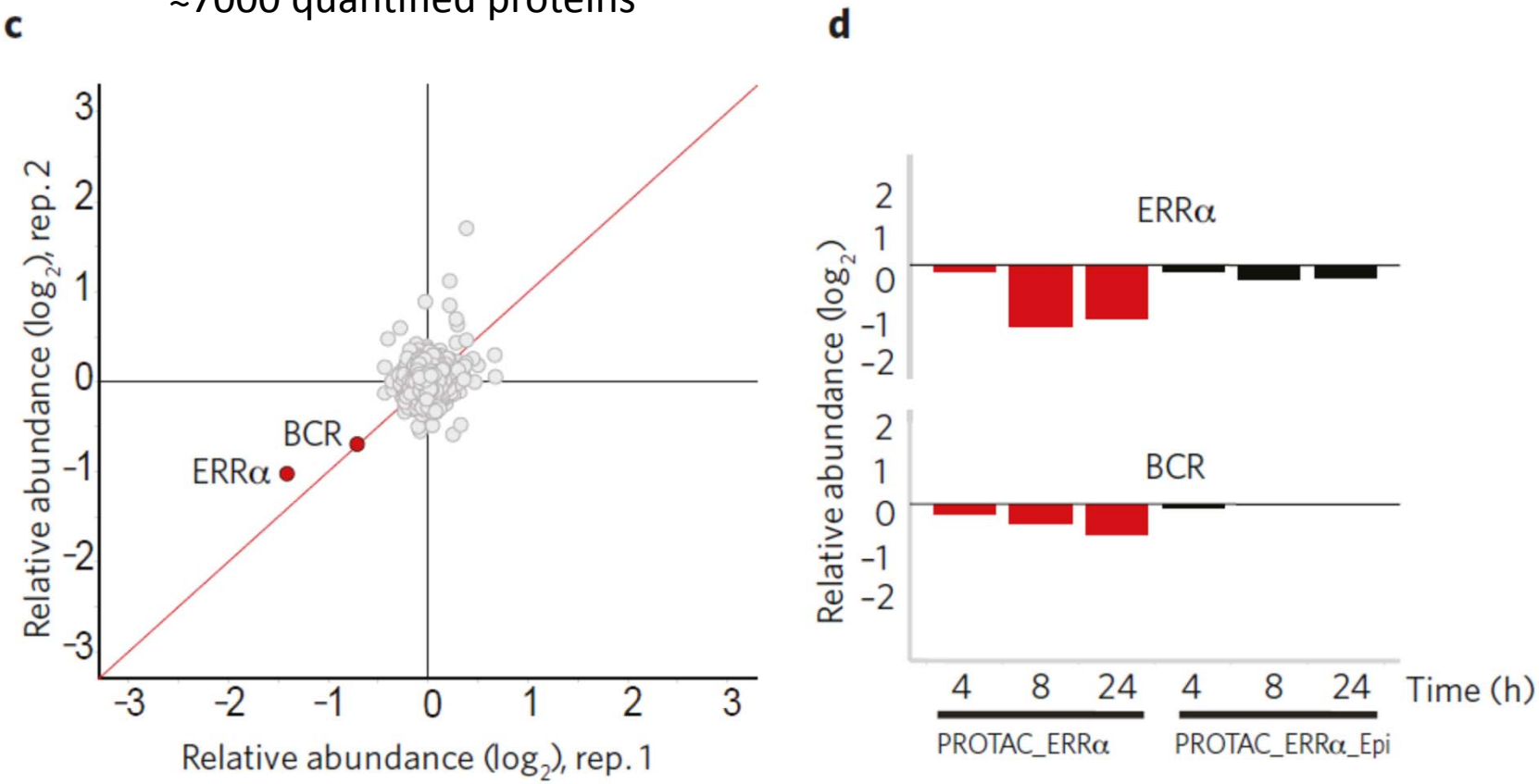

Only RIPK2 and the unrelated MAPKAPK3 were underrepresented in PROTAC_RIPK2 treated cells

# PROTAC_ERRα: specificity for ERRα degradation

Cellular expression proteomics

MCF-7 treated with PROTAC_ERRα or PROTAC_ERRα_epi

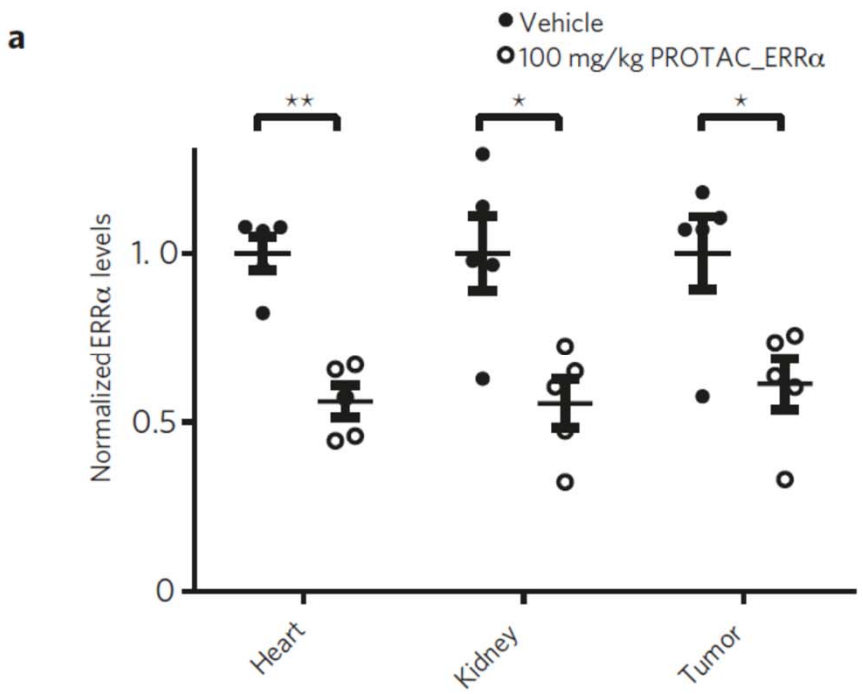
≈7000 quantified proteins

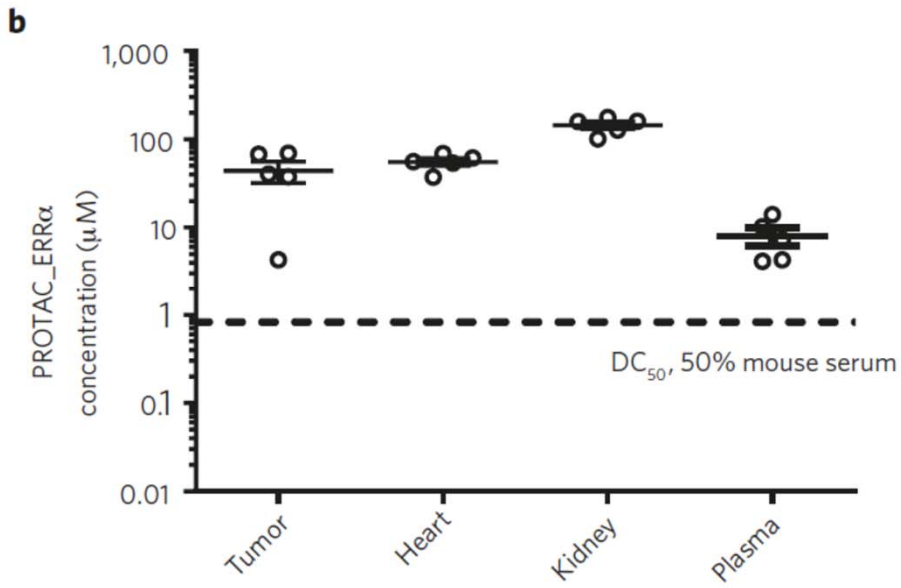



Only ERRα and the unrelated BCR were underrepresented in PROTAC_ERRα treated cells

# PROTAC_ERRα: *in vivo* activity

In mice bearing MDA-MB-231 tumors
PROTAC_ERRα (100 mg/Kg) or vehicle, 3x daily, i.p.




Reduced ERRα levels in different tissues of PROTAC_ERRα-treated mice

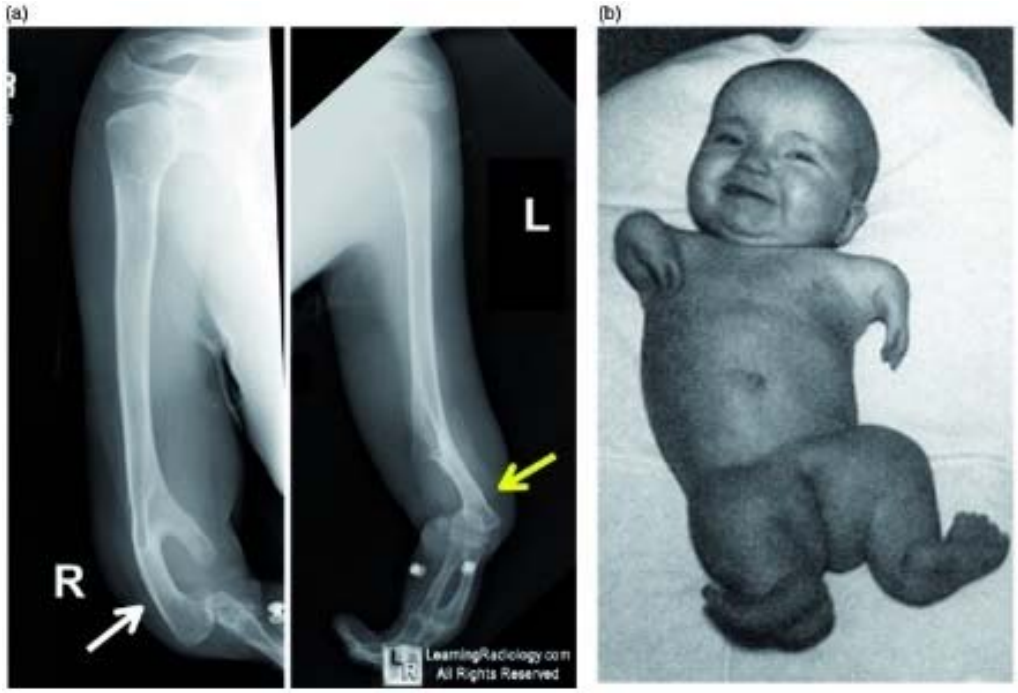
**Protein knockdown**

1) **PROTACs**

2) **Phtalimide conjugation**

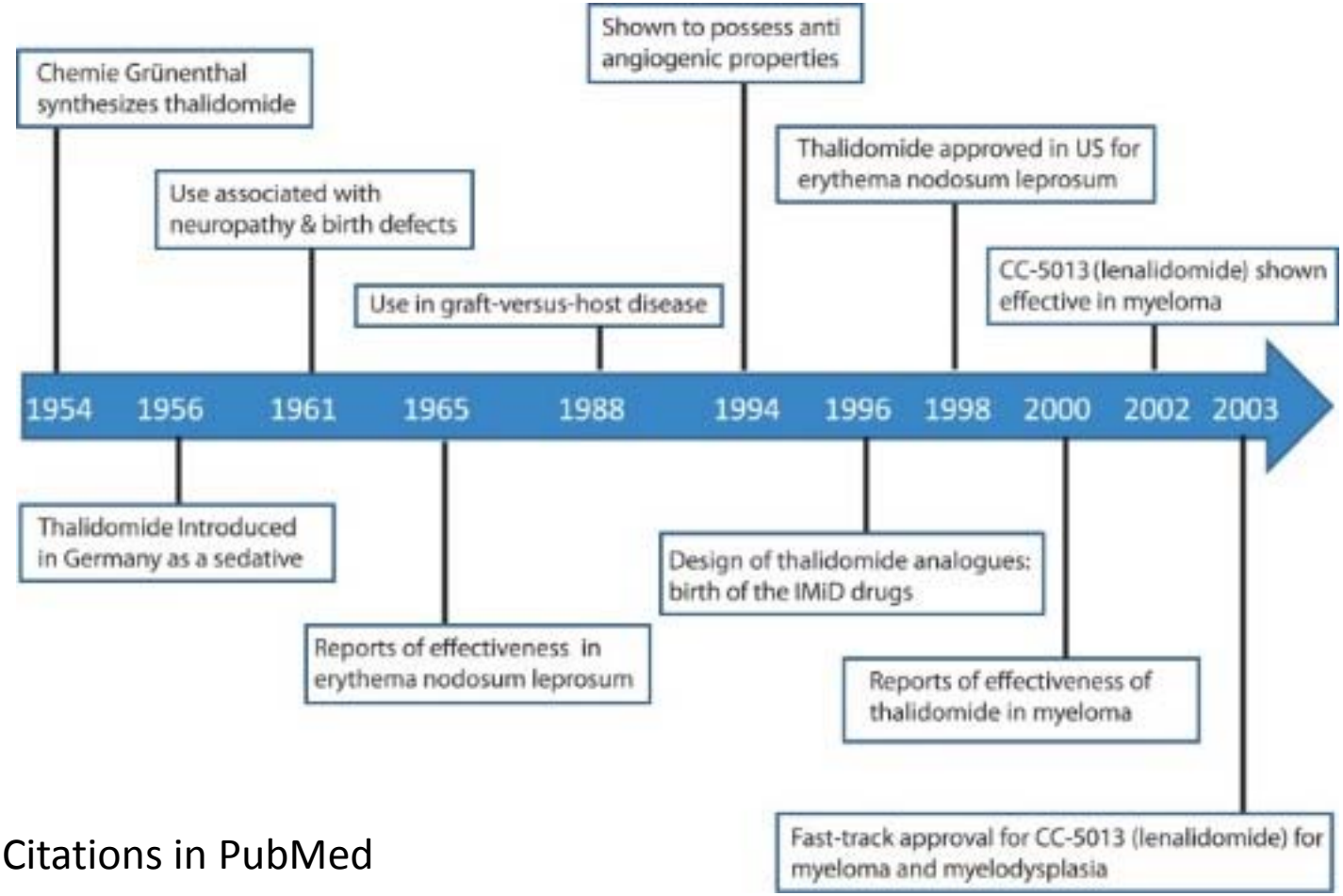
# Thalidomide: the rise and fall… and rise

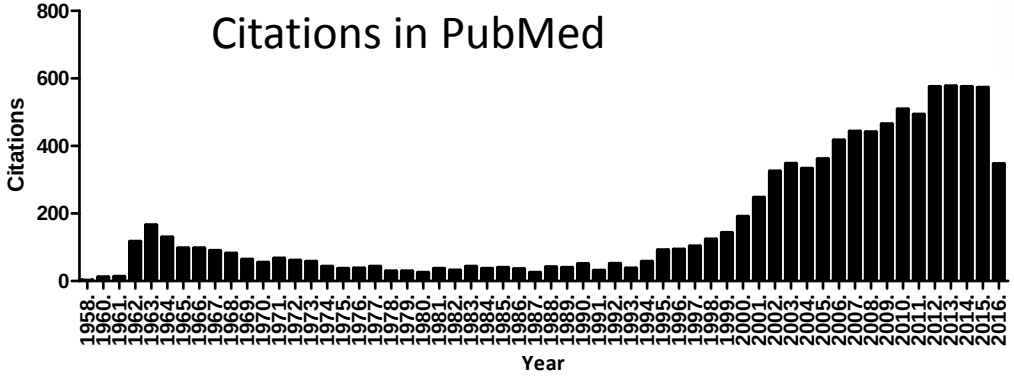
- Synthetic glutamic-acid derivative
- Sedative and anti-emetic activity
- Prescribed also to pregnant women for morning sickness
- In 1961, reports of birth defects led to market withdrawal




Bartlett et al. Nat Rev Cancer 2004
Rehman et al. Ther Adv Hematol 2011

# Thalidomide: the rise and fall… and rise




Rehman et al. Ther Adv Hematol 2011

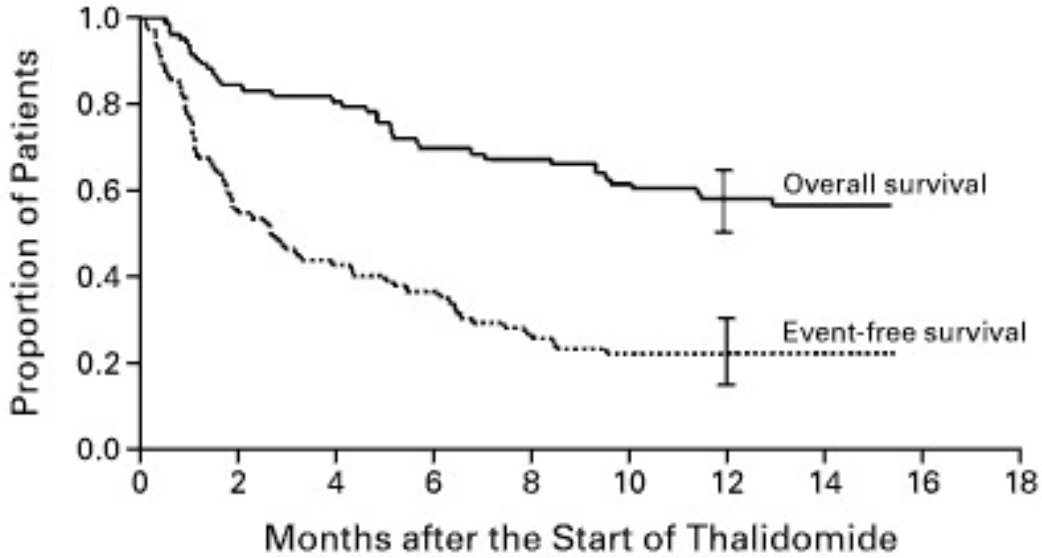
The NEW ENGLAND JOURNAL of MEDICINE

November 18, 1999



# ANTITUMOR ACTIVITY OF THALIDOMIDE IN REFRACTORY MULTIPLE MYELOMA

SEEMA SINGHAL, M.D., JAYESH MEHTA, M.D., RAMAN DESIKAN, M.D., DAN AYERS, M.S., PAULA ROBERSON, PH.D., PAUL EDDLEMON, B.S., NIKHIL MUNSHI, M.D., ELIAS ANAISSIE, M.D., CARLA WILSON, M.D., PH.D., MADHAV DHODAPKAR, M.D., JEROME ZELDIS, M.D., AND BART BARLOGIE, M.D., PH.D.




| No. at Risk | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Overall survival | 84 | 78 | 69 | 64 | 58 | 56 | 51 | 34 |
| Event-free survival | 84 | 65 | 39 | 32 | 24 | 19 | 18 | 11 |

SCIENCE VOL 327 12 MARCH 2010

# Identification of a Primary Target of Thalidomide Teratogenicity

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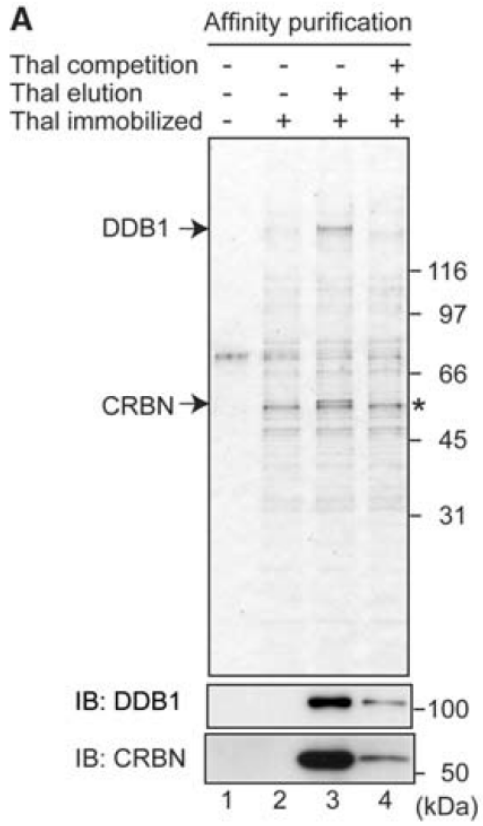
Thalidomide binds to cereblon (CRBN), a component of a cullin-RING ubiquitin ligase complex

CRBN binding mediates thalidomide teratogenicity



SCIENCE VOL 343 17 JANUARY 2014

# Lenalidomide Causes Selective Degradation of IKZF1 and IKZF3 in Multiple Myeloma Cells

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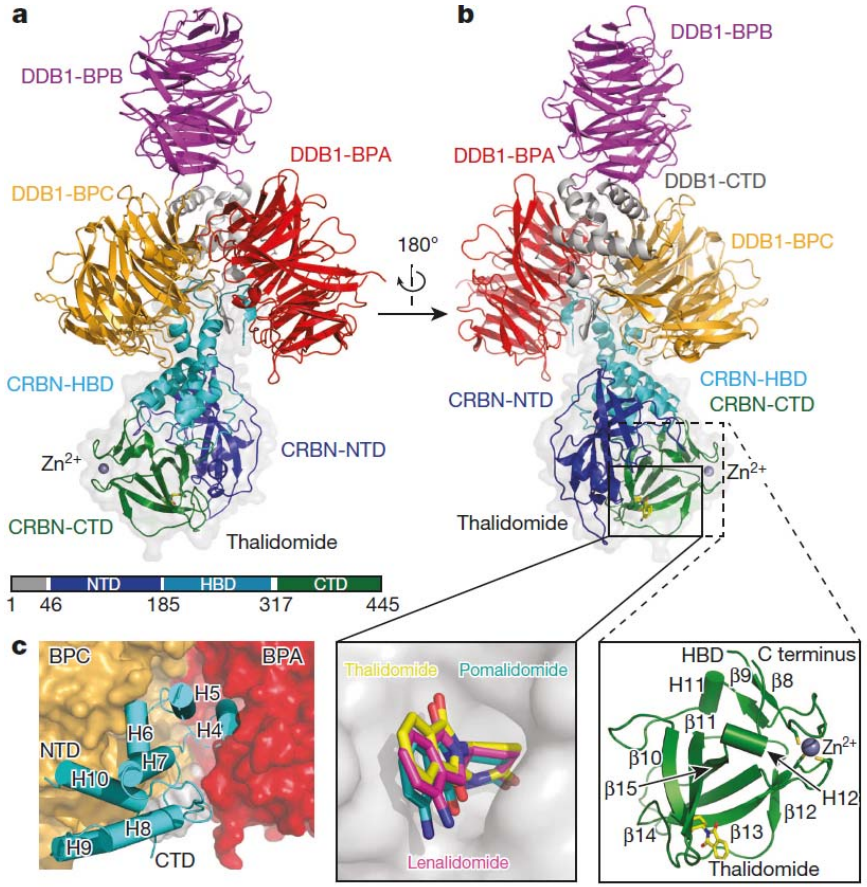
SCIENCE VOL 343 17 JANUARY 2014

# The Myeloma Drug Lenalidomide Promotes the Cereblon-Dependent Destruction of Ikaros Proteins

Gang Lu,[1] Richard E. Middleton,[1,2] Huahang Sun,[1,2] MarkVic Naniong,[1,2] Christopher J. Ott,[1] Constantine S. Mitsiades,[1] Kwok-Kin Wong,[1,2] James E. Bradner,[1] William G. Kaelin Jr.[1,3]*

# Structure of the DDB1–CRBN E3 ubiquitin ligase in complex with thalidomide

Eric S. Fischer[1,2], Kerstin Böhm[1,2], John R. Lydeard[3], Haidi Yang[4], Michael B. Stadler[1,2,5], Simone Cavadini[1,2], Jane Nagel[4], Fabrizio Serluca[4], Vincent Acker[6], Gondichatnahalli M. Lingaraju[1,2], Ritesh B. Tichkule[4], Michael Schebesta[4], William C. Forrester[4], Markus Schirle[4], Ulrich Hassiepen[6], Johannes Ottl[6], Marc Hild[4], Rohan E. J. Beckwith[4], J. Wade Harper[3], Jeremy L. Jenkins[4] & Nicolas H. Thomä[1,2]



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DRUG DEVELOPMENT

# Phthalimide conjugation as a strategy for in vivo target protein degradation

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# BRD4 as a target

- Transcriptional coactivator

- Binds to acetylated Lys of:
  - histone proteins
  - transcription factors

- Its downregulation leads:
  - *MYC* downregulation
  - antiproliferation response

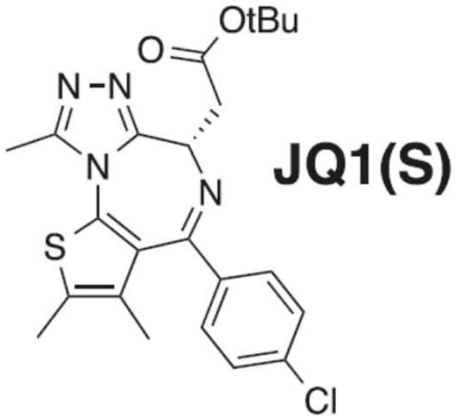
- Implicated in:
  - Cancer
  - Inflammation
  - Heart disease

- Desireble target for selective degradation

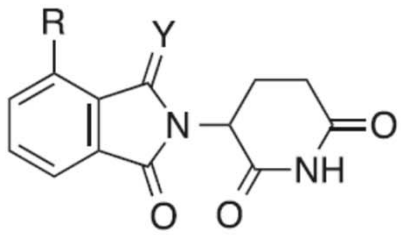
# dBET1 to target BRD4

JQ1 displaces BRD4 from chromatin
Carboxyl group tolerates chemical substitution

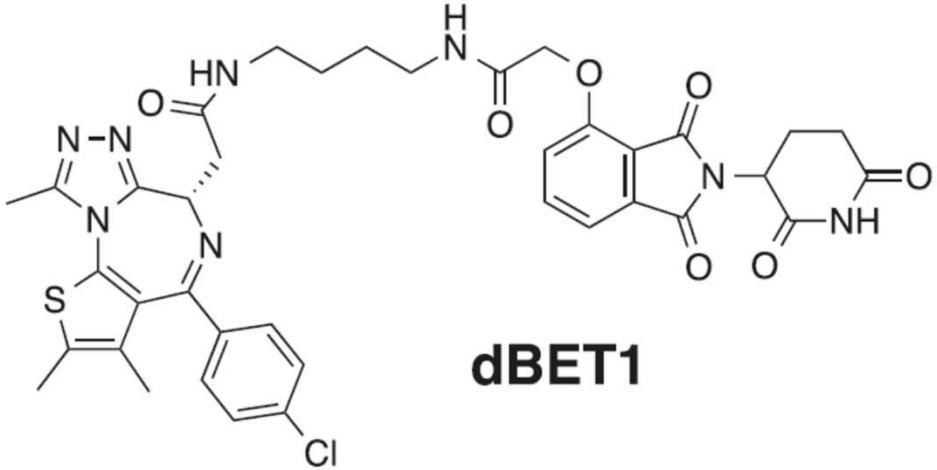
Thalidomide binds CRBN ligase
Aryl ring tolerates chemical substitution




**JQ1(S)**




thalidomide: Y=O, R=H
lenalidomide: Y=$CH_2$, R=$NH_2$
pomalidomide: Y=O, R=$NH_2$

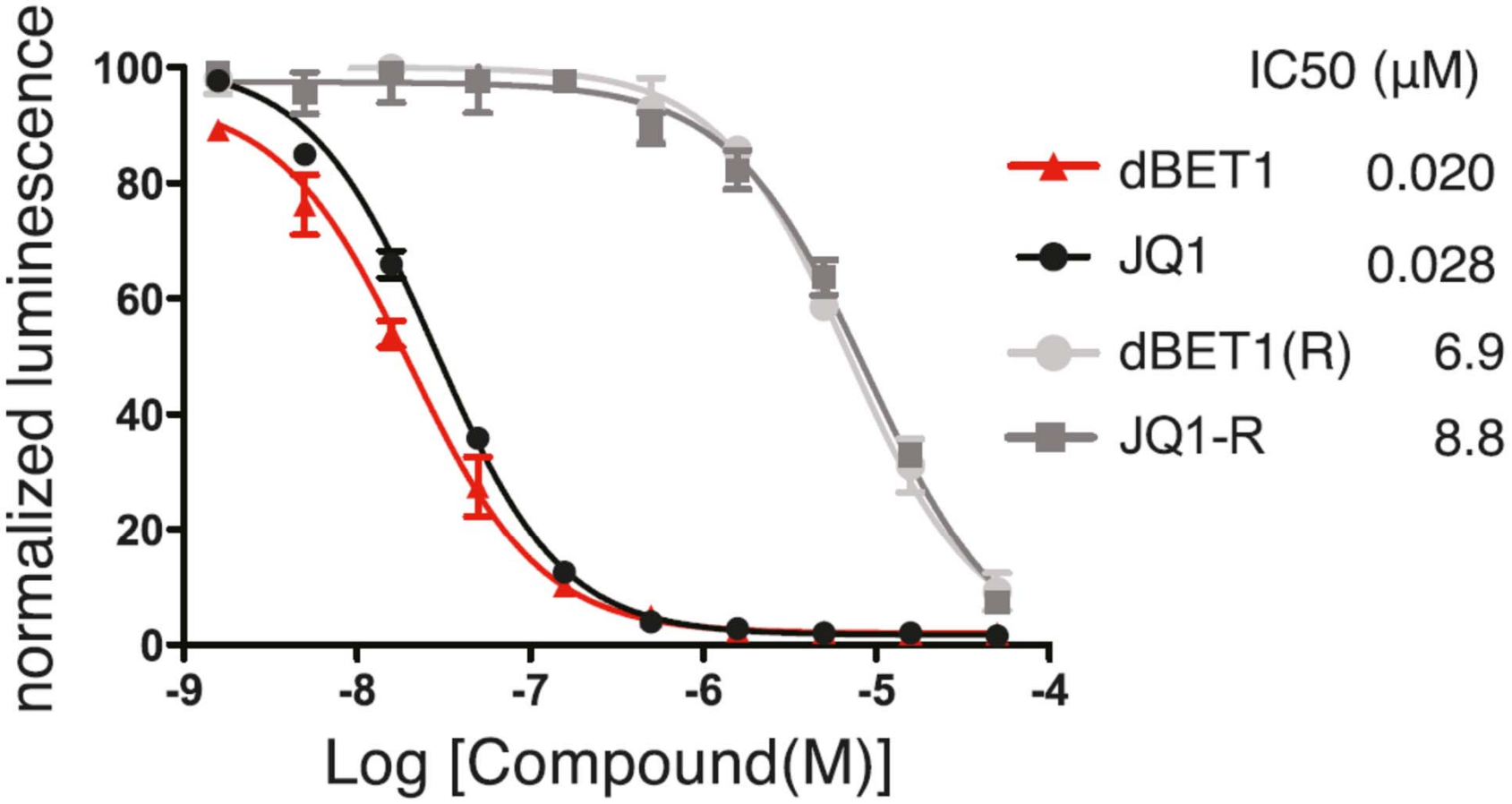



**dBET1**

→ dBET1 was designed to have preserved BRD4 and

# dBET1 binding to BRD4: affinity

BRD4 displacement assay




→ dBET1, not the stereochemical control dBET1(R), has preserved BRD4 binding

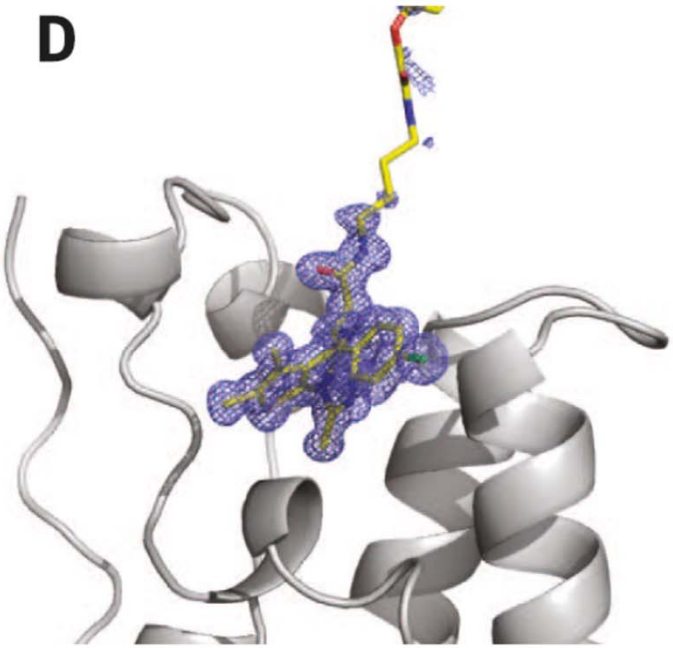
# dBET1 binding to BRD4: structure

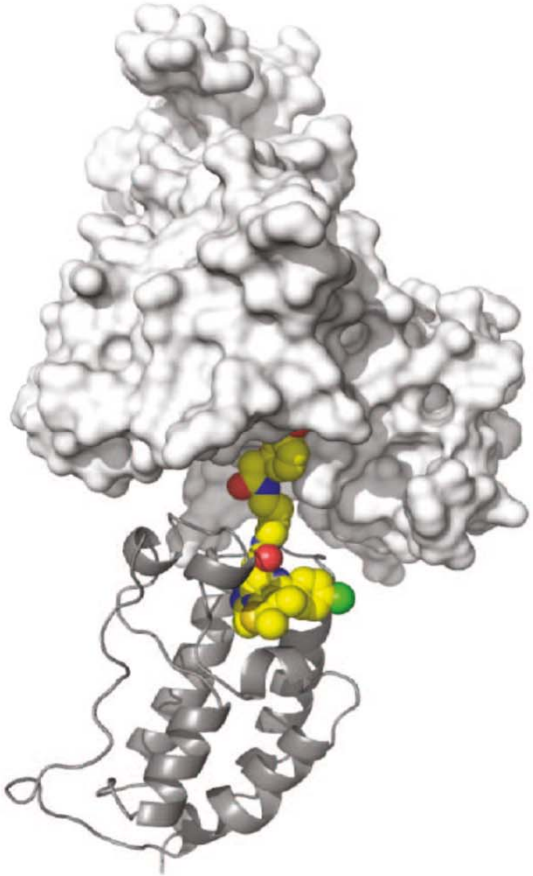
High-resolution crystal structure (1.0 Å) of dBET1 bound to BRD4:

Molecular recognition similar to JQ1

Docking of D into published structure of CRBN bound to thalidomide:

dBET1 can bridge BRD4 and CRBN without destructive steric interactions

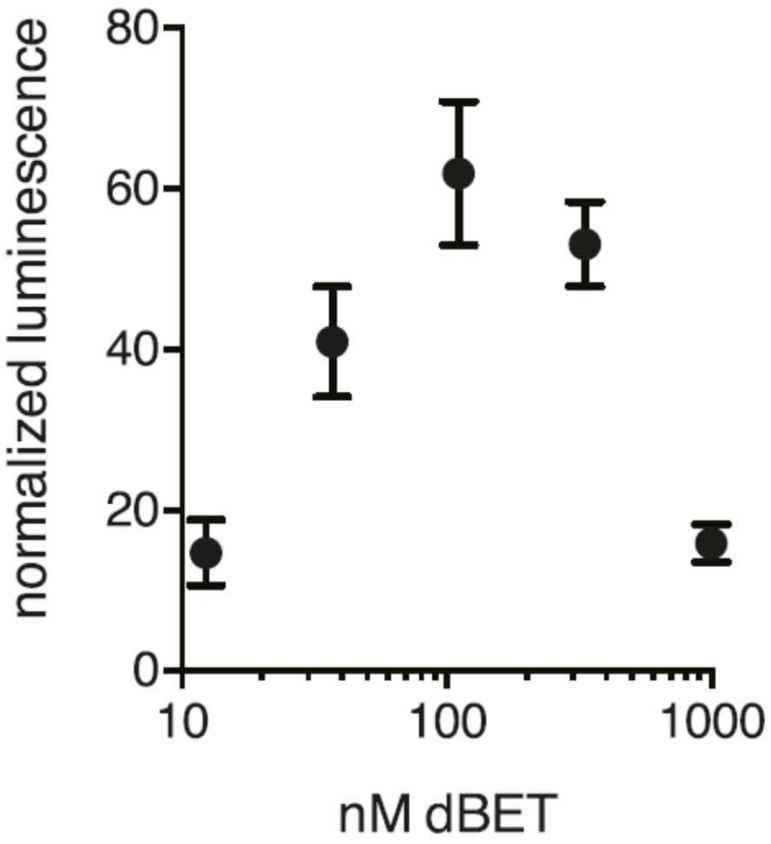


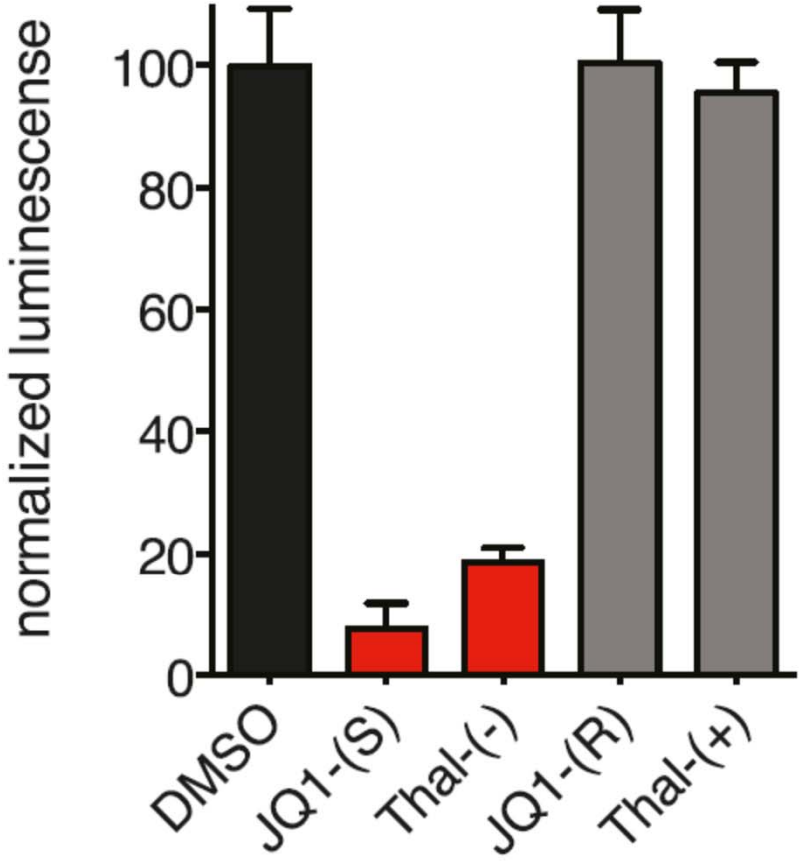



# dBET1 binding to BRD4: ternary complex formation

## Homogeneous proximity assay

Luminescence arises from proximity of CRBN complex and BRD4-bound acceptor beads



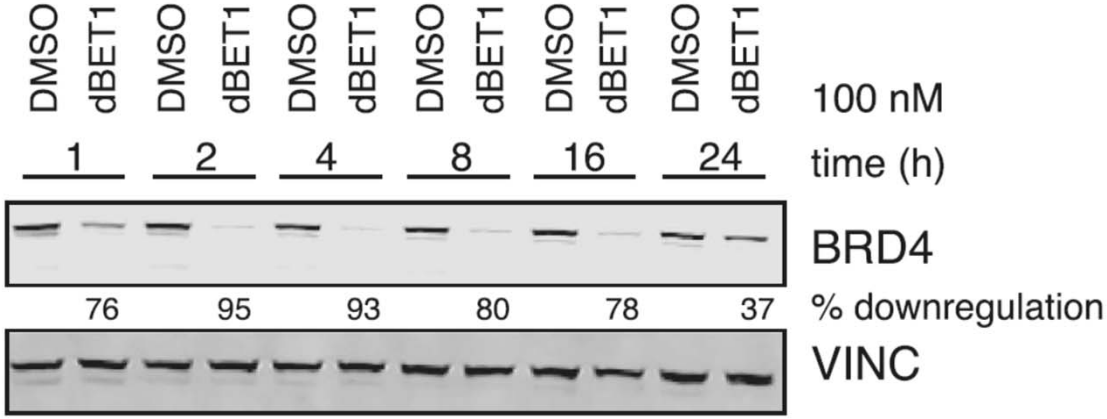




Biphasic response

Free JQ1 or thalidomide inhibits ternary complex formation in a stereospecific manner

# dBET1: effect on cells

In MV4;11 cells (human AML cell line)

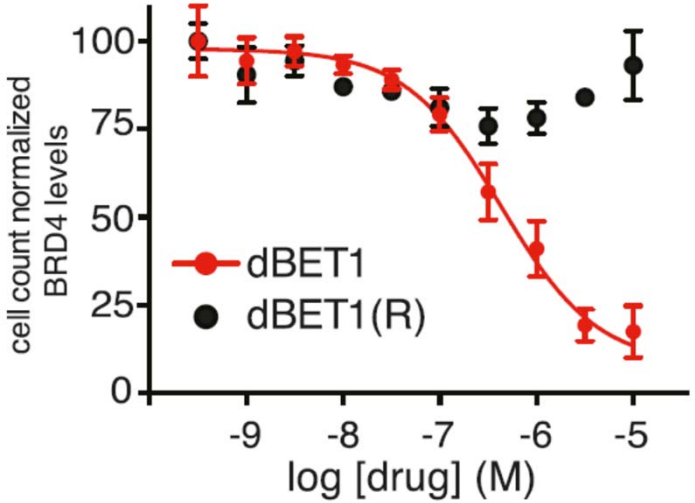

RIPK2 levels

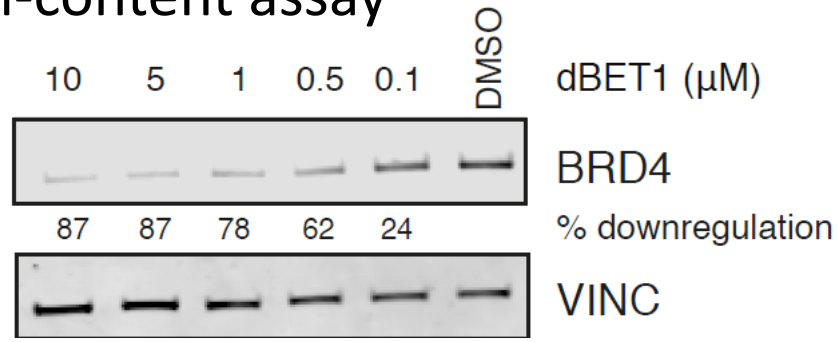
Early degradation

Partial recovery of BRD4 levels at 24h: dBET1 instability?

In SUM149 cells (human breast cancer cell line)

Based on cell-count normalized, high-content assay



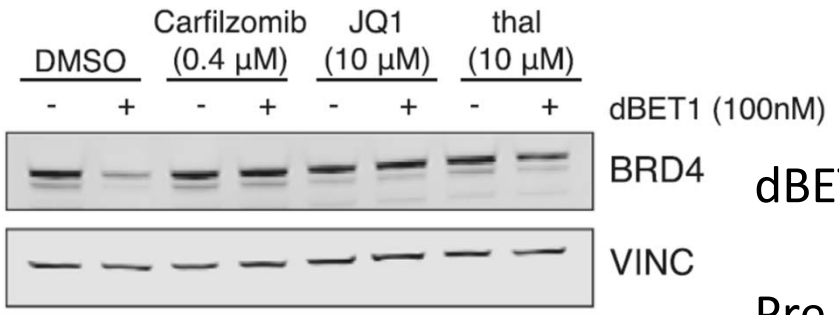




Half maximal effective dose ($EC_{50}$): 430 nM

dBET1(R) inactive

# dBET1: mechanism of action

In MV4;11 cells (human AML cell line)

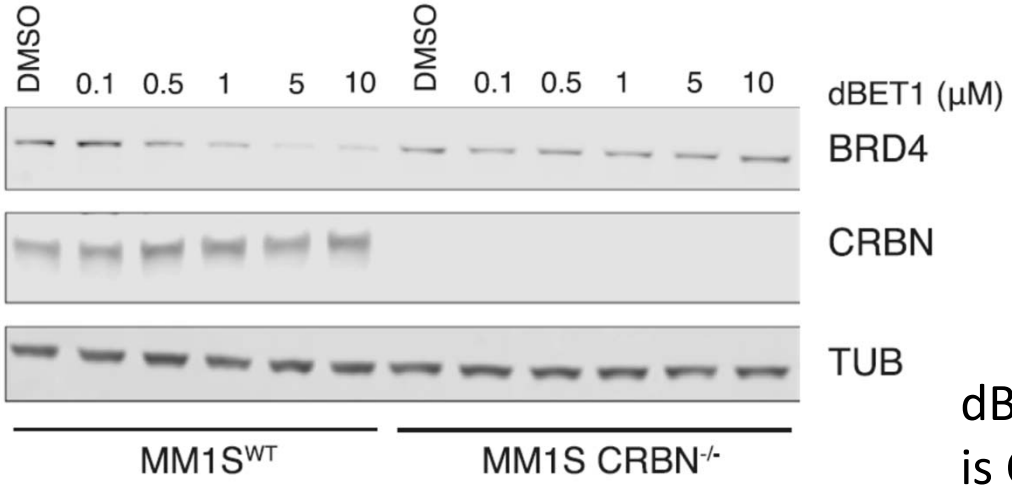



Carfilzomib: proteasome inhibitor

dBET1 and proteasome required

Pre-treatment with JQ1 or thalidomide prevent dBET1-mediated BRD4 downregulation
→ Requirement for both BRD4 and CBN engagement

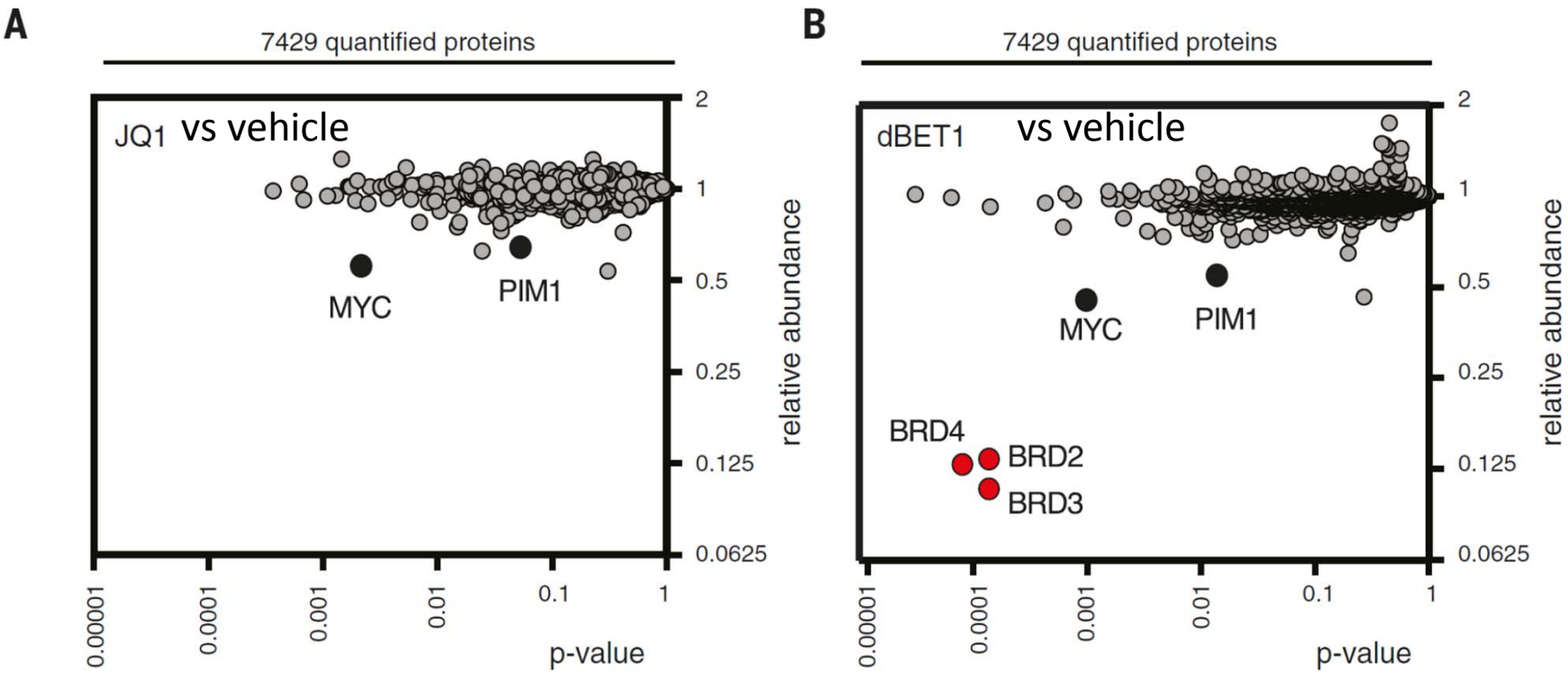
In MM1.S cells (human MM cell line, either WT or CRBN$^{-/-}$)




dBET1-mediated BRD4 downregulation is CRBN-dependent

# dBET1: specificity

Cellular expression proteomics
MV4;11 treated with dBET1, JQ1, or vehicle for 2 h
Multiplex quantitative MS analysis with isobaric mass tags



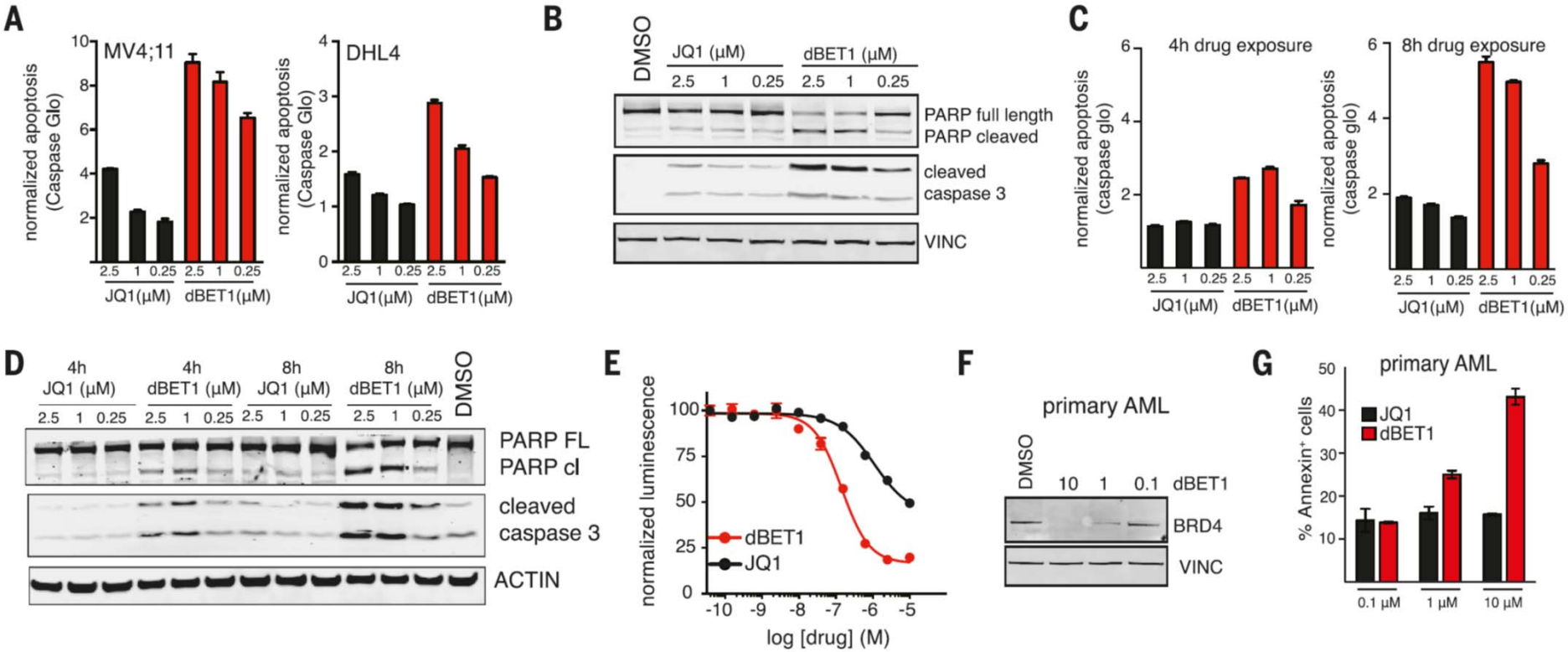

JQ1 resulted in MYC and PIM1 downregulation

dBET1 resulted in MYC, PIM1 and BRD2, 3 and 4 downregulation

Changes in BRD4 at protein, not transcript level, as confirmed by immunoblot and RT-PCR

# dBET1: anti-proliferative response *in vitro*

MV4;11 cells: human AML cell line
DHL4: human lymphoma cell line

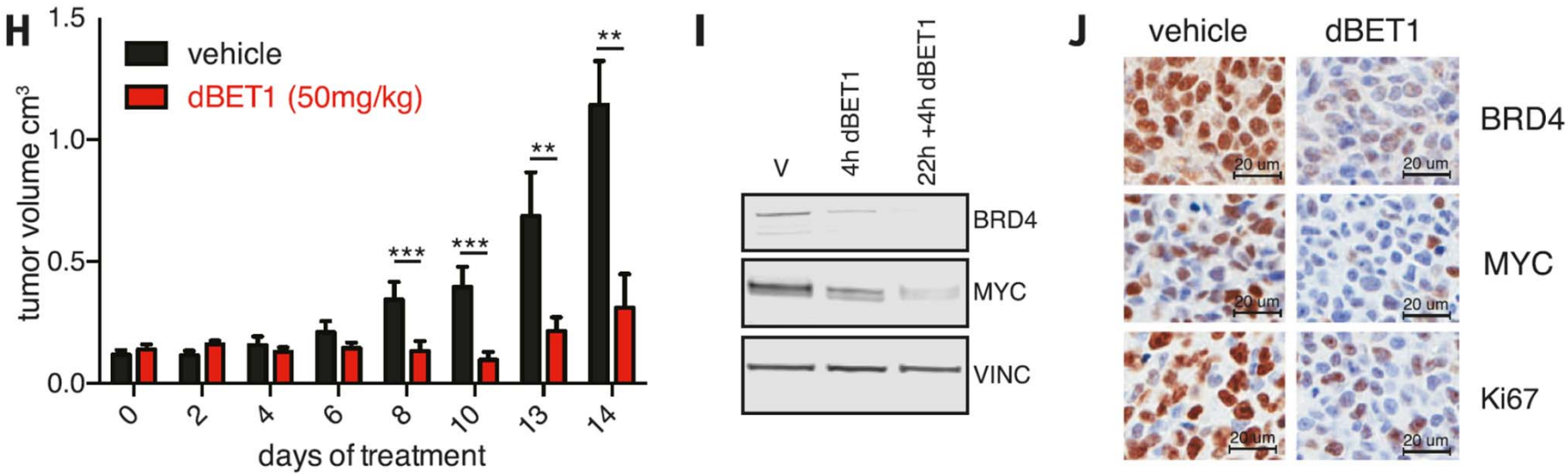



dBET1 treatment results in increased apoptosis with respect to JQ1 in different cell lines and in primary AML blasts

# dBET1: anti-proliferative response *in vivo* I

## Murine hind-limb xenograft model of MV4;11 AML

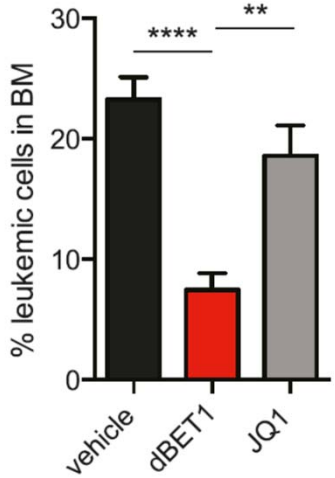
dBET (50 mg/kg) or vehicle 1x day i.p. for 14 d




dBET1 treatment results in reduced tumor mass, BRD4/MYC levels and proliferation

## Murine disseminated leukemia MV4;11 AML model

dBET or JQ1 (both at 63.8 µmol/kg) or vehicle 1x day i.p. for 19 d




dBET1 and JQ1 treatments result in reduced tumor burden

# Catalytic *in vivo* protein knockdown

**Advantages:**

- Potentially applicable to broad spectrum of endogenous proteins
- Catalytic activity makes PROTACs more potent than the target-binding proteins from which they are derived
- Slower recovery from inhibition, because *de novo* synthesis of the target is required

**Disadvantages:**

- PROTAC linker lenght and composition have to be optimized for each target
- Limited to cytosolic proteins (?)
- Possible poor oral bioavailability of PROTACs

Bondeson et al. Nat Chem Biol 2015
Winter et al. Science 2015
Deshaies. Nat Chem Biol 2015
Toure & Crews Angew Chem Int Ed 2015
Ohoka et al. Curr Cancer Drug Targets 2016