TRAFTAC

A generalizable method for transcription factor degradation

230413

Literature seminar #1

M1 Takeuchi

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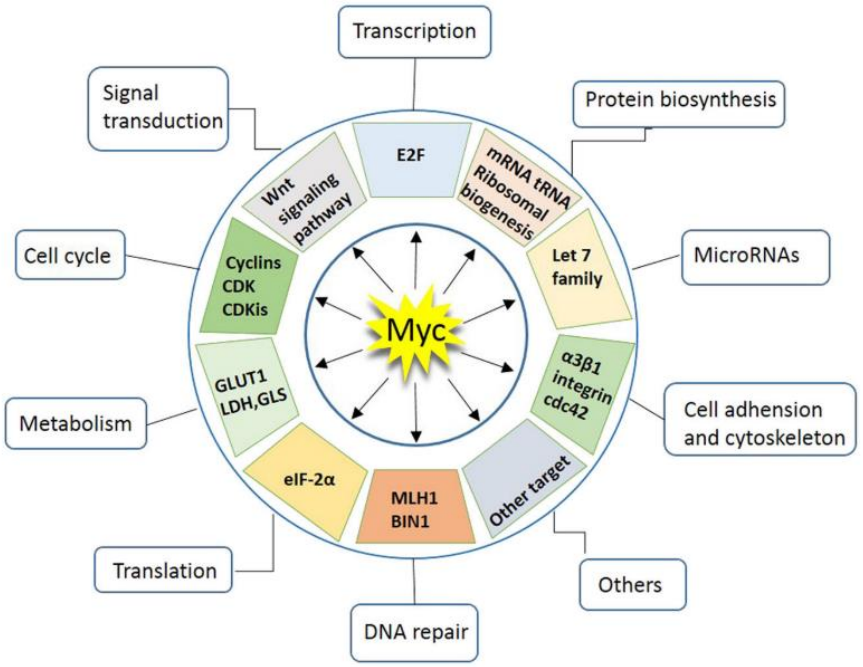
Transcription factor (TF) and disease

表3　転写因子異常と疾患

疾患群	転写因子	疾　患　名
血液	SCL	小児 T 細胞急性リンパ性白血病
	AML1	急性骨髄性白血病 M2
	MLL	11q23 染色体異常
	PAX-5	リンパ腫
	RARa	急性前骨髄性白血病
癌	MYC	子宮頸癌，大腸癌，乳癌，肺小細胞癌，リンパ腫など
	EWS	黒色腫，軟部肉腫
	RB	網膜芽細胞腫，骨肉腫
	WT1	ウィルムス腫瘍
	p53	大腸癌，乳癌，肺癌，胃癌，Li-Fraumeni 症候群など
	BRCA1	乳癌
内分泌	GR	原発性グルココルチコイド不応症
	Pit-1	先天性下垂体ホルモン複合欠損症
神経	AR	伴性劣性球脊髄性筋萎縮症
	PAX-6	遺伝性無虹彩症
	PAX-3	Waardenburg 症候群 I，III 型
ウイルス	HTLV-1 TAX	CD4 陽性 T 細胞白血病，HAM
	HIV-TAT	エイズ，Kaposi 肉腫
	EB-ZTA	Burkitt リンパ腫，上咽頭癌

- TF coordinates the correct gene expression levels for maintaining normal cellular functions.
- Dysregulation of TF activity implicates in numerous cancers.

c-Myc activation in cancer



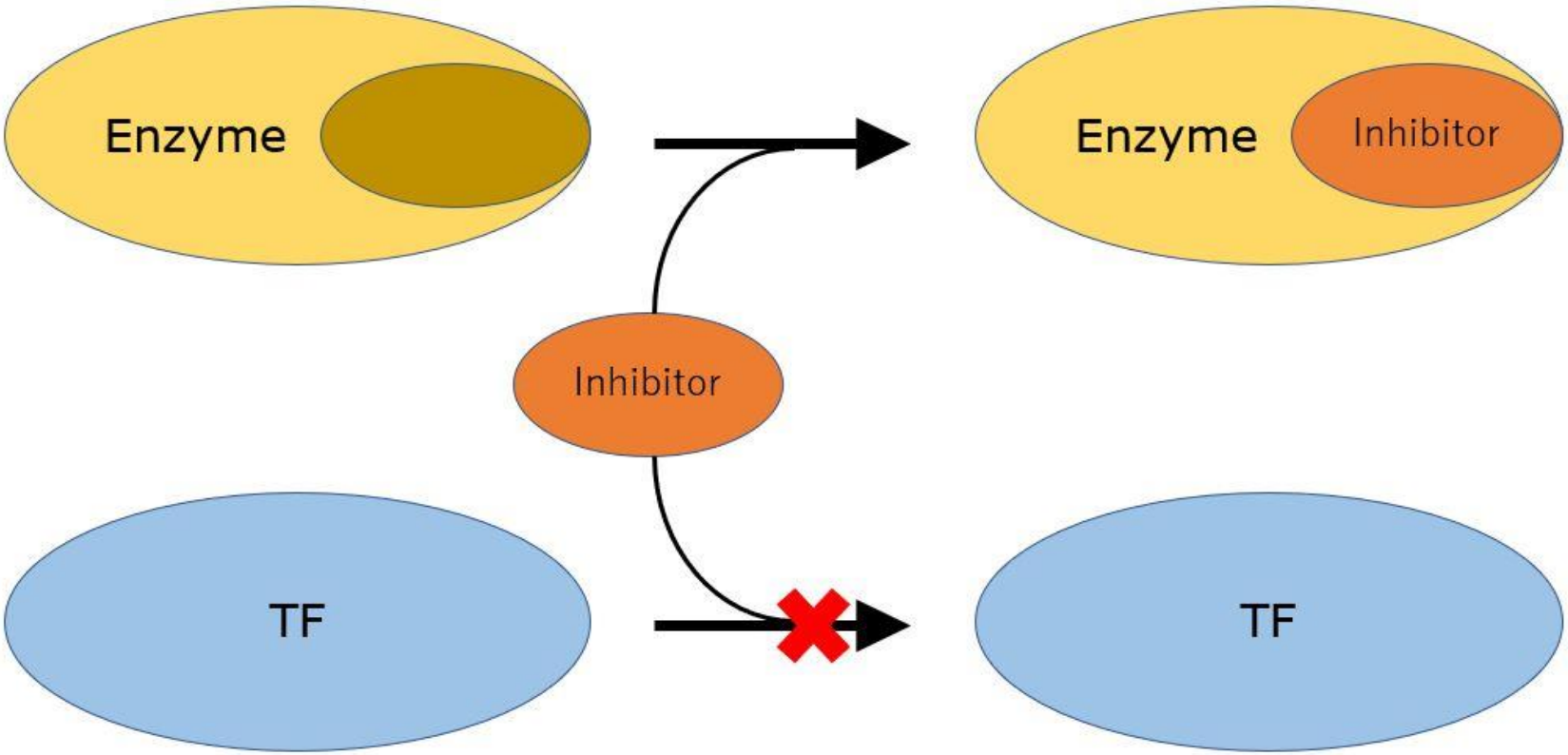
Mechanisms of MYC activation in cancer.

Mechanism	Tumors
Amplification	Ovarian, esophagus, uterine, breast
Translocation	B-cell lymphomas, including Burkitt lymphoma
Enhancer activation	B-cell lymphomas
Mutation	B-cell lymphomas
Altered protein stability	Multiple cancer types
Increased signalling	Multiple cancer types
Loss of p53	Mammary stem cells

- c-Myc is a super-TF that regulate the transcription of at least 15% of the entire genome.
- Gene amplification is one of the most frequent mechanisms for *c-MYC* activation in human cancers.

Chen, H. *et. al. Signal Transduction and Targeted Therapy.* **2018**, *3(1),* 1-7
Duffy, M. *et. al. Cancer Treatment Reviews.* **2020**, *94,* 102154-102160

Difficulty of direct-targeting TF strategy

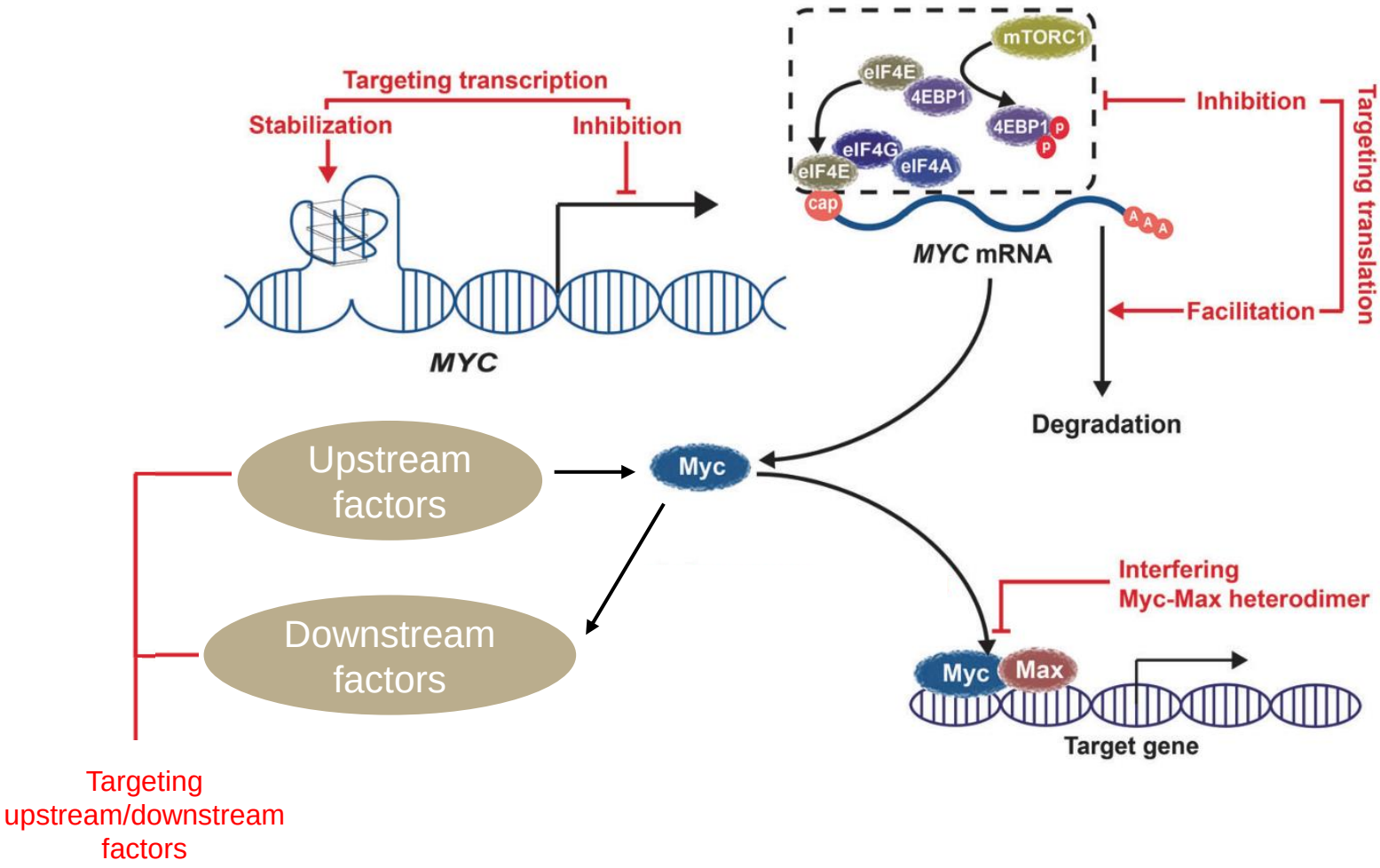


- Enzymes have ligandable pockets and small-molecule inhibitors.
- Development of small-molecule inhibitors for TFs is challenging.

  (← lack of enzymeatic activity and ligandable pockets)

Contents

Existing indirect c-Myc-targeting strategies



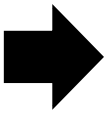
Wang, C. *et. al. Signal Transduction and Targeted Therapy.* **2021**, *6(1),* 1-14

Comparison of each indirect c-Myc-targeting strategy

Target	Classic Compound	advantages	disadvantages
c-MYC gene	Small molecule	・Small molecule	・Off-target ・difficult finding of suitable compounds
c-MYC mRNA	siRNA	・high selectivity ・longer duration of effect ・faster discovery of drug candidates	・low membrane permeability
c-Myc-Max heterodimer	peptide / protein	・suitable for PPI inhibition	・low stability to peptidase ・difficult finding of suitable compounds
Upstream / downstream factors	Small molecule	・Small molecule	・Off-target ・difficult finding of suitable compounds

TF has few small molecule inhibitors.

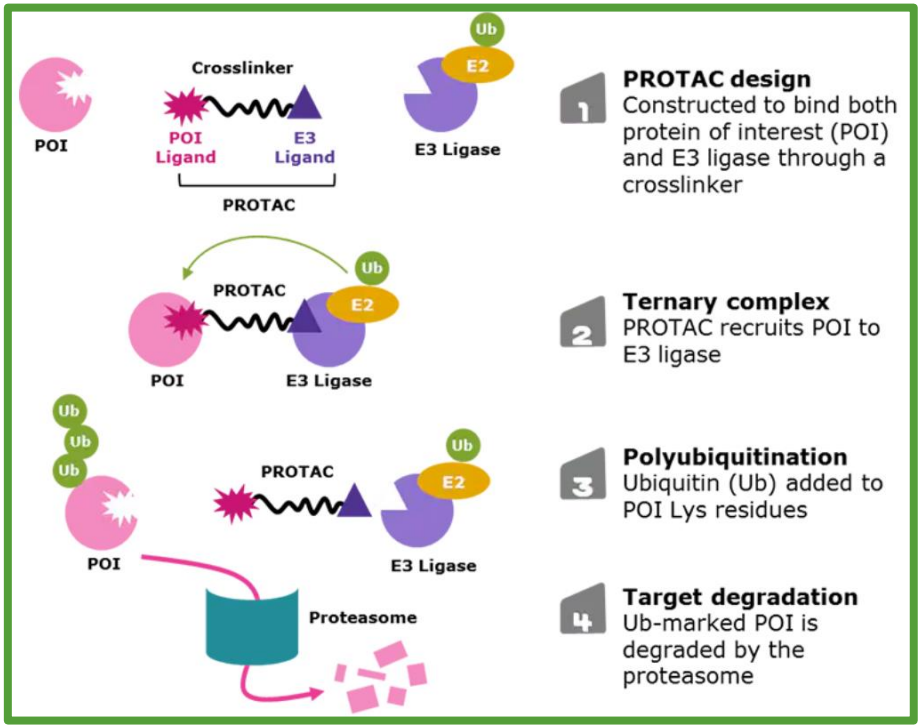
→ Indirect targeting of Myc



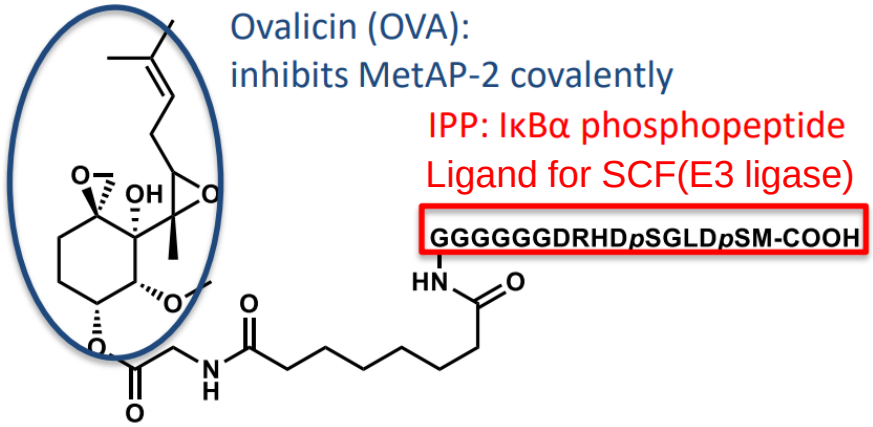
Direct targeting of Myc protein,

PROTAC is difficult.

PROTAC (PROteolysis TArgeting Chimera)

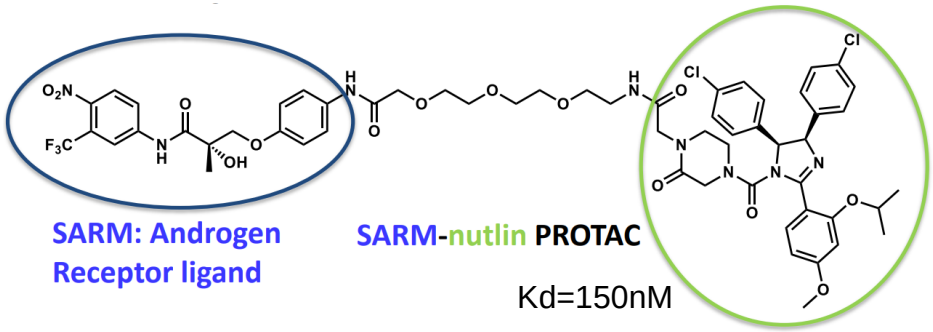


▪Structure of 1st PROTAC



Sakamoto, K M, *et al. Proc. Natl. Acad. Sci. U.S. A.* **2001**, *98*:8554

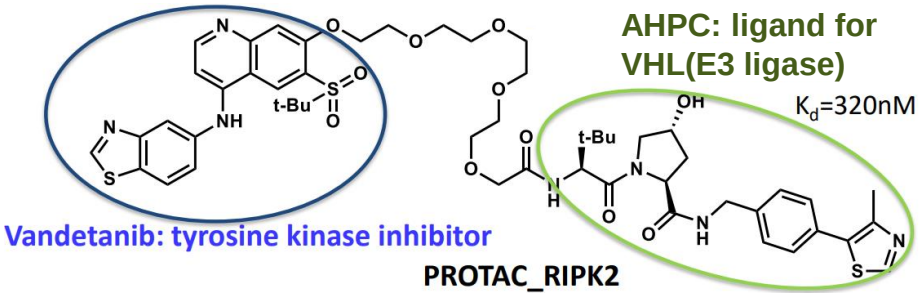
▪Structure of 1st small-molecule PROTAC



Schneekloth, A. R., *et al. Bioorganic & Medicinal Chemistry Letters* **2008,** *18,* 5904-8

Nutlin-3: ligand for MDM2(E3 ligase)

▪Structure of VHL ligand PROTAC



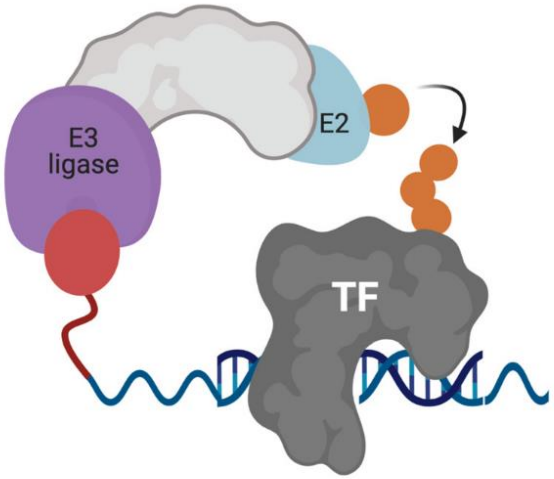
Bondeson, D, P, *et al. Nat. Chem. Biol* **2015,** *11,* 611-617

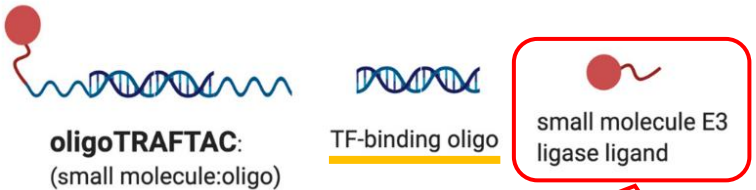
Contents

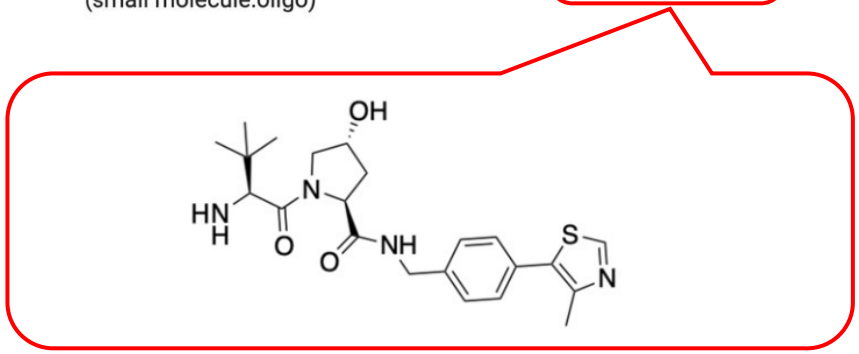
Samarasinghe, K. T. G. *et. al. RSC Chemical Biology.* **2022**, *3(9),* 1144-1153

Oligo-TRAFTAC (TRAnscription Factor TArgeting Chimera)

▪Mechanism

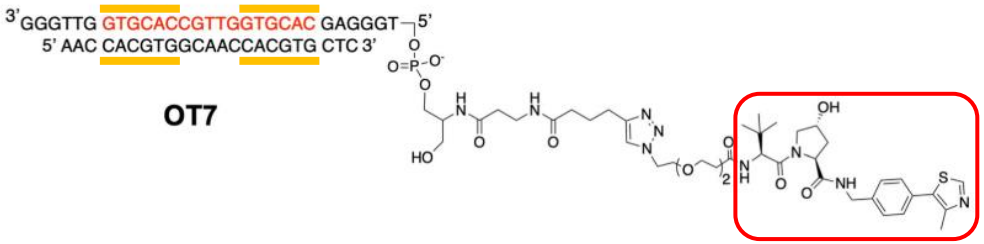




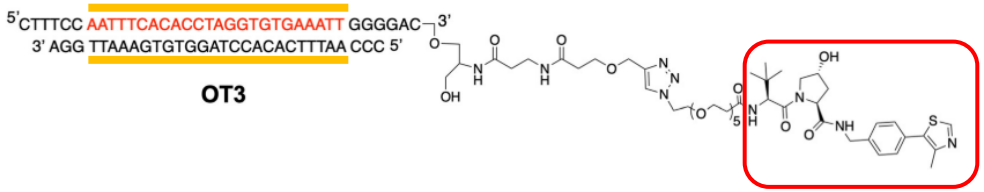


AHPC: VHL (E3 ligase) ligand

▪E-box sequence (CACGTG) (c-Myc)



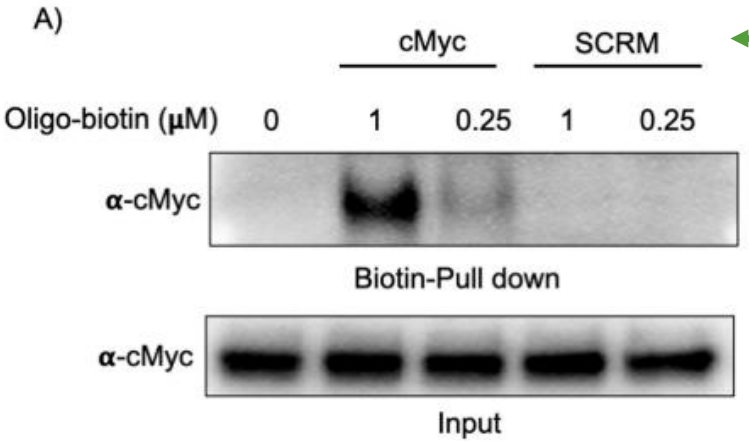
▪Brachyury-binding DNA sequence



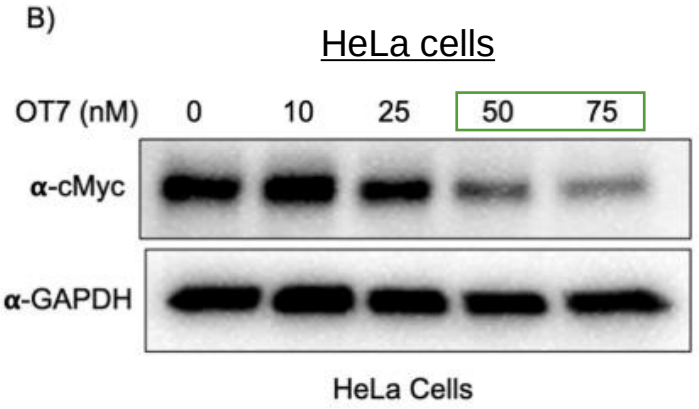
Oligo-TRAFTAC induced c-Myc degradation.

WB

HeLa cell lysates



Target	Oligonucleotide sequence 5' - 3'	Modification	Modification site
c-Myc	TGGGAG **CACGTGGTTGCCACGTG** GTTGGG	Biotin	5'
Scrambled	GTATGT GAGCGGTGGTGGCGTGC CAGCGT	Biotin	5'



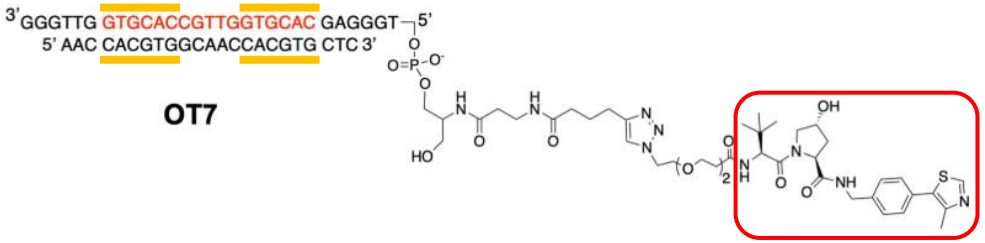
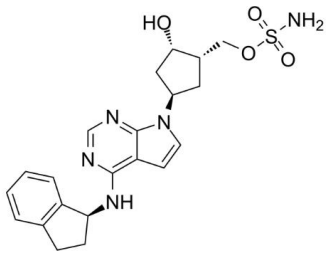


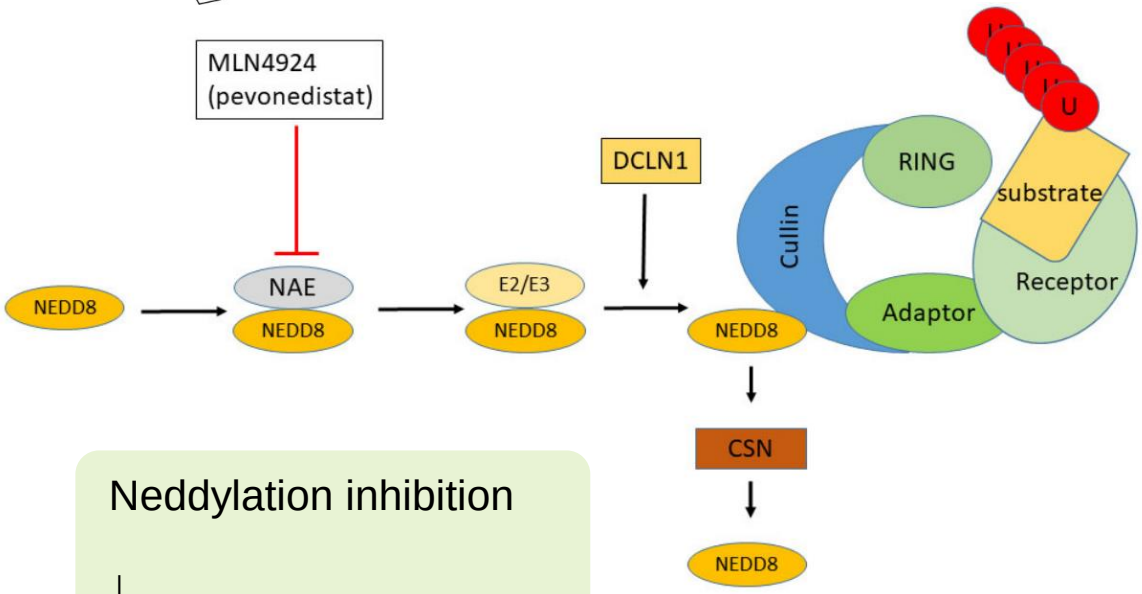
Fig. 2 OligoTRAFTAC induces c-Myc degradation. (A) The oligonucleotide selected for the c-Myc oligoTRAFTAC engages c-Myc. HeLa cell lysates were incubated with biotinylated oligonucleotide, or its scrambled sequence, followed by capture with streptavidin agarose and probing for c-Myc. (n = 2) (B) Dose response of OT7-mediated c-Myc knockdown in HeLa cells. (n = 2)

Neddylation inhibition disrupts cullin RING E3 ligase function.



MLN4924
(pevonedistat)

NAE: NEDD8 Activating Enzyme

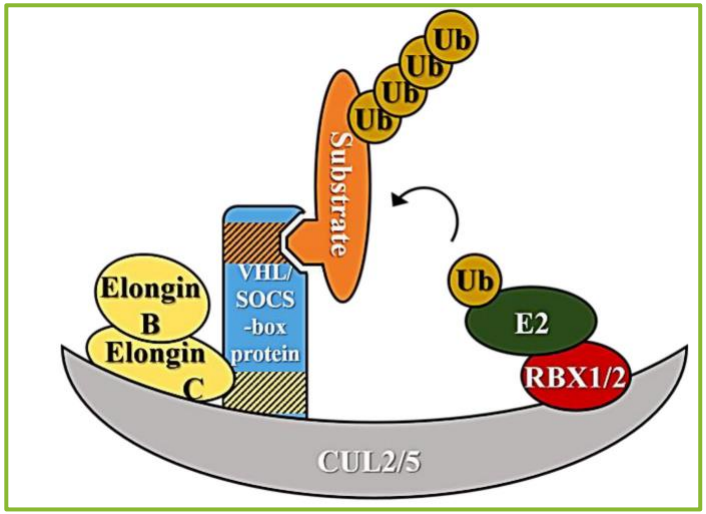


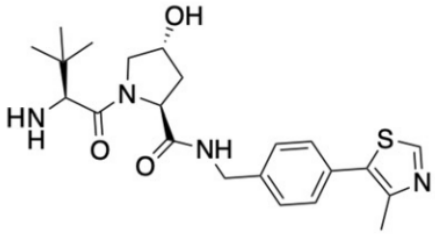
Neddylation inhibition

↓

Disruption of cullin RING E3 ligase function

VHL-CUL2-RBX1 complex



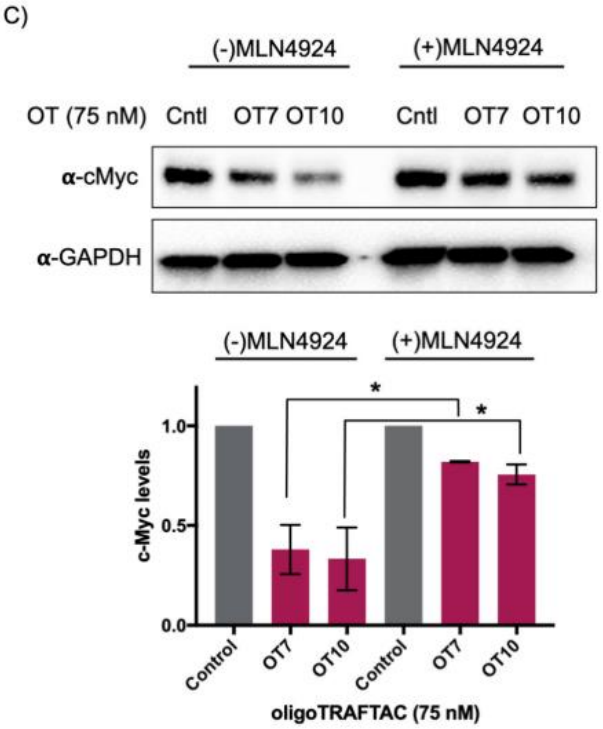


AHPC: VHL (E3 ligase) ligand

Vriend, J., & Nachitigal, M. W. *Cancers.* **2021**, *13(11)*
Fouad, S. *et. al. Frontiers in Physiology.* **2019**, *10(October)*

OT7 induced c-Myc degradation via the proteasomal pathway.

HEK293T cells 1 μM



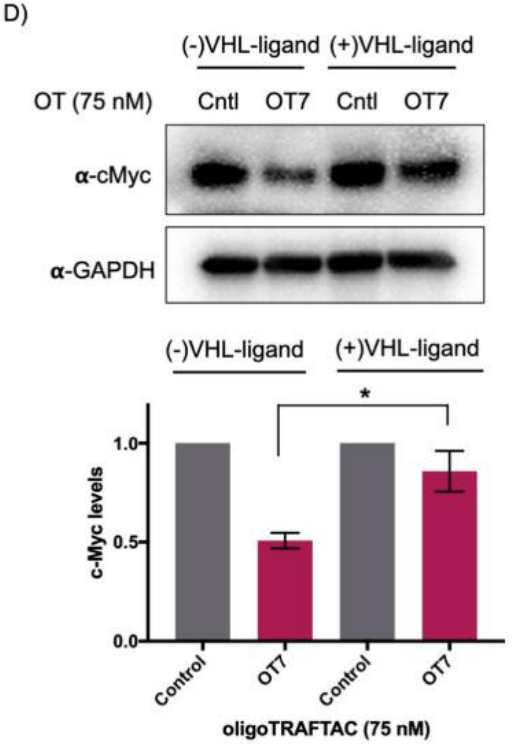
← Neddylation inhibitor

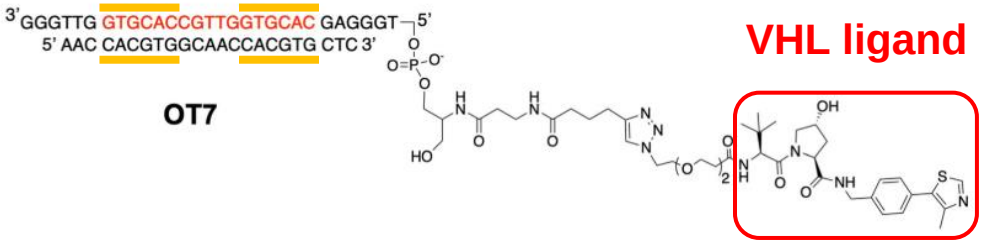
Neddylation inhibition

↓

Disruption of cullin RING E3 ligase function (CUL2)

HEK293T cells 10 μM

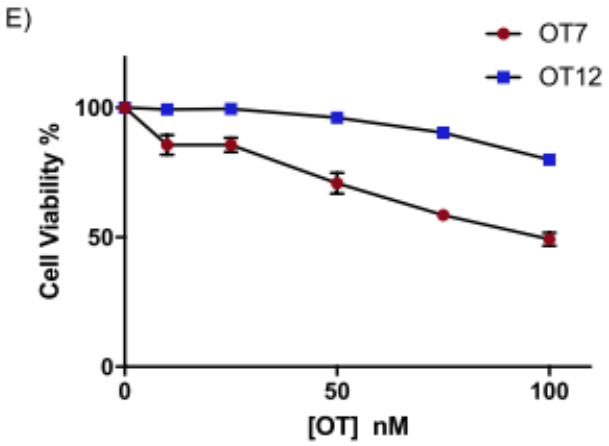




	Modification site
OT7	5‘
OT10	3‘

(C) OligoTRAFTAC-induced c-Myc degradation occurred *via* the proteasomal pathway. HEK293T cells were treated with c-Myc-targeting oligoTRAFTAC with and without the neddylation inhibitor, MLN-4924 (1 μM), and then analyzed for c-Myc levels. ($n = 2$, $*p < 0.05$) (D) HEK293 cells were preincubated with and without 10 μM VHL ligand followed by OT7 transfection and analyzed for c-Myc levels. ($n = 2$, $*p < 0.05$)

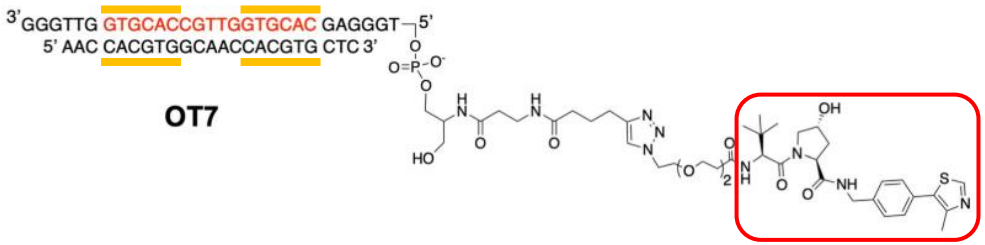
OT7-mediated c-Myc degradation inhibited cell proliferation.



HeLa cells, 48 h

E) HeLa cells were seeded into 96-well plates transfected increasing concentrations of OT7 and OT12. After 48 h, cell viability was monitored using CellTiter-Glo reagent.

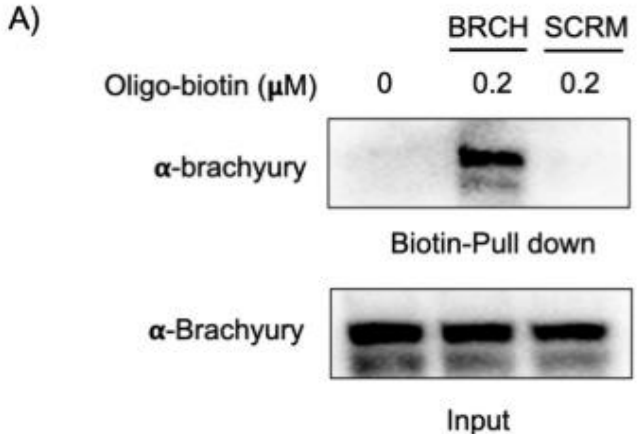
OT#	Target	Oligonucleotide sequence 5' - 3'	Modification	Modification site	Linker PEG, n=
OT7	c-Myc	TGGGAG **CACGTGGTTGCCACGTG** GTTGGG	Alkyne	5'	2
OT12	Scrambled	GTATGT GAGCGGTGGTGGCGTGC CAGCGT	Alkyne	5'	2



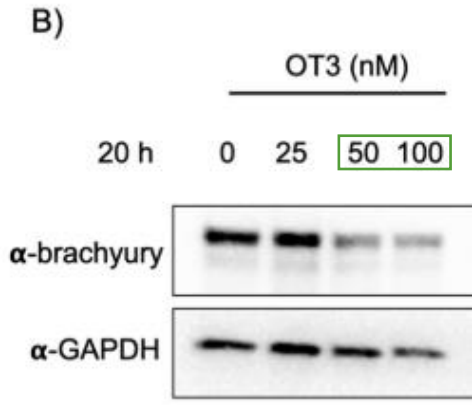
Oligo-TRAFTAC induced brachyury degradation.

WB

Brachyury-GFP expressing HEK293T cell lysates



Target	Oligonucleotide sequence 5' - 3'	Modification	Modification site
Brachyury	CTTTCC**AATTTCACACCTAGGTGTGAAATT** GGGGAC	Biotin	3'
Scrambled	ACGAGACGAGCTCTTAAGTTCCTGGCGTAC TTATAT	Biotin	3'



Brachyury-GFP expressing HEK293T cells

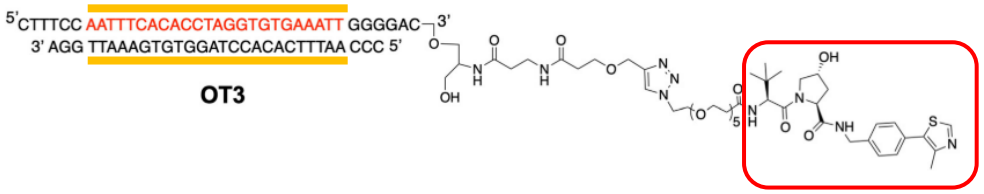
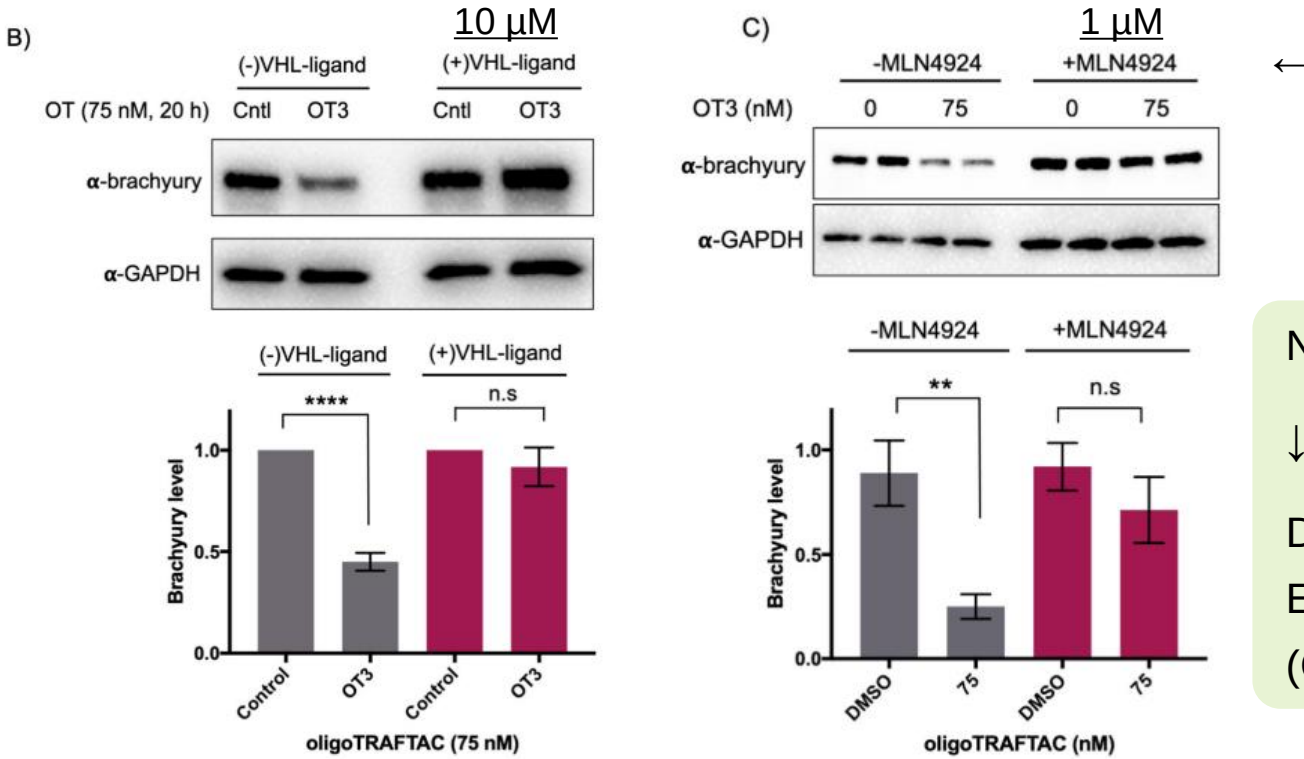


Fig. 3 Brachyury-GFP degradation by oligoTRAFTACs. (A) Brachyury-targeting oligonucleotide used in the oligoTRAFTAC design engaged with brachyury-GFP. Brachyury targeting biotinylated oligonucleotide (BRCH) or its scrambled oligonucleotide (SCRM) incubated with cell lysate and captured by streptavidin agarose beads. (n = 2) (B) Two oligoTRAFTACs with 3′ VHL ligand modifications, OT3 (5 PEG unit linker) and OT4 (2 PEG unit linker) were transfected into HEK293T cells and brachyury-GFP levels were analyzed in lysates prepared after 20 h. (n = 2)

OT3 induced brachyury degradation via the proteasomal pathway.

Brachyury-GFP expressing HEK293T cells



← Neddylation inhibitor

Neddylation inhibition

↓

Disruption of cullin RING E3 ligase function (CUL2)

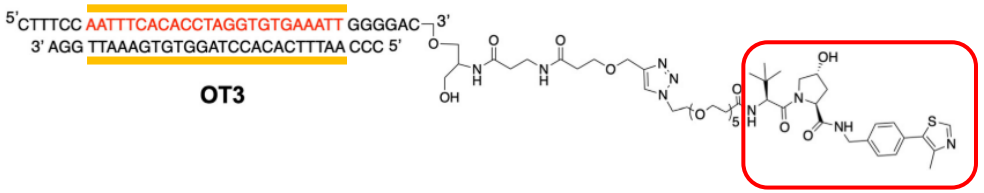
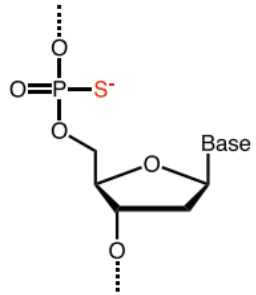


Fig. 4 OligoTRAFTACs induce brachyury-GFP degradation *via* the proteasomal pathway.

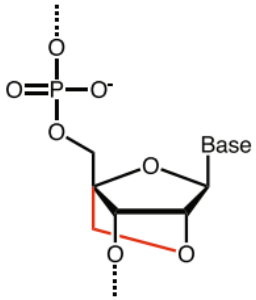
(B) OT3 induced brachyury degradation is VHL-dependent. HEK293T cells were preincubated with and without 10 µM of VHL ligand for 1.5 h prior to OT3 transfection. After 20 h of transfection, cells lysates were prepared and analyzed for brachyury degradation. ($n = 3$, $^{****}p < 0.0001$) (C) OT3 induces brachyury degradation *via* the proteasomal pathway: neddylation inhibitor MLN-4924 was preincubated with cells prior to OT3 transfection. After 20 h of transfection of OT3, cells were harvested and analyzed for brachyury levels. ($n = 2$, $^{**}p < 0.01$)

Xeno Nucleic Acid (XNA)

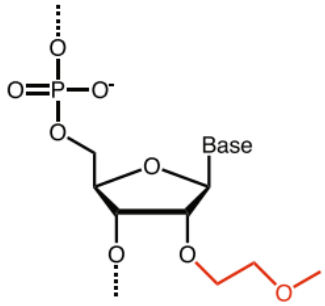
PS



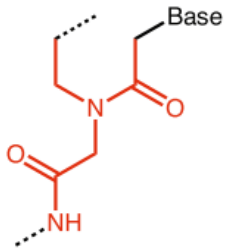
LNA



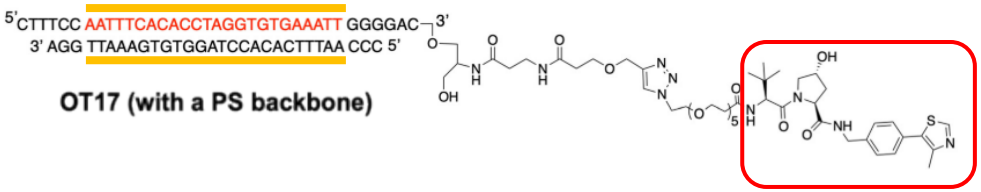
2’ MOE



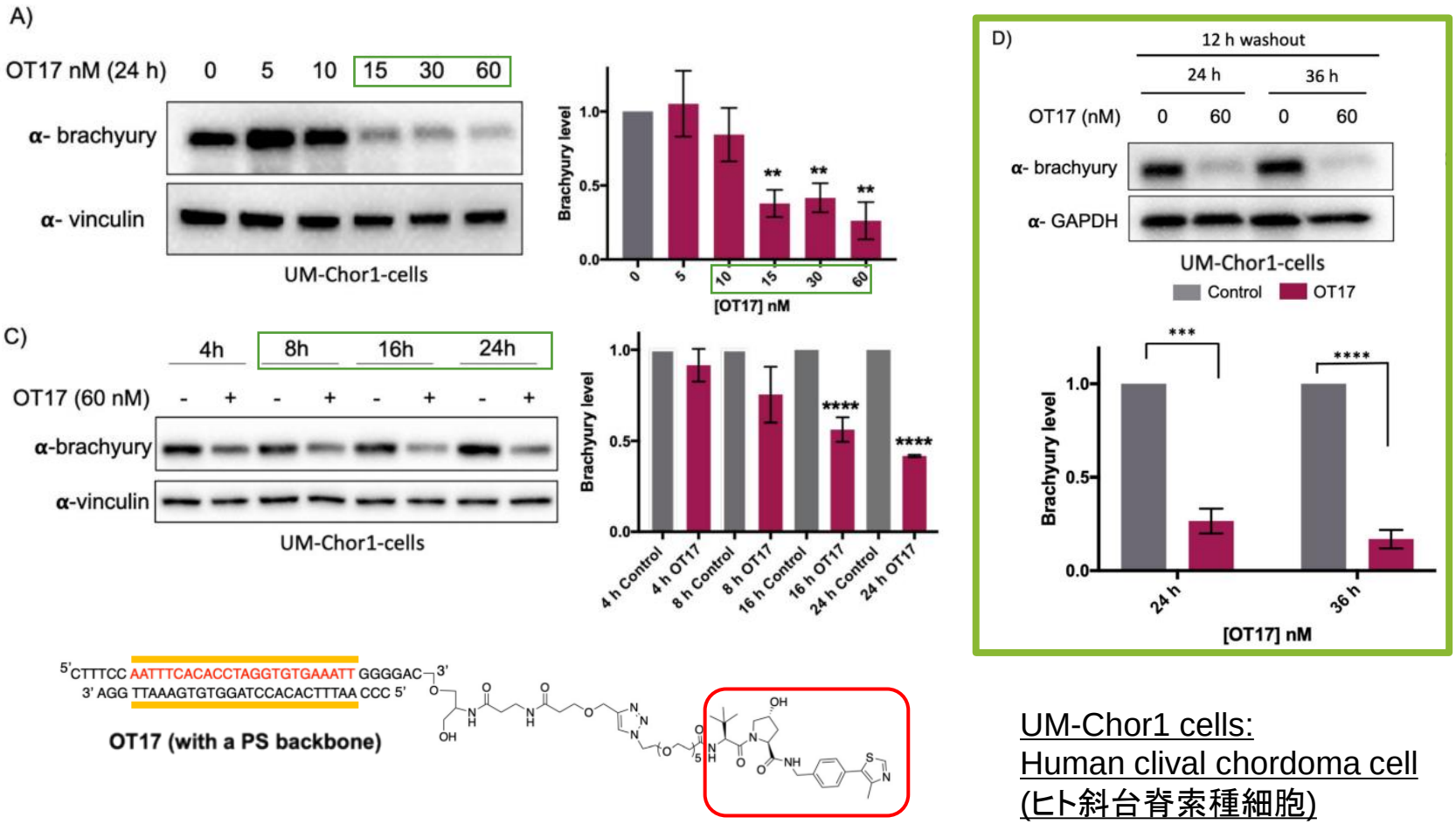
PNA



Increased stability against nucleases



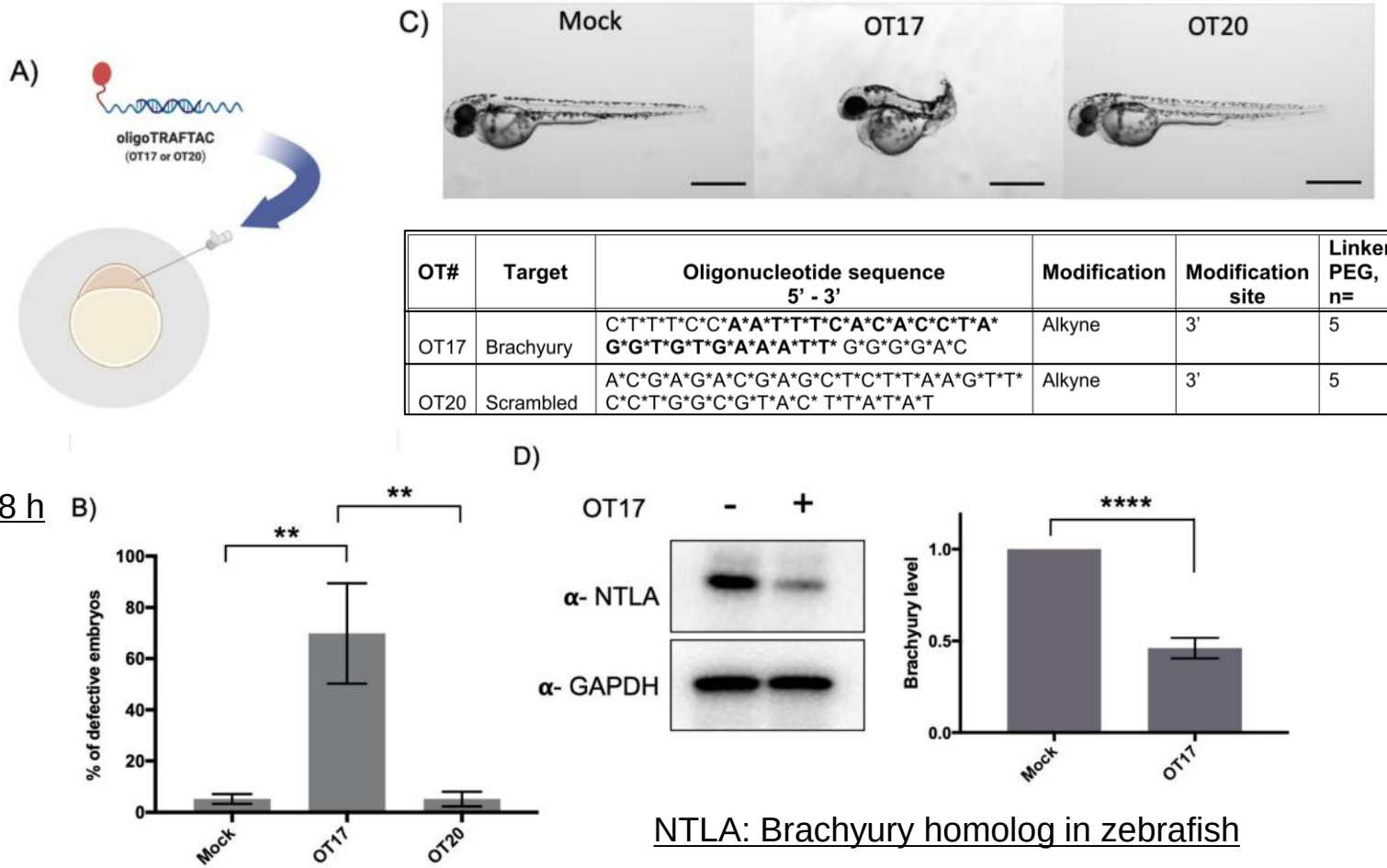
Catalytic brachyury degradability by OT17



UM-Chor1 cells:
Human clival chordoma cell
(ヒト斜台脊索種細胞)

Fig. 5 Endogenous brachyury degradation by oligoTRAFTACs constructed with phosphorothioate backbone. (A) Increasing concentrations of OT17 were transfected into UM-Chor1 cells and harvested after 24 h, subjected to lysis and analyzed for brachyury downregulation. Brachyury levels were normalized to loading control and presented as a bar graph. ($n = 2$, $^{**}p < 0.01$)
C) UM-Chor1 cells were transfected with 60 nM of OT17 and harvested at subsequent different time points as indicated. ($n = 3$, $^{****}p < 0.0001$) (D) Washout experiment: transfection medium was removed after 12 h of OT17 transfection and UM-Chor1 cells were incubated for another 12 h or 24 h in fresh complete cell culture medium. ($n = 2$, $^{***}p < 0.001$, $^{****}p < 0.0001$)

Microinjection of OT17 induced tail deformation.

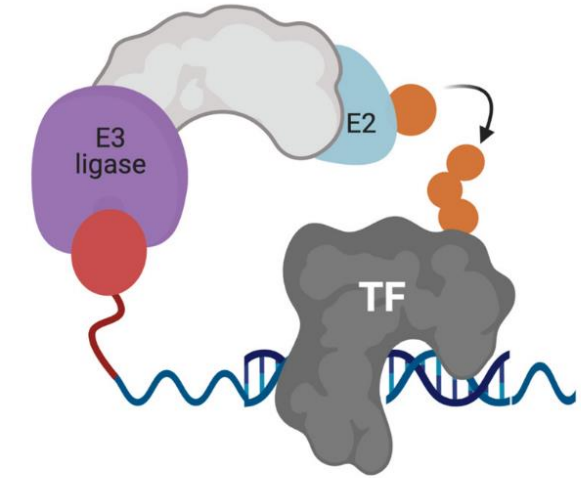


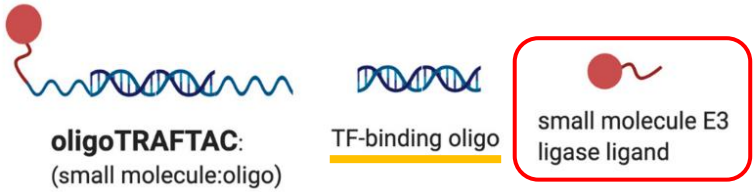
OT#	Target	Oligonucleotide sequence 5' - 3'	Modification	Modification site	Linker PEG, n=
OT17	Brachyury	C*T*T*T*C*C***A*A*T*T*T*C*A*C*A*C*C*T*A*** ***G*G*T*G*T*G*A*A*A*T*T*** G*G*G*G*A*C	Alkyne	3'	5
OT20	Scrambled	A*C*G*A*G*A*C*G*A*G*C*T*C*T*T*A*A*G*T*T* C*C*T*G*G*C*G*T*A*C* T*T*A*T*A*T	Alkyne	3'	5

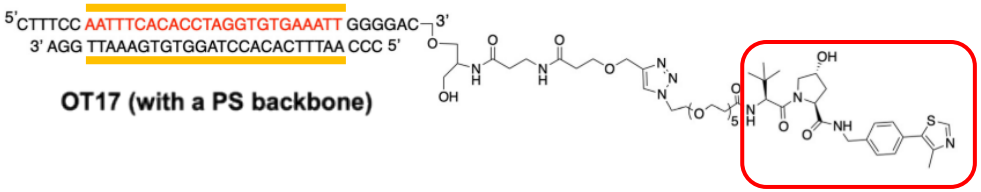
Fig. 6 Microinjection of brachyury-targeting oligoTRAFTAC into zebrafish embryos demonstration of *in vivo* activity. (A) Schematic representation of OT17 and OT20 microinjection into zebrafish embryos. (B) Quantitation of the defective embryos in mock, OT17 and OT20 injected groups. Mock, OT17 and OT20 (180 picoliters from 25 μM of oligoTRAFTACs, or mock equivalent) were microinjected into embryos (number of embryos in each group for three independent experiments; mock-47, 50, 43; OT17-49, 52, 61; OT20-75, 74, 45). After 48 h, the number of defective tails in each group was recorded and presented as percentage in a bar graph. ($n = 3$, $^{**}p < 0.001$) (C) Images of representative zebrafish from the cognate treatment groups. Pictures were captured after 48 h post microinjection of mock, OT17 and OT20. Scale bar 500 μm. ($n = 3$) (D) Brachyury levels in zebrafish embryos after OT17 (180 picoliters from 25 μM of oligoTRAFTACs, or mock equivalent) injection. Embryos were collected at 8–10 somite stage, subjected to lysis, and probed for brachyury levels. ($n = 3$, $^{****}P < 0.0001$).

Contents

Summary







- PS-modified oligo-TRAFTAC improved its in vivo stability and demonstrated oligo-TRAFTAC activity in zebrafish.
- Oligo-TRAFTAC can be rapidly designed for many non-ligandable DNA-binding TFs. (Chemical biology tool and potential therapeutic strategy)

Samarashnghe, K. T. G. *et. al. RSC Chemical Biology.* **2022**, *3(9),* 1144-1153