

1 **Structural basis for inhibition of the Tob-CNOT7 interaction by a fragment**
2 **screening approach**

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5
6 **Supplemental Material**

7
8 **Materials & Methods**

9 **Purification of TobN138 and CNOT7 for inhibitor screening**

10 Human TobN138 cDNA was cloned into a modified pET-28b vector containing an N-
11 terminal His₆-tag and a SUMO cleavage site between the N-terminal tag and the inserted cDNA.
12 *Escherichia coli* Rosetta2 (DE3) transformed with plasmid containing Tob was grown in 2xYT
13 medium at 37°C. Isopropyl β-D-thiogalactopyranoside (IPTG) was added to the *E. coli* culture (1
14 mM) when OD₆₀₀ reached to 0.6. Cells were grown for additional 24hr at 20°C and then
15 harvested by centrifugation. The harvested cells were suspended in buffer A (20 mM Tris-HCl
16 pH 7.9, 500 mM NaCl, 5 mM imidazole). Cells were homogenized with an ultrasonic cell-
17 disruptor instrument (Tommy, Japan). The soluble fraction was separated by centrifugation and
18 loaded into a nickel affinity chromatography (His-Trap HP, GE Healthcare). Protein was eluted
19 with a gradient of 5-500 mM imidazole over 10 column volumes. The fractions containing
20 TobN138 were pooled and subjected to a digestion with SUMO protease in buffer B (20 mM
21 Tris-HCl pH 7.9, 150 mM NaCl) for 12h at 4 °C. The digested products were loaded again in a
22 nickel affinity chromatography to remove the cleaved His₆-tag. The fractions containing
23 TobN138 were further purified on a size exclusion chromatography column (Hiload 16/600

1 Superdex 200 PG, GE Healthcare) equilibrated with buffer B. The fractions containing TobN138
2 monomer were used for the following assays.

3 Human CNOT7 cDNA was cloned into a modified pET-28b vector containing an N-
4 terminal His6-tag. *E. coli* Rosetta2 (DE3) transformed with plasmid containing CNOT7 was
5 grown in 2xYT medium at 37°C. IPTG was added to the *E. coli* culture (1 mM) when OD600
6 reached to 0.6. Cells were grown for additional 24hr at 20°C and then harvested by
7 centrifugation. The harvested cells were suspended in buffer A. Cells were homogenized with an
8 ultrasonic cell-disruptor instrument (Tommy, Japan). The soluble fraction was separated by
9 centrifugation and loaded into a nickel affinity chromatography (His-Trap HP, GE Healthcare).
10 Protein was eluted with a gradient of 5-500 mM imidazole over 10 column volumes. The
11 fractions containing CNOT7 were pooled and further purified on a size exclusion
12 chromatography column (Hiload 16/600 Superdex 200 PG, GE Healthcare) equilibrated with
13 buffer B. The fractions containing CNOT7 monomer were used for the following assays.

14

15 **Screening of fragments that bind to Tob**

16 Fragment screening for Tob was conducted with a Biacore T200 instrument (GE
17 Healthcare, Piscataway, NJ) using a CM5 sensor chip. First, Tob was immobilized on a CM5
18 sensor chip covalently by the amine coupling method in running buffer A (25 mM HEPES pH
19 7.5, 150 mM NaCl, 10 mM MgCl₂ and 0.005% Tween 20). The immobilized level of Tob was
20 about 7500 Response units (RU). The chemical compound library was supplied by the Drug
21 Discovery Initiative (DDI), The University of Tokyo (<http://www.ddi.u-tokyo.ac.jp/en/>) and is

1 comprised of approximately 2000 fragment compounds. The compounds of this fragment library
2 from DDI have the molecular weight of ~250 Da and the solubilities at 200 μ M in the buffer
3 containing 5% DMSO. The fragments were injected at 200 μ M and a flow rate of 30 μ l/min in
4 running buffer B (25 mM HEPES pH 7.5, 150 mM NaCl, 10 mM MgCl₂, 1 mM DTT, 0.005%
5 Tween 20 and 5% DMSO). The binding level of each fragment was corrected with a standard
6 solvent correction (4–6% DMSO in eight steps) in the Biacore T200 evaluation software.

7

8 **Screening of compounds that bind competitively to Tob against CNOT7**

9 Competitive screening for Tob was conducted with a Biacore T200 instrument (GE Healthcare,
10 Piscataway, NJ) using a CM5 sensor chip. Tob was immobilized on a CM5 sensor chip
11 covalently by amine coupling method in the running buffer A. Immobilized level of Tob was
12 about 1000 RU. The fragments at 100 μ M and CNOT7 at 0.3 μ M were injected in the running
13 buffer B and subsequently regeneration buffer (1 M Arg-HCl, pH 4.4) was injected at flow rate
14 of 30 μ l/min. As a positive control, CNOT7 at 0.3 μ M was also injected in the first and final
15 cycles. After solvent correction, the response of each samples was normalized to the positive
16 control in the Biacore T200 evaluation software. The % inhibition is calculated by the following
17 equation; the % inhibition =

18 $[1 - (\text{the response with compounds at } 100 \mu\text{M}) / (\text{the response without compounds})] \times 100$

19

20 **Cloning, expression and purification for crystal screening and *in vitro* assays**

1 To obtain the plasmids encoding GST-TobN138 and GST-CNOT7, the corresponding
2 region of Tob and CNOT7 was separately PCR-amplified and cloned into the pGEX-6P-1 vector
3 (Novagen) via BamHI and XhoI. The plasmids were then individually transformed into *E. coli*
4 BL21 (DE3), which was expressed as a glutathione S-transferase fusion protein. Cells were
5 cultured to an OD600 of 0.5 and induced with 0.5 mM IPTG for 18 h at 16 °C. After cell lysis,
6 the GST-tagged TobN138 protein was applied to a glutathione affinity column (GE Healthcare)
7 and eluted with PBS buffer. The glutathione S-transferase tag was then removed with
8 PreScission Protease at 4 °C, and fractions containing the purest protein were pooled and
9 concentrated (Amicon Ultra, Millipore) and buffer was exchanged with buffer A (20 mM Tris–
10 HCl pH 8.0). For TobN138, protein was purified by anion exchange chromatography (Resource
11 Q, GE Healthcare), eluting with a linear gradient of 0–1 M NaCl in buffer A. Fractions
12 containing the purest recombinant TobN138 protein were pooled then subjected to gel filtration
13 chromatography (Superdex-75 HR, GE Healthcare) using buffer B (20 mM Tris pH 8.0, 150 mM
14 NaCl). The purity of resultant TobN138 was greater than ~95% as determined by SDS–PAGE
15 analysis. To obtain the mutations of several key residues in the CNOT7-binding interface of
16 human Tob, including K63A, W93A and D95A, single point mutations were introduced by the
17 PCR method with the human TobN138 expression plasmid as a template using the Easy
18 Mutagenesis System (TransGen) according to the manufacturer’s instructions. The TobN138
19 mutant proteins and CNOT7 were expressed and purified following the protocol for wild-type
20 Tob described above.

21

22 **Crystallization and data collection**

1 The purified recombinant TobN138 protein was concentrated to 20 mg/ml in
2 crystallization buffer (20 mM Tris–HCl pH 8.0). Optimal crystals were obtained using the
3 hanging-drop, vapor-diffusion technique at 293K with reservoir solutions containing 0.1 M
4 Citric acid pH 5.0, 3.0 M Sodium chloride and 3% v/v Ethylene glycol at a 1:1 ratio. To obtain
5 inhibitor-bound complex structures, native TobN138 crystals were soaked at 293 K for 12 h in a
6 solution containing inhibitor 1 or 6 at a final concentration of 1 mM in 0.1 M Citric acid pH 5.0,
7 3.0 M Sodium chloride and 5% DMSO. Crystals were flash-frozen in liquid nitrogen after
8 soaking in a crystallization buffer containing 25 % glycerol as cryo-protectant. Data were
9 collected at 100K on beamline BL17U of the Shanghai Synchrotron Radiation Facility. Data
10 were processed with the HKL2000 suite of programs (Otwinowski and Minor, 1997).

11

12 **Structure determination and refinement**

13 Crystal structures of TobN138 were determined by molecular replacement using the
14 previous crystal structure of Tob from the Tob-CNOT7 complex structure (PDB ID: 2ZI5) as a
15 search model. The structures were refined by alternate cycles of manual rebuilding by Coot
16 (Emsley et al., 2010) and refinement with PHENIX (Adams et al., 2010). Ligands were
17 automatically fit into electron density using the LigandFit module in PHENIX and manually
18 adjusted according to difference electron density maps during the late stages of refinement. Final
19 structures were validated by MolProbity (Chen et al., 2010).

20

21 ***In vitro* GST pull-down assays**

1 To obtain GST-tagged CNOT7, proteins were purified as mentioned above without
2 removing the GST tag and exchanged with binding buffer (PBS supplemented with 5%
3 glycerol). GST pull-down assays were performed in a final volume of 100 μ l of the binding
4 buffer using 5 μ M of purified GST-tagged CNOT7 and TobN138 (wild type and mutants). The
5 purified GST-tagged CNOT7 were incubated with glutathione beads for 30 min at 4 $^{\circ}$ C before
6 adding TobN138 (wild type, K63A and D95A mutants) followed by another 2 hrs incubation at 4
7 $^{\circ}$ C. After washing five times with the binding buffer, the beads were analysed on a 13% SDS-
8 PAGE gel.

9

10 **ITC experiments**

11 The binding affinities of TobN138 (wild type and K63A mutant) for CNOT7 were
12 assayed with an ITC-200 microcalorimeter (MicroCal) device at room temperature. CNOT7 was
13 placed in the reaction cell at a concentration of 0.05 mM in buffer C (20 mM HEPES pH 7.5,
14 150 mM NaCl with 5% DMSO). For each binding assay, TobN138 (wild type and K63A mutant)
15 at a concentration of 0.45 mM were titrated into CNOT7 samples. The titration consisted of an
16 initial injection of 0.4 μ L followed by 20 injections of 1.5 μ L every 150 s. The titration data and
17 binding plot were analyzed with MicroCal Origin.

18

19 ***In vitro* deadenylation assays**

1 The purified and concentrated CNOT7 proteins (Figure 8a) were diluted in the
2 deadenylase buffer and incubated with a poly(A) RNA substrate at 37 °C for 40 min. The wild
3 type and mutant human Tob proteins and human CNOT7 were purified as described and dialyzed
4 against the deadenylase buffer (50 mM Tris–HCl, 150 mM NaCl, 10% glycerol, 1 mM DTT, 5
5 mM MgCl₂ at pH 7.5). These proteins were incubated at 37 °C for 30 minutes with a
6 synthesized poly(A) RNA substrate (5'-UCUAAAUAAAAAAAAAAAAAAAAAAAAAA-3'; final
7 concentration 0.5mM) labeled with fluorescein isothiocyanate at the 5' end (Morita et al., 2007).
8 After addition of formamide, the reaction mixtures were fractionated on a 7 M urea-25%
9 sequence polyacrylamide denaturing gel. The products were analyzed and quantified with a
10 fluorescence imager, Typhoon Trio (GE).

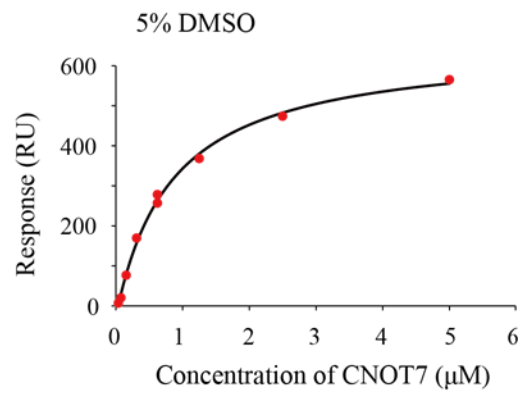
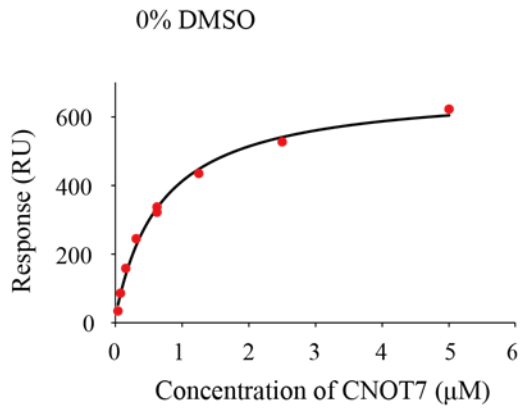
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1 **Figure S2**

2



3

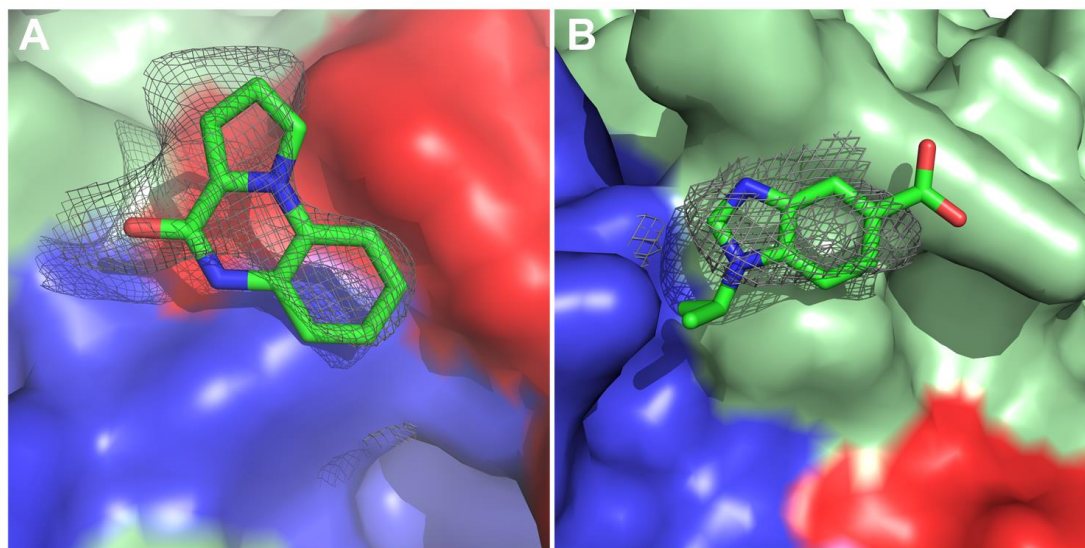
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5 Tolerance of Tob stability in a buffer containing 0% DMSO (top) and 5% DMSO (bottom)

6

7

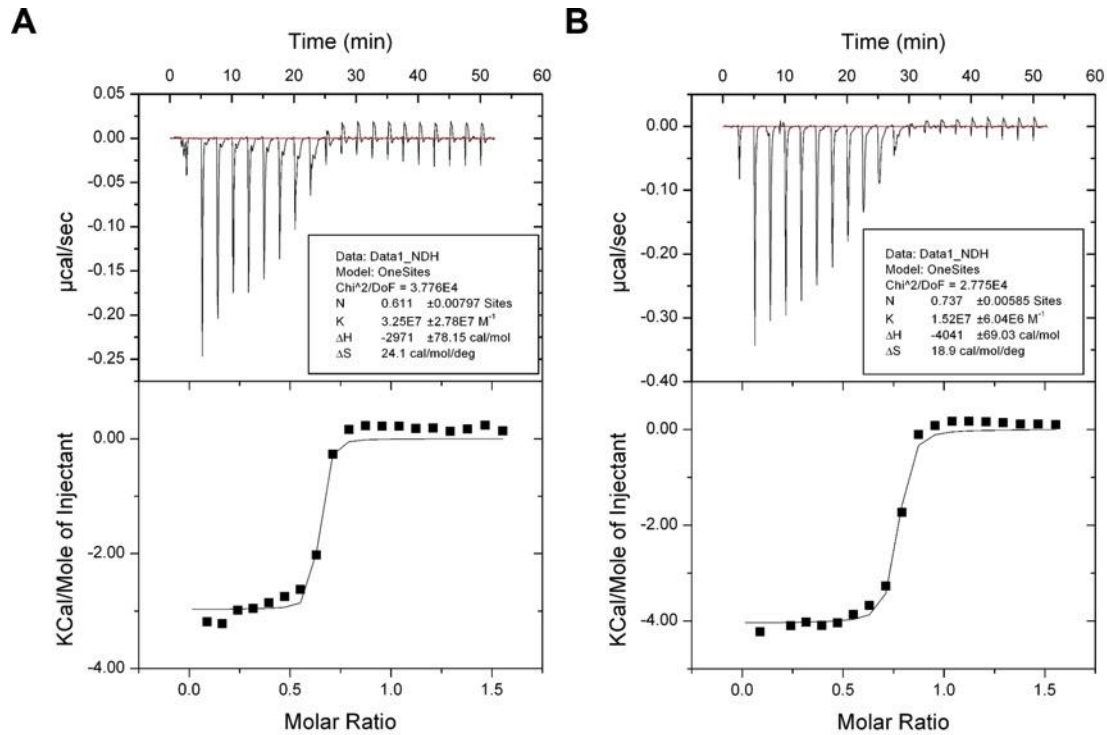
1 **Figure S3**



4 Electron density for (a) inhibitor 1 and (b) inhibitor 6. 2mFo-DFc composite omit maps are shown
5 contoured at 1σ and coloured as a grey mesh. A surface representation of Tob is also shown and coloured
6 according to the scheme in Figure 4 (Tob: pale green; Box A: red; Box B: blue).

7

1 **Figure S4**



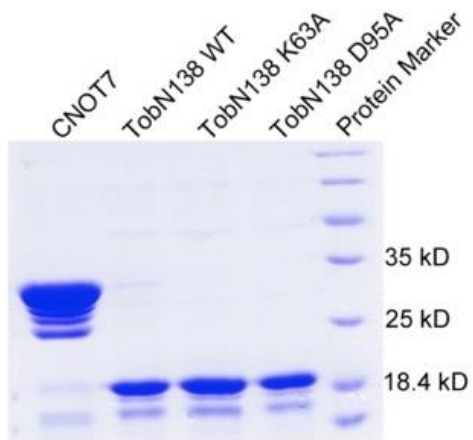
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3 CNOT7 binding of TobN138 wild type and K63A mutant assayed with ITC. (a) CNOT7 titrated

4 with TobN138 wild type. (b) CNOT7 titrated with TobN138 K63A mutant.

5

1 **Figure S5**

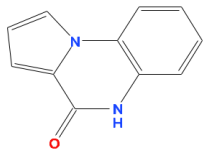
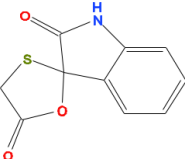
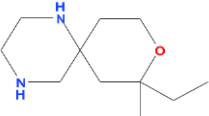
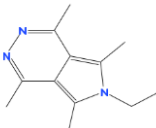
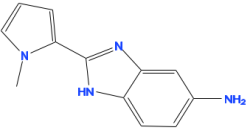
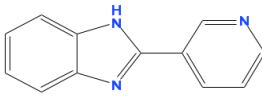
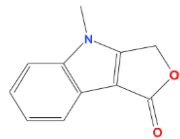
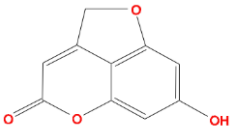
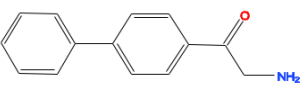
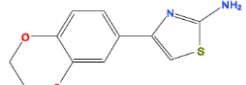
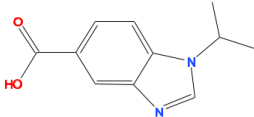
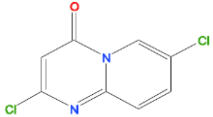
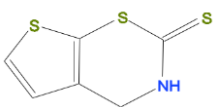
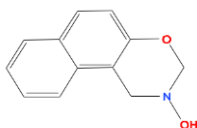
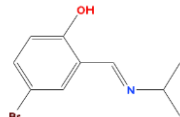
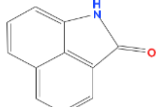
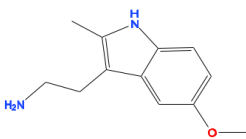
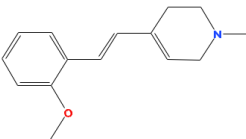
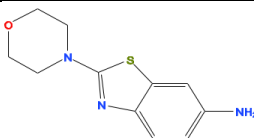
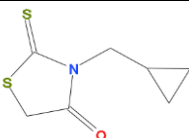


2

3 Protein profile of CNOT7, wild-type TobN138 and K63A, D95A mutants. The migration of a
4 molecular weight size marker is indicated. According to the results of SDS-PAGE and UV
5 spectrophotometer, proteins were diluted to the same concentration for deadenylase assay.

6

- 1 **Table S1: List of hit compounds.** The rate of inhibition of the Tob-CNOT7 interaction is shown
- 2 for each compound at 100 μM .
- 3

Compound		% inhibition	Compound		% inhibition
1		96.4	11		29.9
2		61.2	12		29.5
3		60.2	13		29.2
4		49.1	14		27.4
5		46.2	15		27
6		43.0	16		26.2
7		40.0	17		26.2
8		35.1	18		26.1
9		34.3	19		24.4
10		31.0	20		23.9

1 **Table S2: Data collection and refinement statistics**

Parameter	Tob-i1	Tob-i6
Data collection		
Space group	<i>P</i> 6 ₁ 22	<i>P</i> 6 ₁ 22
Cell dimensions a/b/c (Å)	65.0/65.0/163.7	65.1/65.1/162.9
Resolution (Å)	50-2.31 (2.35-2.31)	50-2.3 (2.35-2.30)
Average I/σ (I) ¹	52.6 (1.4)	32.6 (5.9)
Total reflections	110,471	180,073
Unique reflections	8,867	9,738
Completeness (%) ¹	94.4 (88.5)	99.6 (99.2)
Redundancy	12.5 (10.6)	18.5 (17.5)
<i>R</i> _{merge} (%) ^{1,2}	9.4 (59.6)	7.8 (48.9)
Structure refinement		
Resolution (Å)	50-2.3	50-2.3
<i>R</i> _{work} / <i>R</i> _{free} (%) ³	23.2/27.8	21.4/26.5
R.m.s.d. bond lengths (Å) ⁴	0.009	0.007
R.m.s.d. bond angles (°) ⁴	1.479	1.020
Ramachandran plot		
Favored (%)	98.3	94.7
Outliers (%)	0	0
MolProbity score	2.19	2.20

¹ Numbers in parentheses correspond to the highest-resolution shell.

² $R_{\text{merge}} = \sum_i |I_i - \langle I \rangle| / \sum_i I_i$, where I_i is an individual intensity measurement and $\langle I \rangle$ is the average intensity for all the reflections.

³ $R_{\text{work}}/R_{\text{free}} = \sum ||F_o| - |F_c|| / \sum |F_o|$, where F_o and F_c are the observed and calculated structure factors, respectively.

⁴ R.m.s. deviations relate to the Engh and Huber parameters.

1 **Table S3: Interaction properties between Tob and CNOT7 calculated by ITC.**

Tob	K_d (M⁻¹)	N (sites)
Wild-type	3.25E7 ±2.18E7	0.611 ±0.00797
K63A	1.52E7 ±6.04E6	0.737 ±0.00585

2

3

4

5

6

7