Supporting Information

Yeast display evolution of a kinetically efficient 13-amino acid substrate for lipoic acid ligase

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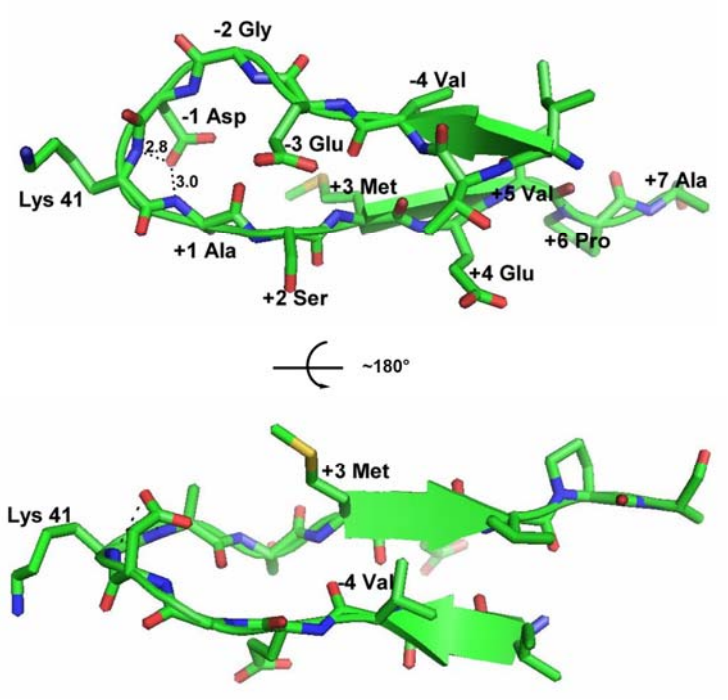


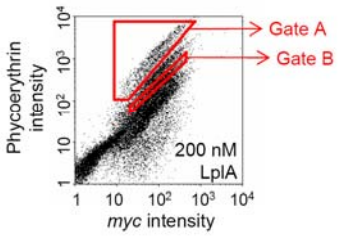
Figure S1. NMR structure of the E2p domain of *E. coli* pyruvate dehydrogenase (PDB 1QJO).[1] β-strands 4 and 5 are shown with the lipoylation site at Lys41. (Top) Hydrogen bonds between the sidechain of -1 Asp and the backbone NH groups of Lys41 and +1 Ala are indicated by dashed lines. (Bottom) β-strands 4 and 5 are shown in a different orientation. +3 Met and -4 Val sidechains point in the same direction. Based on this, we suggest that the sidechains of +3 Tyr and -4 Phe in our engineered LAP2 sequence may stack together.

A

Clones after Round 2	
Clone Name	*Sequence*
LAP2.1	MEMNKVWRDLAA
LAP2.2	IDQDKFWRELGS
LAP2.3	LEGDKVWLEVRS
LAP2.4	IEVDKVQHDLLS
LAP2.5	FDEHKLWYELAA
LAP2.6	LDVDKFREEVAS

Clones after Round 3	
Clone Name	*Sequence*
LAP3.1	LERNKVWYEIKA
LAP3.2	IEVDKSWLELRS
LAP3.3	MELDKAWVEVWS
LAP3.4	IDIDKIWYEFGS
LAP3.5	FENDKIWHDIWA
LAP3.6	IQGDKIWTELDS
LAP3.7	FEYDKVWVDLPA

B



Clones after Round 4 (Gate A)	
Clone Name	*Sequence*
LAP4.1	FELDKVWFDVDS
(2x) LAP4.2	FEIDKVWHDFPA
(5x) LAP4.3	FEHEKVWYDLCA
(3x) LAP4.4	FEINKVWFELLA
Clones after Round 4 (Gate B)	
Clone Name	*Sequence*
B1	VEHDKVFYEFDS
B2	IEIDKVWHDLYS
B3	LEIDKVWHELDS
B4	IELYKVWYEIDA
B5	LEEDKIWYEFEA
B6	VERDKVWYDISS
B7	MERAKVWYELEA

C

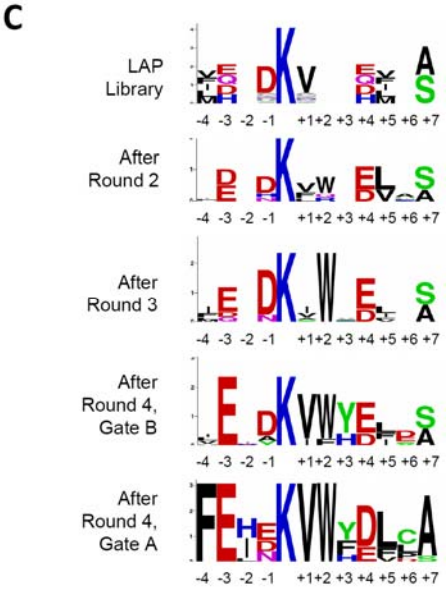


Figure S2. LAP sequences after each round of selection. (**A**) Sequences of LAP clones after rounds 2 and 3. Lipoylated lysine is underlined. (**B**) Comparison of clones obtained from two different sorting gates in round 4. Several clones from the higher gate (Gate A) appeared multiple times. (**C**) Diagrams illustrating amino acid frequencies at specific positions in the original library (based on library design), and after rounds 2-4 (based on sequences of isolated clones). Generated using http://weblogo.berkeley.edu/

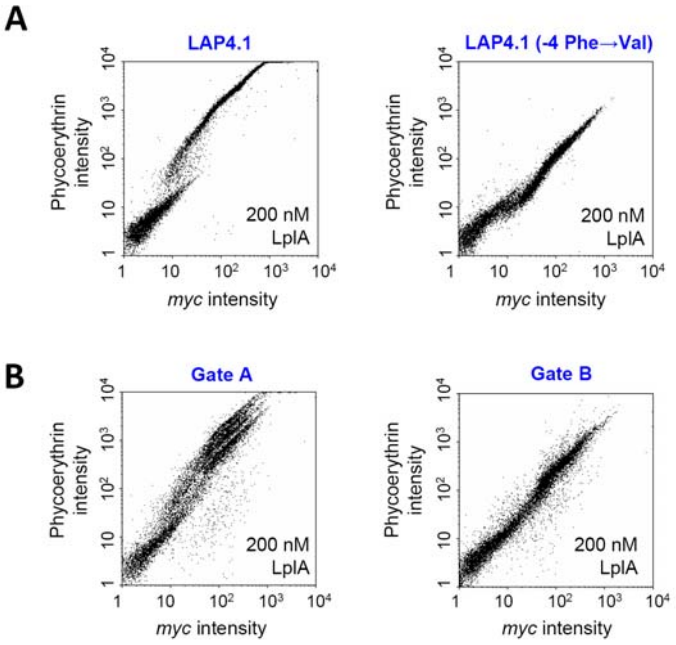


Figure S3. Contribution of -4 Phe to LAP recognition by LplA. (**A**) The -4 Phe→Val mutant of LAP4.1 was prepared, and compared to LAP4.1 in a yeast cell surface lipoylation assay with 200 nM LplA. (**B**) For comparison, the same assay was performed with Gate A and Gate B yeast pools, obtained from the fourth round of selection.

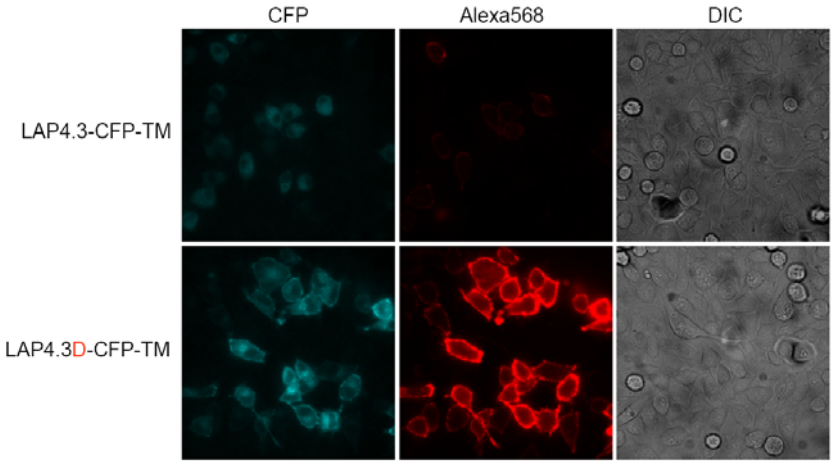
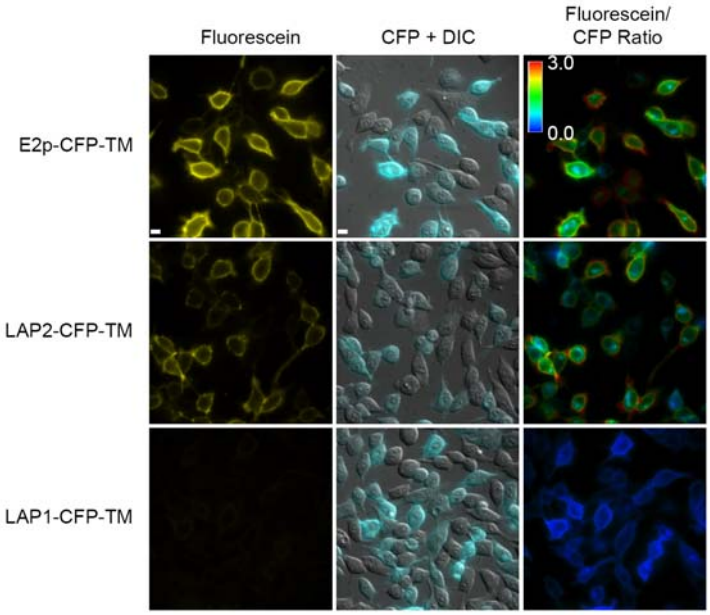


Figure S4. Cell surface lipoylation of LAP4.3 vs. LAP4.3D. HeLa cells expressing either LAP4.3-CFP-TM or LAP4.3D-CFP-TM were lipoylated with 1 μM LplA for 10 minutes. Lipoylation was detected with Alexa568-conjugated anti-lipoic acid antibody.

A



B

	Fluorescein/CFP ratio	R^2
LAP1-CFP-TM	0.0452	0.5768
LAP4.1-CFP-TM	0.8704	0.5233
LAP4.2-CFP-TM	1.3297	0.3818
LAP4.3D-CFP-TM	1.0158	0.283
LAP2-CFP-TM	1.345	0.3396
E2p-CFP-TM	1.3718	0.6728

Figure S5. Cell surface lipoylation of LAP sequences and E2p. (**A**) HEK cells expressing CFP-TM fusions to E2p, LAP2, or LAP1 were labeled with 1 μM LplA for 10 minutes, before staining with anti-lipoic acid antibody followed by fluorescein-conjugated secondary antibody.[2] The right column shows fluorescein/CFP ratio images, reflecting lipoylation efficiency. Red indicates a fluorescein/CFP intensity ratio of ~3.0 or greater. Scale bar, 10 μm. (**B**) HEK cells expressing CFP-TM fusions to various LAP sequences or E2p were labeled and imaged as in (A). Single cell mean fluorescein/CFP intensity ratios were tabulated for >160 cells from >18 fields of view. These ratios were plotted, and the slopes and R^2 values are shown in the table.

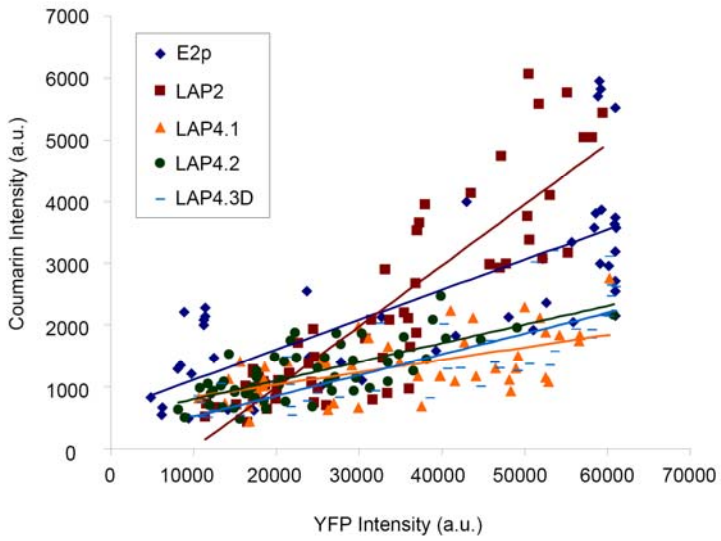


Figure S6. Comparison of LAP sequences for intracellular protein labeling with a coumarin fluorophore ligase.[3] LAP sequences or E2p were fused to Yellow Fluorescent Protein (YFP) and expressed in the nuclei of HEK293T cells. The fusion proteins were labeled for 10 minutes with 7-hydroxycoumarin using an engineered coumarin fluorophore ligase.[3] To evaluate labeling efficiency, the mean coumarin intensity was plotted against the mean YFP intensity, for single cells. A high coumarin/YFP ratio signifies high labeling yield. We note that LAP2-YFP expression levels were comparable to E2p-YFP expression levels in this assay.

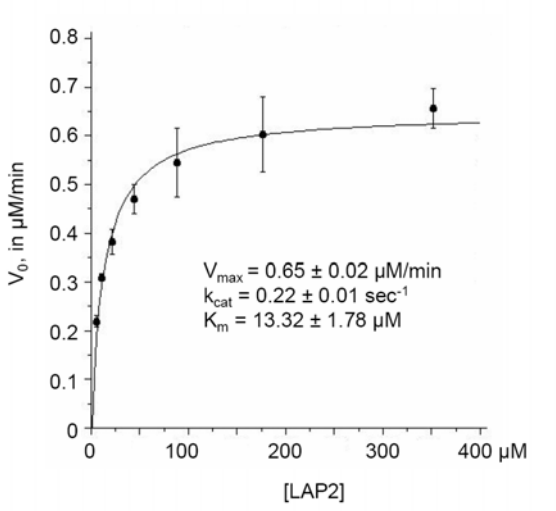


Figure S7. LAP2 kinetics. Various concentrations of synthetic LAP2 peptide (not a fusion protein) were lipoylated with 50 nM LplA, 750 µM lipoic acid, and 3 mM ATP, and initial reaction rates were measured by HPLC. The Michaelis-Menten curve shows the intial rates plotted as a function of LAP2 concentration. Measurements were performed in triplicate. Error bars, ± 1 s.d.

Peptide	Forward oligos
LAP4.1	5'CTAGCGGATTTGAACTTGATAAAGTATGGTTTGATGTCGATTCAC
LAP4.2	5'CTAGCGGATTCGAGATTGATAAAGTATGGCATGATTTCCCTGCAC
LAP4.3D	5'CTAGCGGATTTGAGCATGAGAAAGTTTGGTATGATCTCGATGCGC
LAP2	5'CTAGCGGCTTCGAGATCGACAAGGTGTGGTACGACCTGGACGCCC
LAP2-C	5' CTAGCGGCTTCGAGATCGACAAGGTGTGGTACGACCTGGACGCCTAAGAG

Peptide	Reverse oligos
LAP4.1	5'AATTGTGAATCGACATCAAACCATACTTTATCAAGTTCAAATCCG
LAP4.2	5'AATTGTGCAGGGAAATCATGCCATACTTTATCAATCTCGAATCCG
LAP4.3D	5'AATTGCGCATCGAGATCATACCAAACTTTCTCATGCTCAAATCCG
LAP2	5'AATTGGGCGTCCAGGTCGTACCACACCTTGTCGATCTCGAAGCCG
LAP2-C	5'GATCCTCTTAGGCGTCCAGGTCGTACCACACCTTGTCGATCTCGAAGCCG

Table S1. Forward and reverse primers used in cloning LAP-HP1 fusion proteins.

Complete author list for Reference 30

(30) Los, G. V.; Encell, L. P.; McDougall, M. G.; Hartzell, D. D.; Karassina, N.; Zimprich, C.; Wood, M. G.; Learish, R.; Ohana, R. F.; Urh, M.; Simpson, D.; Mendez, J.; Zimmerman, K.; Otto, P.; Vidugiris, G.; Zhu, J.; Darzins, A.; Klaubert, D. H.; Bulleit, R. F.; Wood, K. V. *ACS Chem. Biol.* **2008**, *3*, 373-382.

Reference List

(1) Jones, D. D.; Stott, K. M.; Howard, M. J.; Perham, R. N. *Biochemistry* **2000**, *39*, 8448-8459.

(2) We note that the surface expression levels of TM fusions to LAP peptides are ~2-fold lower than TM-fused E2p. However, expression levels of intracellular proteins are similar, whether fused to a LAP sequence or E2p.

(3) Baruah, H., Uttamapinant, C., White, K. A., Fernández-Suárez, M., Puthenveetil, S., Thompson, S, and Ting, A. Y. Ref Type: Unpublished