Cellular-Based Selections Aid Yeast-Display Discovery of Genuine Cell-Binding Ligands: Targeting Oncology Vascular Biomarker CD276

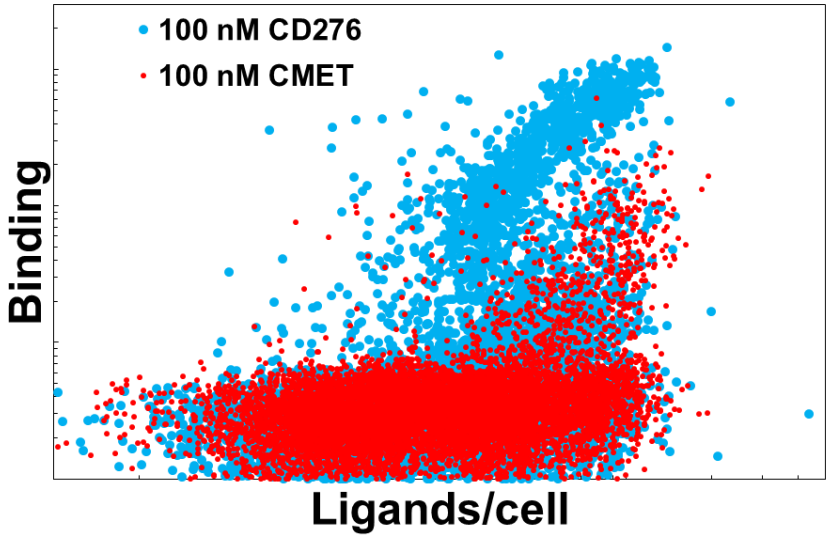
Lawrence A. Stern[1], Patrick S. Lown[1], Alexandra C. Kobe[1], Lotfi Abou-Elkacem[2], Juergen K. Willmann[2], and Benjamin J. Hackel[1]

[1] Department of Chemical Engineering and Materials Science, University of Minnesota–Twin Cities, Minneapolis, MN

[2]Department of Radiology, Stanford University, Palo Alto, CA

Corresponding Author:
Benjamin J. Hackel
421 Washington Avenue SE
356 Amundson Hall
Minneapolis, MN 55455
612.624.7102
hackel@umn.edu

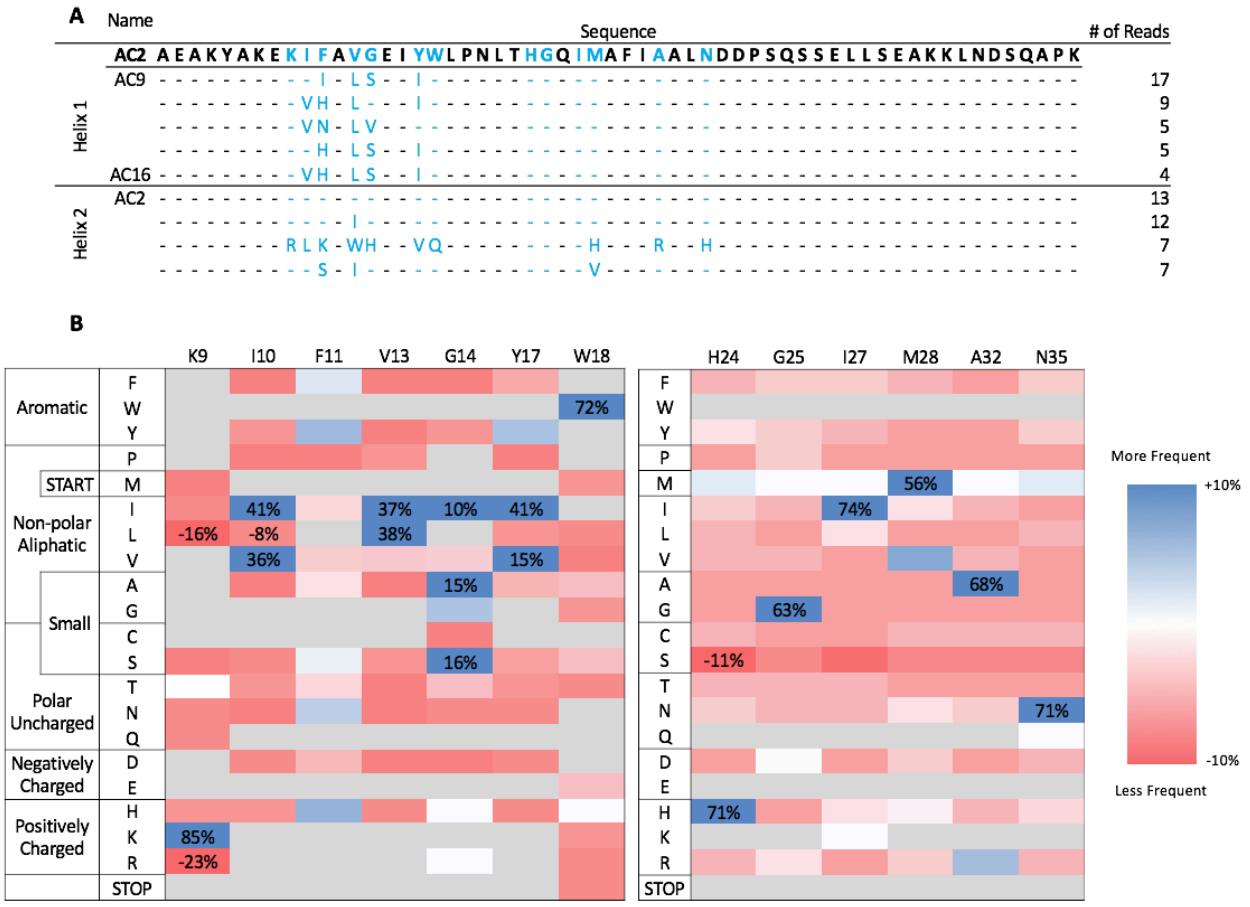
Supplemental Figures



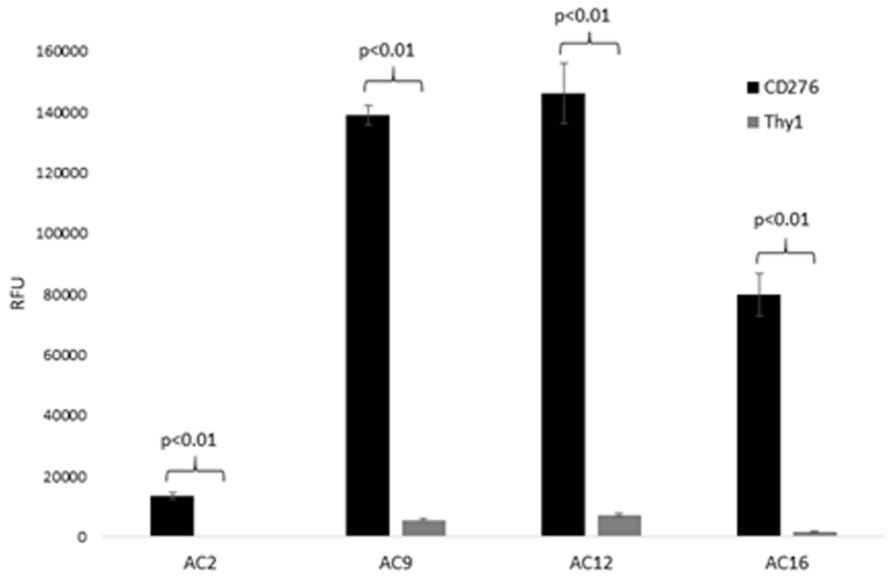
Supplemental Figure 1. *Assessment of specificity of fibronectin population against soluble CD276 extracellular domain.* The population of fibronectin domains selected by magnetic bead sorting against soluble CD276 extracellular domain was assessed for target specificity. The population was labeled by either 100 nM soluble CD276 extracellular domain (blue) or 100 nM soluble CMET extracellular domain (red).

		Library	
		Helix 1	Helix 2
Site	K9	HIKLMNQRS	K
	I10	ADFHILNPSTVY	I
	F11	ADFHILNPSTVY	F
	V13	ADFHILNPSTVY	V
	G14	ACDFGINSTVY	G
	Y17	ADFHILNPSTVY	Y
	W18	AEGKLMRSTVW	W
	H24	H	ACDFGHILNPRSTVY
	G25	G	ACDFGHILNPRSTVY
	I27	I	ACDFGHILNPRSTVY
	N28	N	ACDFGHILNPRSTVY
	A32	A	ACDFGHILNPRSTVY
	N35	N	ACDFGHILNPRSTVY

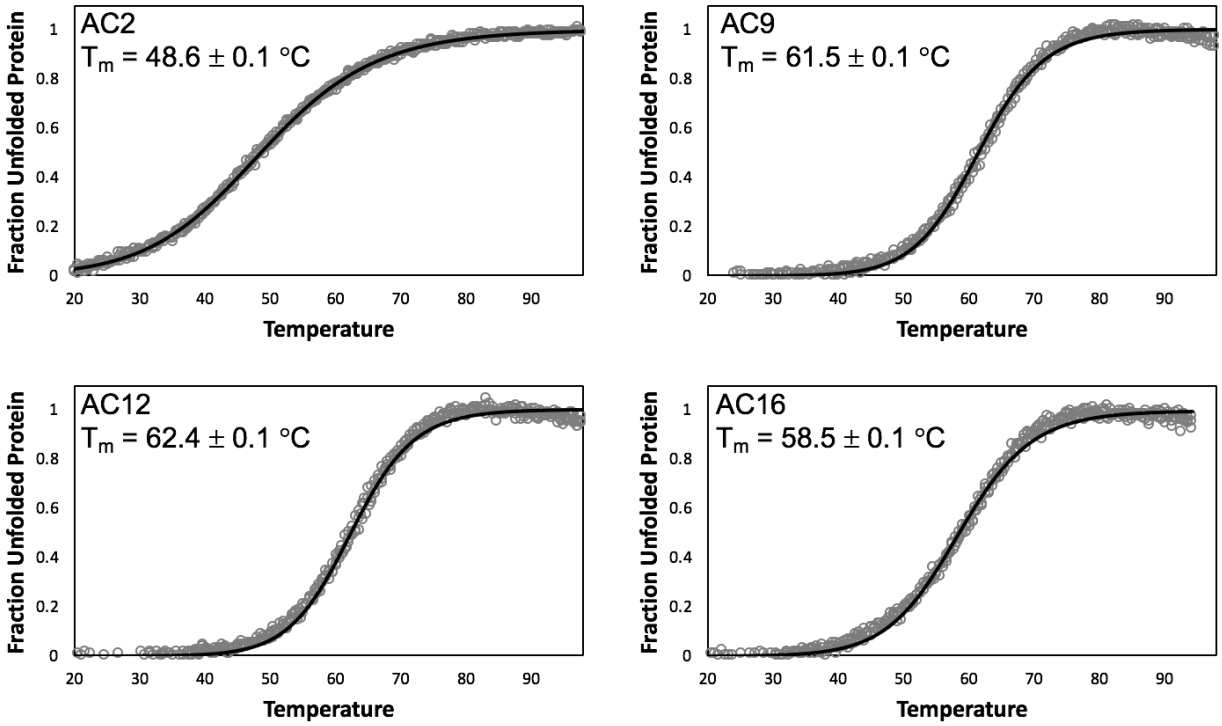
Supplemental Figure 2. *Affibody helix walking library designs.* Amino acid diversity for each site of the sub-library.



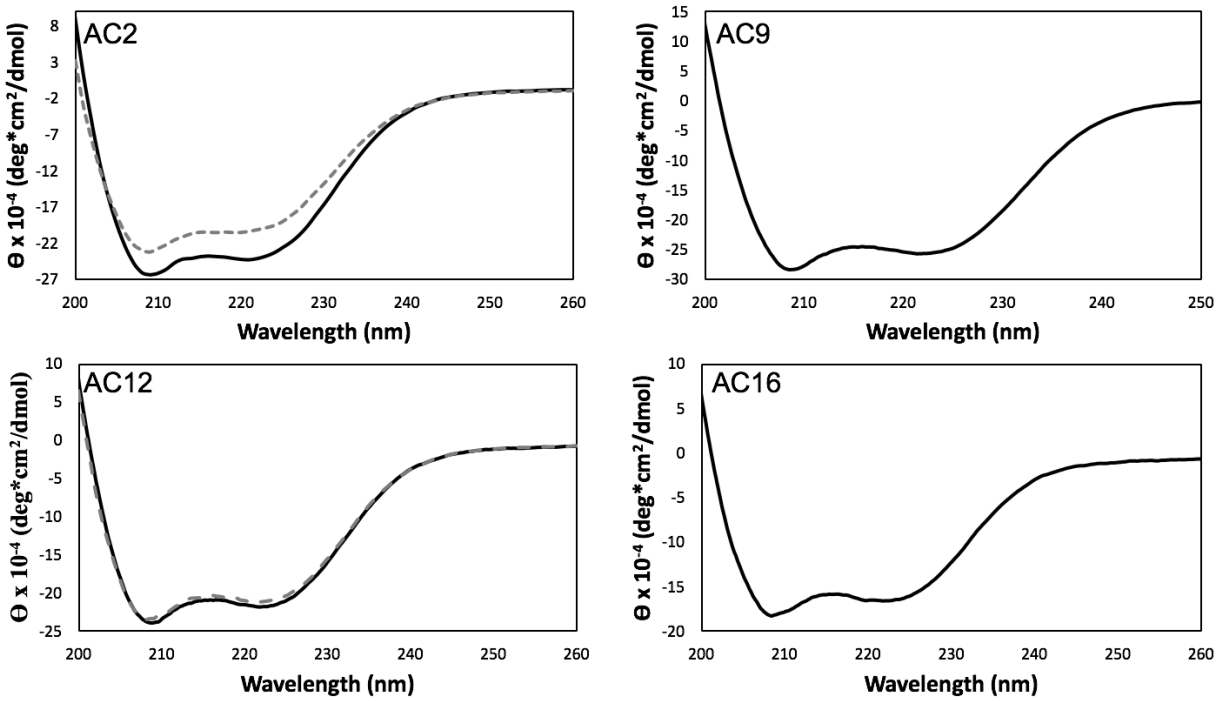
Supplemental Figure 3. *Deep sequencing of sorted single-helix libraries reveals substantially improved mutants.* (A) Top unique sequences of each single-helix library listed by number of reads. Parental AC2 is supplied as a reference. Blue lettering indicates diversified residues in the helix-walking libraries and dashes indicate parental amino acids at the indicated position. Clone names are supplied for sequences that were pursued for characterization. (B) Sitewise amino acid enrichments for the helix one (left) and helix two (right) libraries. Amino acid frequencies were calculated by grouping, counting, and quad-root dampening identical sequences. Values shown are change in amino acid frequency in sorted populations compared to theoretical amino acid diversity of the naïve library. Amino acids not allowed by library design are shown in grey, except in cases where they are substantially enriched or depleted.



Supplemental Figure 4. *Specificity of affibody variants.* Purified parental and evolved affibody variants were used to label either MS1-CD276 or MS1-Thy1 cells at saturating conditions (10 μM for AC2, 1 μM for all others). Binding was detected by fluorophore tagged anti-His_6 antibody via flow cytometry. Fluorescence was quantified as the difference between affibody labelled cells and control cells labelled with secondary antibody with n = 3 trials (68% confidence interval indicated). AC2 showed no significant binding to MS1-Thy1 cells relative to control. All other variants, while showing significant binding to MS1-Thy1 cells, bound significantly less strongly to MS1-Thy1 cells relative to MS1-CD276 cells.



Supplemental Figure 5. *Thermal denaturation curves of affibody variants.* Purified affibody variants were scanned at a wavelength of 220 nm during heating from 20 to 98 °C (1 °C/min). The midpoint of thermal denaturation (T_m) was calculated by linear least-squares regression using a two-state protein unfolding model.



Supplemental Figure 6. *Thermal denaturation curves of affibody variants.* Purified affibody variants were analyzed by circular dichroism spectroscopy in triplicate between 200 and 260 nm wavelengths before (solid) and after (dashed) thermal denaturation and cooling. No spectral data after cooling collected for AC9 or AC16.

Supplemental Table 1. Error-Prone PCR Oligonucleotide Sequences

W5	5'-CGACGATTGAAGGTAGATACCCATACGACGTTCCAGACTACGCTCTGCAG-3'
W3	5'-ATCTCGAGCTATTACAAGTCCTCTTCAGAAATAAGCTTTTGTTCGGATCC-3'
BCHPEP5	5'-CTGGAGGTTACCAACGCAACTCCGAACTCTCTGACTATTTCTTGG-3'
BCHPEP3	5'-CGGAACAGTGAATTCCTGGCTCGGGGAGTTACCACCAGTTTCGCCGTAGGTGATACGGTA-3'
DEHPEP5	5'-TACCGTATCACCTACGGCGAAACTGGTGGTAACTCCCCGAGCCAGGAATTCACTGTTCCG-3'
DEHPEP3	5'-TACAGCGTACACGGTAATGGTATAATCCTGGCCCGGTTTCAGACCGCTGATGGTCGC-3'
FGHPEP5	5'-AAACCGGGCCAGGATTATACCATTACCGTGTACGCTGTA-3'
FGHPEP3	5'-GTCGATTTCGGTGCGATAATTGATGCTGATTGG-3'
ABY1F-b	5'-TTCTGGTGGTGGTGGTTCTGCTAGCGCCGAAGCAAAATAC-3'
ABY1R	5'-GGTCAGGTTCGGCAG-3'
ABY2F	5'-CTGCCGAACCTGACC-3'
ABY2R-b	5'-TTTCTTCGCCTCAGACAGGAGTTCAGAGCTCTGGGACGGGTC-3'

Supplemental Table 2. Isolated Affibody Sequences and Frequencies

Isolated Affibody Sequences	CD276-Targeted	Frequency
Soluble Target	AEAKYAKEKIFAVGEIYWLPNLTHGQIMAFIAALNDDPSQSSELLSEAKKLNDSQAPK	3/5
	AEAKYYKELHNAIVSIRVLPNLTVDQITAFIRALVNDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYSKEWFNAYVSIWGLPNLTVDQKSAFSYALDDDPSQSSELLSEAKKLNDSQAPK	1/5
Soluble Target + Lysate	AEAKYNKEWKYAYFSIVALPNLTGTQVHAFIQALHNDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYSKEWFTAYYQIGYLPNLTEYQRYAFVKALYDDPSQSSELLSEAKKLNDSQAPK	2/5
	AEAKYYKELHNAIGVIRNLPNLTPIQKVAFAIALANDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYAKEYANAMVEIVCLPNLTLSQSGAFIAALLDDPSQSSELLSEAKKLNDSQAPK	1/5
Soluble Target + Cell Panning	AEAKYTKEKANAIVQILVLPNLTVSQLHAFLSALHNDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYSKEWFNAYVSIWGLPNLTVDQKSAFSYALDDDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYAKERLKAWLEIVELPNLTYTQLHAFIRALSDDPSQSSELLSEAKKLNDSQAPK	2/5
	AEAKYNKEKFNAIASIFNLPNLTHTQKTAFIVALNDDPSQSSELLSEAKKLNDSQAPK	1/5
Depleted Cell Panning	AEAKYTKEMYTAFDEIAQLPNLTQVQKVAFIVALWNDPSQSSELLSEAKKLNDSQAPK	1/4
	AEAKYSKEKADAILSILLLPNLTRAQVVAFMHALHNDPSQSSELLSEAKKLNDSQAPK	1/4
	AEAKYAKEFSSALVEILTLPNLTVRQSSAFIRALHDDPSQSSELLSEAKKLNDSQAPK	2/4
Cell Panning	AEAKYAKESSDAWHEIVQLPNLTHGQIHAFIRALHDDPSQSSELLSEAKKLNDSQAPK	2/5
	AEAKYAKEFSSALVEILTLPNLTVRQSSAFIRALHDDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYSKERLRAWMEITGLPNLTKPQRIAFILALRDDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYYKELHNAIYSIRWLPNLTRVQKAAFLRALANDPSQSSELLSEAKKLNDSQAPK	1/5
	Thy1-Targeted	
Cell Panning	AEAKYSKELHDAVDSIIALPNLTGHQMDAFISALINDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYAKEMDAAIIVILQLPNLTGYQMAAFIDALADDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYNKEMPTADYVIRQLPNLTLSQKQAFIHALHDDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYYKEQDDAIDEILSLPNLTGLQMRAFIVALYDDPSQSSELLSEAKKLNDSQAPK	1/5
	AEAKYNKEMDTAFDEILALPNLTGFQMDAFIYALSNDPSQSSELLSEAKKLNDSQAPK	1/5

Supplemental Table 3. Isolated Fibronectin Domain Sequences and Frequencies

Isolated Fibronectin Domain Sequences

	CD276-Targeted	**Frequency**
Soluble + Cell Panning	SSDSPRNLEVTNATPNSLTISWDAPCNTDTSGYRITYGETGGNSPSQESTVPGSNSATISGLKPGQDYTITGYAVSYGDYWWSNPISINYRTEIDKPSQ	4/9
	SSGSPRNLEVTNATPNSLTISWDAPCNTDTSGYRITYGETGGNSPSQESTVPGSNSATISGLKPGQDYTITGYAVSYGDYWWSNPISINYRTEIDKPSQ	3/9
	SSDSPRNLEVTNATPNSLTISWDAPCNTDASGYRITYGETGGNSPSQESTVPGSNSATISGLKPGQDYTITGYAVSYGDYWWSNPISINYRTEIDKPSQ	1/9
Depleted Cell Panning	SSDSPRNLEVTNATPNSLTISWDDSYDRAYYYRITYGETGGNSPSQEFTVPGTTNATISGLKPGQDYTITVYAVSYVNYAYYRSNPISINYRTEIDKPSQ	1/3
	SSDSPRNLEVTNATPNSLTISWDAPYYVYTYGYRITYGETGGNSPSQEFTVPGYNTATISGLKPGQDYTITVYAVSYHNTRYYSSNPISINYRTEIDKPSQ	1/3
	SSDSPRNLEVTNATPNSLTISWDAPCRYYAYGYRITYGETGGNSPSQEFTVPGNTNATISGLKPGQDYTITVYAVSNINYRYYASNPISINYRTEIDKPSQ	1/3
	Thy1-Targeted	
Depleted Cell Panning	SSDSPRNLEVTNATPNSLTISWDAPYVYTFGYRITYGETGGNSPSQEFTVPGTNSATISGLKPGQDYTITVYAVSYDNYKYAHSNPISINYRTEIDKPSQ	1/1
Cell Panning	SSDSPRNLEVTNATPNSLTISWDAPDDGYTNGYRITYGETGGNSPSQEFTVPGSNNTATISGLKPGQDYTITVYAVSYTSYAYYLSNPISINYRTEIDKPSQ	1/1

Supplemental Table 4. Sequences for Depletion Model System

HS	AEAKYTKEKANAIVQILVLPNLTVSQLHAFLSALHNDPSQSSELLSEAKKLNDSQAPK
HN	AEAKYAKESSYASLYIGILPNLTHSQYYAFIYALQDDPSQSSELLSEAKKLNDSQAPK
LS	AEAKYNKELANAALSIVYLPNLTGDQKSAFWLALQDDPSQSSELLSEAKKLNDSQAPK
LN	AEAKYAKERHRAWMEITGLPNLTRPQRIAFILALRDDPSQSSELLSEAKKLNDSQAPK
A5	AEAKYAKENFNATSEIYYLPNLTHFQRSAFSNALFDDPSQSSELLSEAKKLNDSQAPK