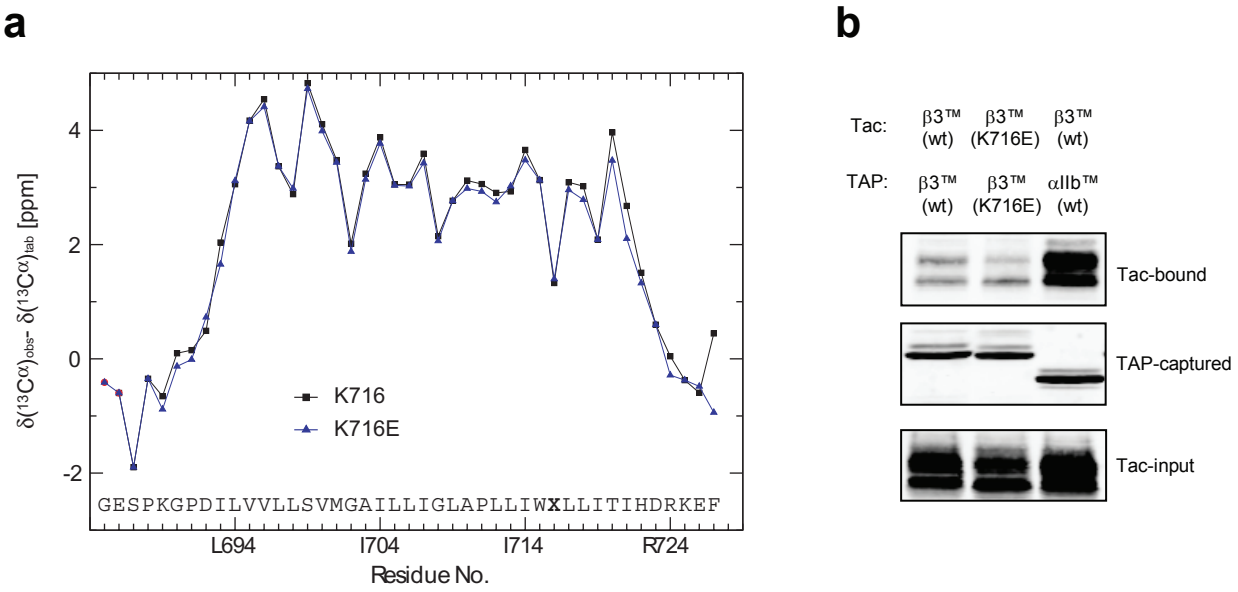
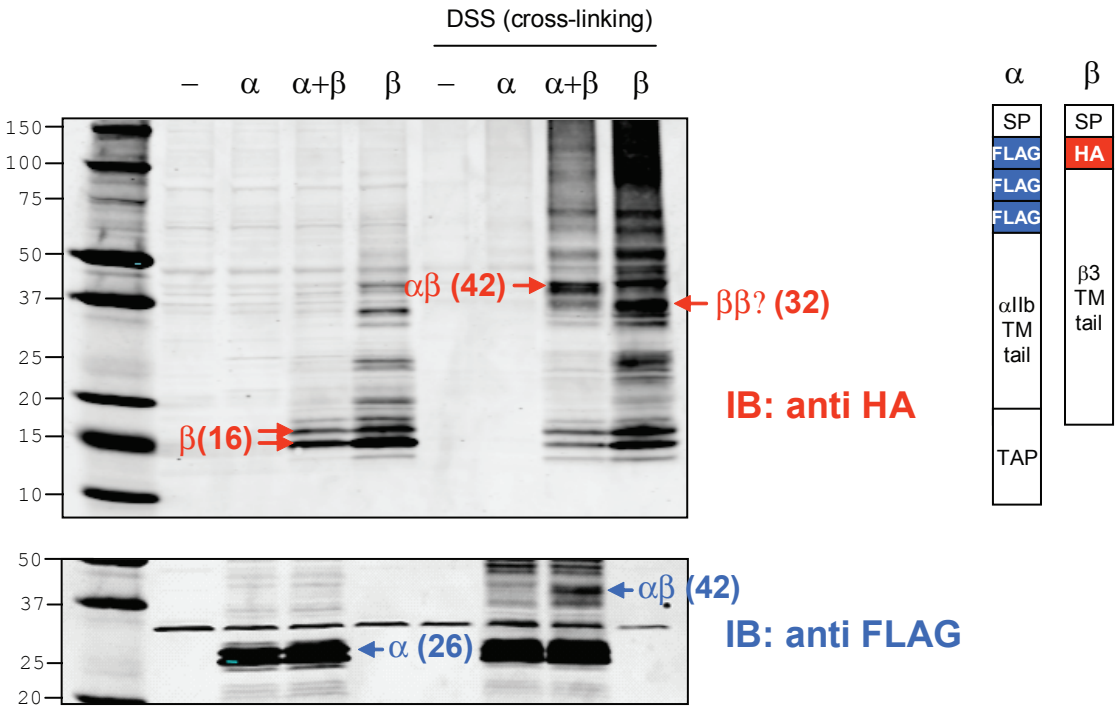
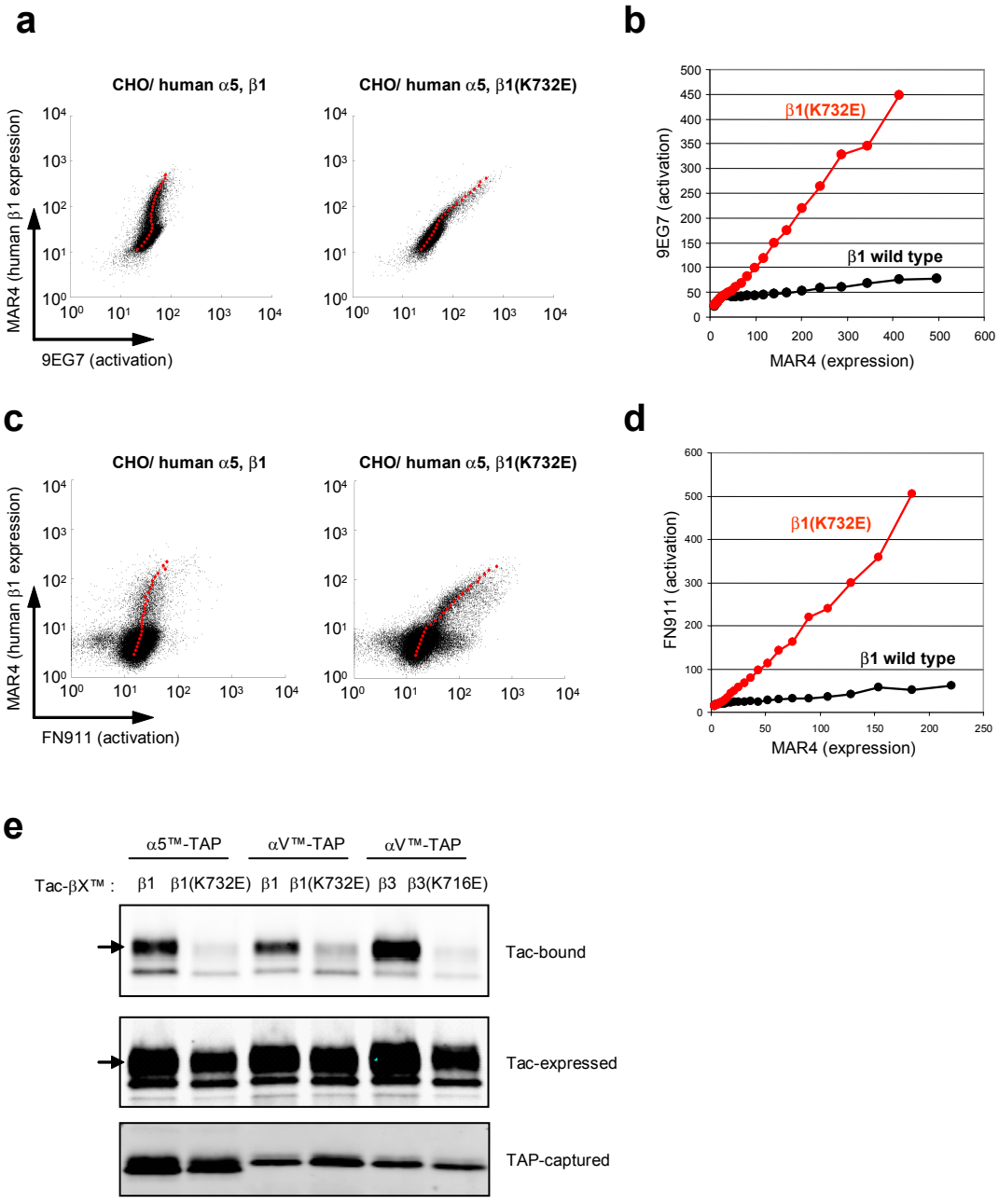


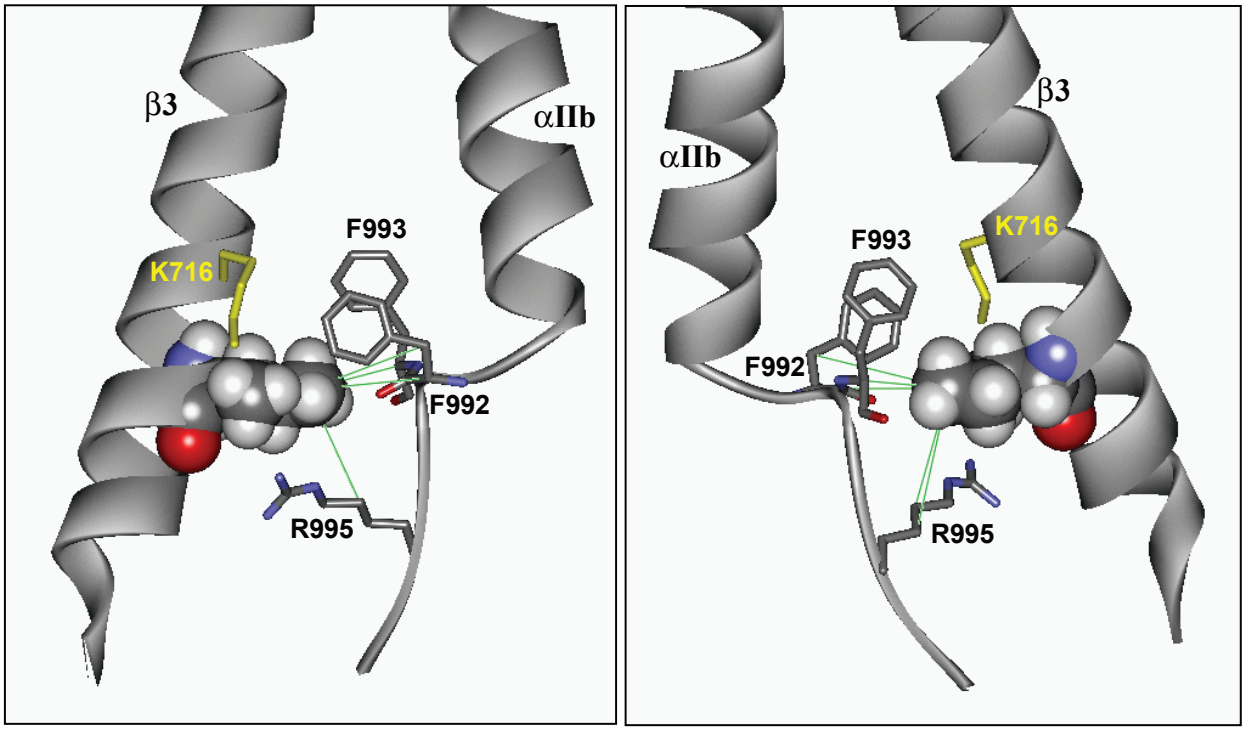
sFigure 1. K716E mutation in β 3 TMD doesn't induce structural changes. a, Comparison of $^{13}C^{\alpha}$ secondary chemical shifts between the TMD region of wild-type integrin β 3 and β 3(K716E)TMD. Secondary $^{13}C^{\alpha}$ chemical shifts, defined as the difference between the observed and tabulated random-coil $^{13}C^{\alpha}$ shift of a residue, correlate with the backbone conformation. The minor shift differences between the β 3 variant demonstrate the absence of any significant rearrangements in secondary structure conformation or content. Chemical shifts were measured in bicelles composed of 350 mM 1,2-dihexanoyl-sn-glycero-3-phosphocholine, 70 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine, and 35 mM 1-palmitoyl-2-oleoyl-sn-glycero-3-[phospho-L-serine]. **b,** To test whether the K716E mutant self - associates, TMD interaction between K716E mutants was tested using affinity capture (lane 2) as in Fig. 2a. There was no evidence of increased self-association.



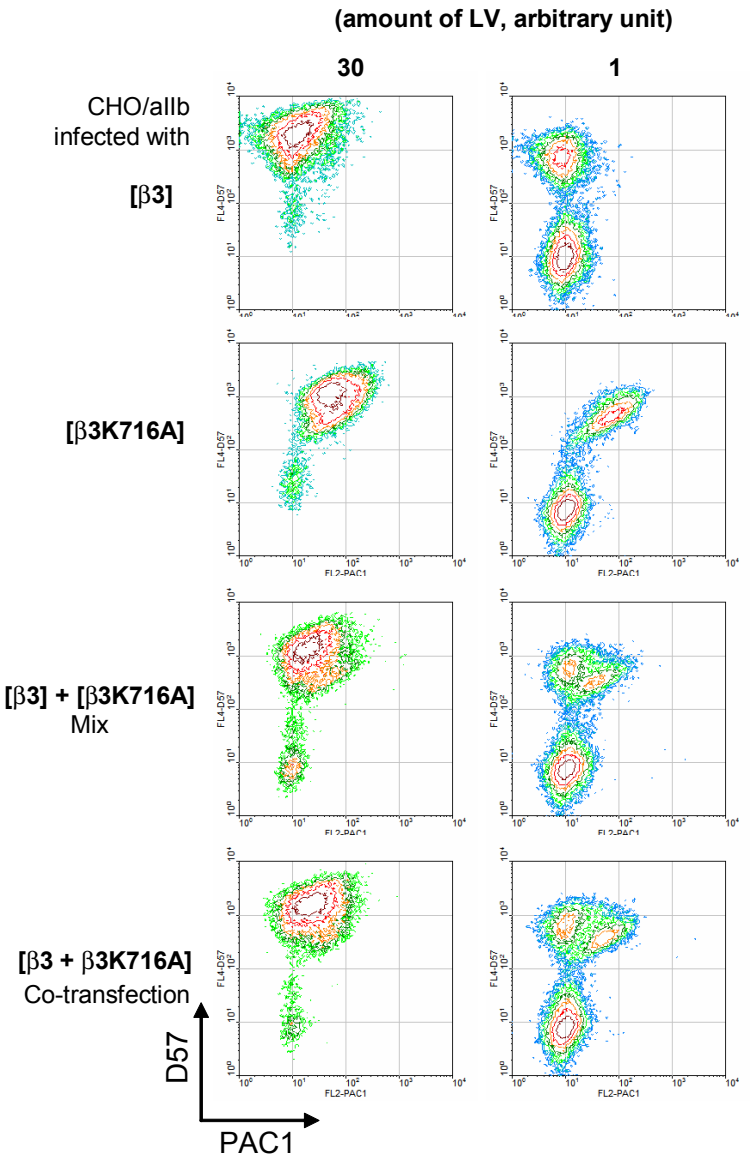
sFigure 2. α and β TMD-tails form 1:1 predominantly heterodimers when co-expressed in mammalian cells. CHO cells were transiently transfected with N-terminal FLAG-tagged αIIb TMD-tail construct and/or HA-tagged β3 TMD-tail construct (diagram right) as indicated. At 24 h after transfection, cells were washed with phosphate-buffered saline, and treated with membrane-permeable cross-linking reagent, disuccinimidyl suberate (DSS), at 2 mM for 5 min at room temperature. Cells were washed with tris-buffered saline three times for quenching, harvested with SDS-PAGE loading buffer containing mercaptoethanol, and boiled. Samples were analyzed with western blot using anti HA antibody (upper) and FLAG antibody (lower). Note that ~ 42 kD band, the size of 1:1 complex of α and β TMD-tail constructs, are detected by both HA antibody and FLAG antibody only when both α and β constructs are expressed, and is the dominant species (second lane from the right in figure).



sFigure 3. Effects of mutation in snorkeling residues in various integrins. a, cDNA encoding human integrin α5 construct was co-transfected into CHO cells incombination with either wild type human integrin β 1 or the snorkeling lysine mutant β 1(K732E). Surface expression of integrin β 1 was measured by human specific anti β 1 antibody (MAR4) conjugated with phycoerythrin, and affinity of the integrin was measured by the activation-specific integrin β1 antibody (9EG7). **b,** The dot plots in panel a were analyzed as in Fig. 2c. **c,** CHO cells were transfected as in panel a, and stained with MAR4 antibody and the binding of a fibronectin fragment (FN 9-11) as previously described[16]. **d,** The dot plots in panel c were analyzed as in Fig. 2c. **e,** α and β TMD-tail constructs of various integrins were co-transfected into CHO cells as indicated, and their interactions on cell surface were analyzed as previously reported[16] and described in Fig. 2a .



sFigure 4. Structure of the inner membrane region of bicelle-embedded integrin αIIbβ3 TMD complex.. The structure shown is derived from the average structure of an ensemble of 20 structures calculated without an αIIb(R995)- β3(D723) structural constraint. It exhibits a negligible backbone r.m.s.d. of 0.32 Å to the average structure calculated with such a restraint (PDB entry 2k9j). Ile739 is depicted as a space filling model and the intersubunit distance (NOE) restraints on the position of its side chain are indicated by green lines between the carbon atoms that are covalently-bonded to the hydrogen nuclei that give rise to the NOE. Note that the Ile719 side chain can sterically hinder interaction of Lys716 (yellow) ε-NH_3^+ with the backbone carbonyls of the α IIb Phe residues (red).

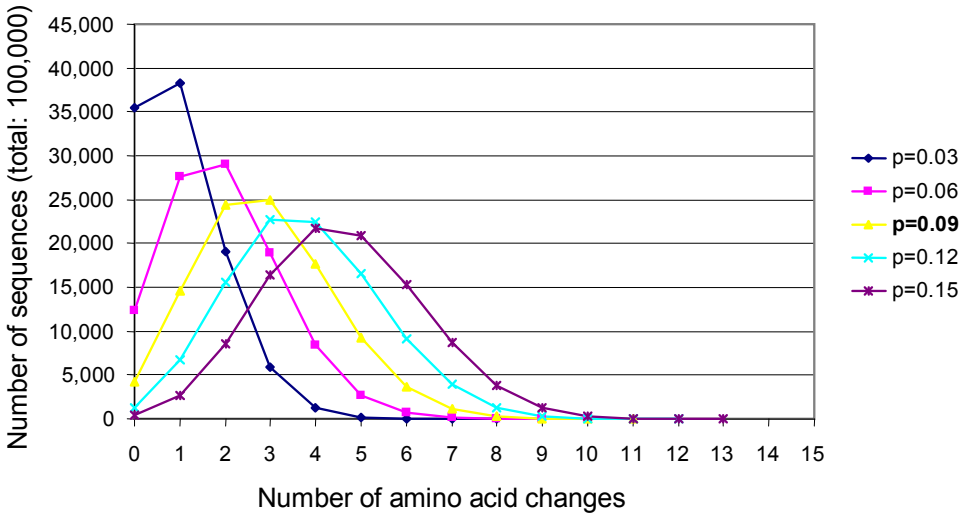


sFigure 5. Lentivirus for expression library screening. CHO/αIIb cells were infected with integrin β3 lentiviral particles ([β3]), β3(K716A) lentiviral particles ([β3K716A]), or 1:1 mixture of both β3 and β3 (K716A) particles ([β3]+[β3K716A]). In addition, to mimic packaging of a mixture of cDNAs as might occur with a library, cells were infected with lentiviral particles, [β3+ β3K716A], which were generated by co-transfecting those two lentiviral vectors in a 1:1 ratio into the packaging cell line. D57 binding (surface expression) and PAC1 binding (activation) were analyzed by flow cytometry. Cells infected with higher tighter (left panels) show higher infection efficiency, but each cell is infected with more than one type of virus so that the cells show an intermediate phenotype when infected with the mixture. However, cells infected at a lower titer (right panels) show ~ 10% infection efficiency but exhibit two different population with 1:1 ratio when infected with either the mixture, [β3]+[β3K716A], or [β3+ β3K716A]. This shows that the two genomes in each LV particle are identical, otherwise [β3+ β3K716A] particles would lead to three different phenotypes at a 1:2:1 ratio. Also note that cells were infected with single viral particle, when titers are used that result in infection of ~ 10% of the cells.

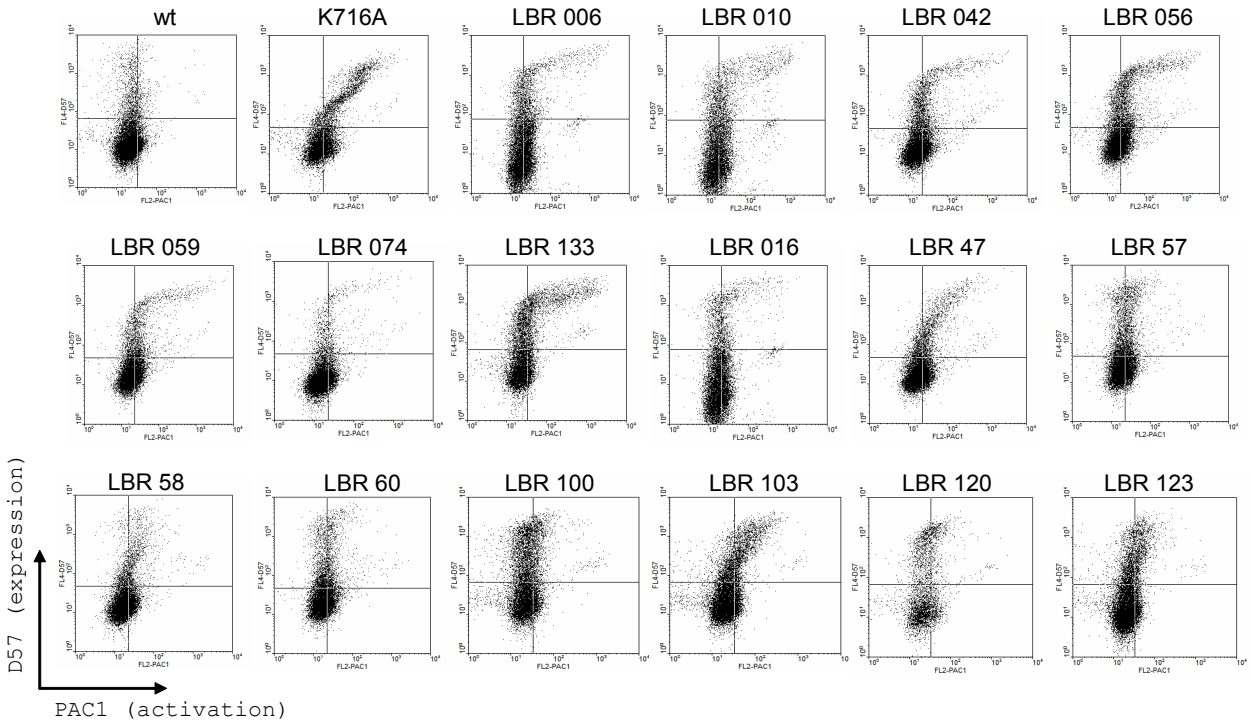
a

(NheI)
cattgggctagccGCCCTGCTCATCTGGgcaCTCCTCATCACCATCCACGACCGAAAAGAA
I G L A **A L L I W** A **L L I T I H D R K E**
ttcgctaaatttgaggaagaacgcgccagagcaaaatgggacacagccaacaacccactg
F A K F E E E R A R A K W D T A N N P L
tataaagaggccacgtctaccttcaccaatat**cacgtaccggggcacttaa**tctagaccc
Y K E A T S T F T N I T Y R G T - (XbaI)
ggatccccgctgatcagcctcgactgtgccttcta
(BamHI) (BGH reverse)

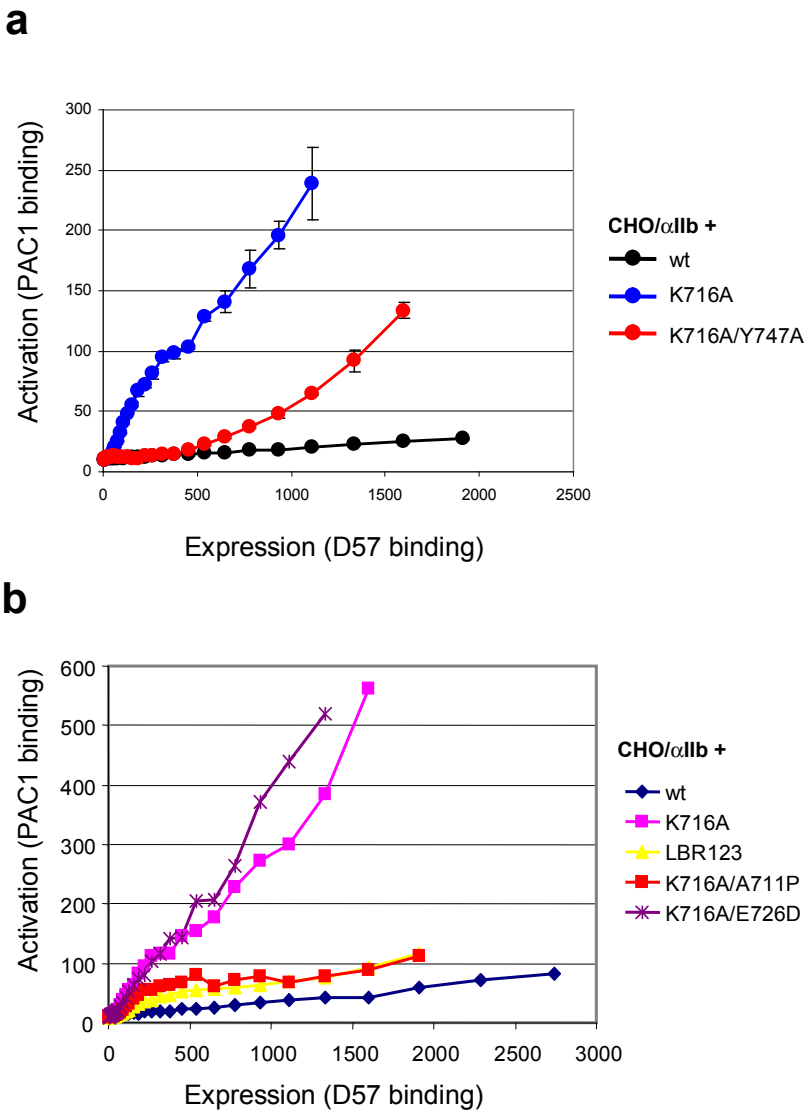
b



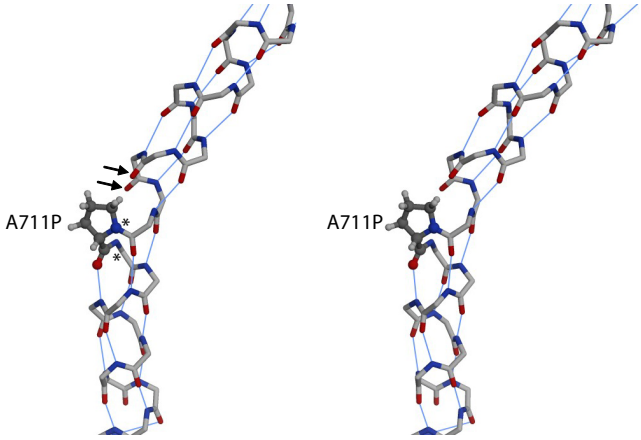
sFigure 6. Randomized β3 library design. **a,** Nucleotide and amino acid sequences of C-terminal region of human integrin β3(K716A) in LV vector are shown. The randomized forward primer, random β3(F), binding site are shown in blue with the randomized sequences indicated as bold. The reverse primer, β3-BamH1(R), binding site is shown in red. These two primers were used to generate randomized β3 fragments. **b,** A total of 100,000 differ ent randomized primers were generated in a computer simulation by using different contamination levels (p) of incorrect nucleotides in the randomized region; for example, when p = 0.15, the percent of correct nucleotide in each position is 85% and that of each of the three other bases is 5%. The number of sequences which have various numbers of amino acid substitutions were counted and plotted for each p value. In our random library, contamination level p=0.09 was chosen, since >85% of unique single amino acid substituitons are reperesented in a library containing ≥ 300,000 recombinants (sTable 1).



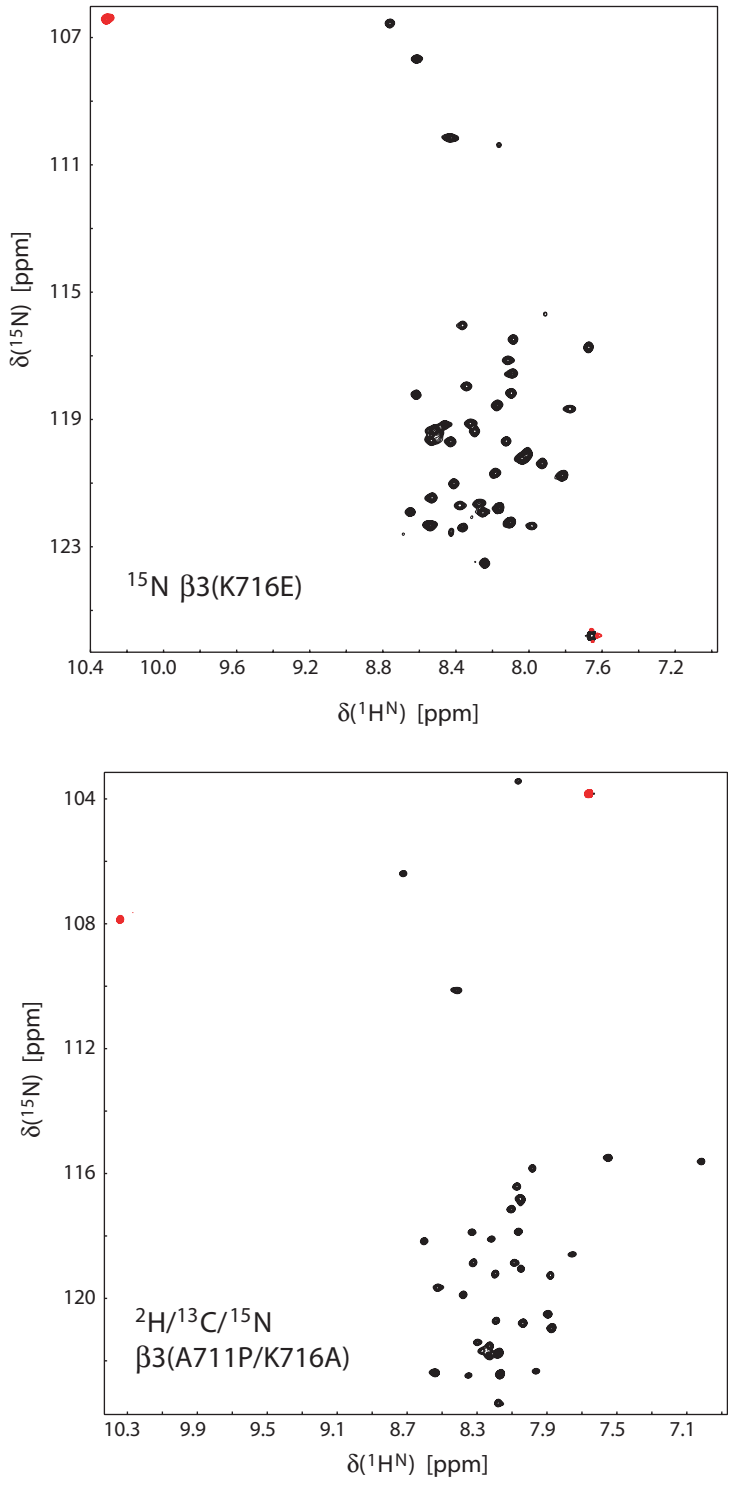
sFigure 7. Flow cytometry analysis of compensating mutations. The individual compensating clones generated by ligating the genomic PCR products into integrin β3 (see Fig. 3b) were transiently transfected to CHO/αIIb cells. 18 hour after transfection, cells were detached and tested for their binding to D57 (y axis) and PAC1 (x axis) by using flow cytometry. Dot plots correspond to compensating clones sequenced (Fig. 3e).



sFigure 8. Talin binding deficient β 3(Y747A), reduces the activating effect of β 3(K716A) a, CHO/αIIb cells were transfected with wild type or mutant integrin β3, and their binding to PAC1 and D57 were analyzed. **b,** β 3(A711P) is responsible for c ompensation. One of compensating clones, LBR123, contains A711P as well as a conservative E726D substitution. Only β 3(A711P) compensated.



sFigure 9. A711P disrupts helix-stabilizing hydrogen bonds. Stereogram of NMR structure of β3 TMD (K716A/A711P) embedded in phospholipid bicelles (PBD ID: 2l91). The side chain of A711P residue is displayed with ball-and-stick model. For the other residues, only the α-helical backbone residues are shown with hydrogen bonds indicated with light blue line. Arrows indicate carboxyl groups (red) of Ile707 and Gly708, and stars indicate amino groups (blue) of Ala711 and Leu712.



sFigure 10. Illustration of NMR spectral quality. TROSY H-N correlation spectra of the indicated β3 transmembrane mutants recorded in DHPC/POPC/POPS bicelles (q=0.3) in 25 mM HEPES-NaOH, pH 7.4, solution at 35 ºC and a ^{1}H frequency of 700 MHz.

sTable 1. Diversity of the library with a total of 310,000 recombinants predicted by simulation.

# of mutations	Clone # ± stdev	# of unique seq. ± stdev	# of cases	coverage (%)
0	12589.33 ± 94.0	1 ± 0	1	**100**
1	45322.67 ± 217.3	271.2 ± 2.4	315	**86.09524**
2	75480.67 ± 155.8	10399.2 ± 48.8	92610	**11.22902**
3	77539.33 ± 174.1	60194.0 ± 191.8	25282530	**0.238085**
...	**...**	**...**	**...**	**...**

The diversity of random library with 310,000 independent clones was simulated by a custom Perl script. The total clone number (clone #) of wild type sequence, single mutations, double mutations, and triple mutations in the 310,000 random clones were counted. Since there can be overlapping sequences in each case, the number of unique sequences (# of unique seq.) were also counted. The number of unique sequences was compare to the possible number of cases, to calculate the coverage rate. The simulation was performed five times, and results of each trial were averaged and shown with standard deviation.

sTable 2. χ_1 rotamer distribution of wild-type β3 and β3(A711P/K716A)

	wild-type β3					β3(A711P/K716A)			
residue	$^3J_{C'C\gamma}$	$^3J_{NC\gamma}$	type	χ_1	flag[a]	$^3J_{C'C\gamma}$	$^3J_{NC\gamma}$	χ_1	flag[a]
693	3.62	1.54	ILE	-	averaged	3.06	1.62	-	Averaged
694	1.97	1.46	LEU	-	averaged	3.40	1.48	-60	Loose
695	3.58	1.98	VAL	-	averaged	3.76	2.02	-	Averaged
696	3.38	2.11	VAL	-	averaged	-	2.19	-	Averaged
697	2.85	1.28	LEU	-	averaged	3.33	-	-	Averaged
698	4.13	0.35	LEU	-60	tight	3.39	0.77	-60	Loose
700	3.93	2.1	VAL	-	averaged	3.98	1.74	-	Averaged
701	2.03	1.33	MET	-	averaged	2.65	1.29	-	Averaged
704	2.98	2.07	ILE	-	averaged	4.39	1.96	-	Averaged
705	2.63	1.03	LEU	-	averaged	2.70	1.24	-	Averaged
706	3.52	0.85	LEU	-60	loose	-	1.08	-	Averaged
707	0.88	1.91	ILE	180	tight	3.67	-	-	Averaged
709	1.67	1.25	LEU	-	averaged	4.15	0.76	-60	Tight
712	1.96	1.51	LEU	-	averaged	3.48	1.08	-60	Loose
713	3.57	0.91	LEU	-60	loose	3.88	0.96	-60	Loose
714	5.33	1.94	ILE	-	averaged	4.63	2.06	-	Averaged
715	0.01	2.96	TRP	180	tight	1.04	2.73	180	Tight
716	2.46	1.56	LYS	-	averaged	-	-	-	-
717	2.75	1.3	LEU	-	averaged	2.68	1.28	-	Averaged
718	3.41	0.75	LEU	-60	tight	3.78	0.63	-60	Tight
719	2.65	1.54	ILE	-	averaged	3.03	1.62	-	Averaged
720	1.92	1.41	THR	-	averaged	2.49	1.23	-	Averaged
721	3.73	1.68	ILE	-	averaged	3.69	1.47	-60	Loose
722	2.63	1.47	HIS	-	averaged	2.11	1.74	-	averaged

a, A tightly defined rotameric state is considered for $^3J_{NC\gamma}$ in either *trans* (2.4 ± 0.6 Hz) or *gauche* (≤0.8 Hz) conformation and $^3J_{C'C\gamma}$ in either *trans* (4.0 ± 0.6 Hz) or *gauche* (≤1.1 Hz) conformation. Loosely coupled states are considered for $^3J_{NC\gamma}$ in either *trans* (2.4 ± 0.9 Hz) or *gauche* (≤1.0 Hz) conformation and $^3J_{C'C\gamma}$ in either *trans* (4.0 ± 1.0 Hz) or *gauche* (≤1.7 Hz) conformation. In case of borderline values additional combinations are also considered.

sTable 3. Structural statistics for the β3(A711P/K716A) transmembrane segment[a]

R.m.s. deviations from experimental dihedral restraints (deg)[b]	
All *(70)*	0.21 ± 0.12
R.m.s. deviations from experimental residual dipolar couplings (Hz)[c]	
$^1D_{NH}$ *(30)*	1.63 ± 0.10
$^1D_{NC'}$ *(31)*	1.79 ± 0.08
$^1D_{C\alpha C'}$ *(30)*	1.64 ± 0.07
R.m.s. deviations from experimental distance restraints (Å)	
All (*46*)	0.19 ± 0.01
Interresidue sequential (|i – j| =1) (*21*)	0.04 ± 0.00
Interresidue short range (1 < | i – j| < 5) (*25*)	0.26 ± 0.01
Deviations from idealized covalent geometry	
Bonds (Å)	0.004 ± 0.000
Angles (deg)	0.542 ± 0.029
Impropers (deg)	0.495 ± 0.027
Coordinate precision (Å)[d]	
Backbone non-hydrogen atoms	0.26
All non-hydrogen atoms	0.81
Measures of structural quality	
E_{LJ} (kcal mol^{-1})[3] [e]	-131.6
Residues in most favorable region of Ramachandran plot[f]	98.8%

a, Statistics for the 20 calculated simulated annealing structures, encompassing residues D692-D723.

b, None of the structures exhibited D692-D723 ϕ, ψ dihedral angle violations greater than 5°. Torsion angle restraints included 31 ϕ, 31 ψ, and 8 χ_1 angles.

c, R.m.s. deviations are normalized to an alignment tensor magnitude of 10 Hz.

d, Defined as the average r.m.s. difference between the final 20 simulated annealing structures and the mean coordinates.

e, The Lennard–Jones van der Waals energy was calculated with the CHARMM PARAM 19/20 parameters and was not included in the simulated annealing target function.

f, PROCHECK V3.4.4 (Roman A. Laskowski, Malcolm W. MacArthur, David S. Moss and Janet M. Thornton (1993). PROCHECK: a program to check the stereochemical quality of protein structures. J. Appl. Cryst., 26, 283-291)