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Supporting Information

Integrin-Targeting Fluorescent Proteins: Exploration of RGD Insertion Sites

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Materials and methods

All chemicals and compounds have been used without prior purification, if not otherwise stated. The restriction enzymes, T4 ligase, and isopropyl-beta-D-thiogalactopyranoside (IPTG) have been obtained from Fermentas. DNA polymerase Platinum-Taq, and fetal bovine serum (FBS) was purchased from Invitrogen. All oligonucleotides were ordered from eurofinsgenomic. Bio-safe Coomassie Brilliant Blue, 30% acrylamide mix (30% Acrylamide/Bis Solution, 29:1 (3.3%C)), brom phenol blue, 6x DNA loading dye and N,N,N′,N′-tetramethyl-ethane-1,2-diamine (TEMED) were purchased from BioRad. Sodium dodecyl sulfate (SDS), yeast extract, sodium chloride, imidazole, ammonium persulfate (APS), manganese (II) chloride ($MnCl_2$), and Tween20 were ordered from Merck. Glycerol, glycine, guanidine hydrochloride (GndHCl) and ethylenediaminetetraacetic acid (EDTA) were obtained from Calbiochem. All competent *E.coli* cells, benzonase, Bugbuster, phosphate buffered saline (PBS) tablets and Ni-NTA resin were obtained from Novagen. Blue-white select screening reagent, ß-mercaptoethanol, ampicillin, urea, egg lysozyme, nickel(II)sulfate hexahydrate ($NiSO_4$), trypsin, sodium hydroxide (NaOH), tris (2-carboxyethyl) phosphine (TCEP), dithiothreitol (DTT), tris-base, magnesium chloride ($MgCl_2$), and bovine serum albumin were obtained from Sigma. Agar was purchased from Difco. Amicon dialysis and concentration devices were obtained from Millipore. Filters (0.2 µm, 0.45 µm) were obtained from Pall. NucleoSpin Gel and PCR Clean-up kit was ordered from Macherey-Nagel and QIAprep Spin Miniprep kit from Qiagen. MaxiSorb plates were obtained from Nunc. All integrin variants were purchased from Millipore in Triton X-100 formulation. Goat anti-6-His antibody was obtained from Bethyl. Donkey Anti-Goat IgG (HRPO) antibody was bought from Leinco. TMB substrate and stopper solution were obtained from Cell Signaling. Milk powder was bought from Campina. The pHT293 plasmid containing the His-mCitrine was previously developed by us.[1]

<u>*Site-directed mutagenesis of His-mCitrine-Strep:*</u>

The mutations in His-mCitrine-Strep were performed using the site-directed mutagenesis kit from Stratagene and primers listed below.

<u>*Primer to insert or exchange to cysteines or GRGDS / GRADS sequences:*</u>

Primer to insert the GR*G*DS sequence	
0/1_F	aattaaaggaggaaggatctatggggcgcggggactctcaccatcatcatcatcattcttc
0/1_R	gaagaatgatgatgatgatggtgagagtccccgcgccccatagatccttcctcctttaatt
240/241_F	ggagccatccgcaatttgaaaaagggcgcggggactcttaacgtccccggg
240/241_R	cccggggacgttaagagtccccgcgccctttttcaaattgcggatggctcc
49/50_F	aagttcatctgcaccgggcgcggggactctaccggcaagctgccc
49/50_R	gggcagcttgccggtagagtccccgcgcccggtgcagatgaactt
78/79_F	ctaccccgaccacatggggcgcggggactctaagcagcacgacttct
78/79_R	agaagtcgtgctgcttagagtccccgcgccccatgtggtcggggtag
116/117_F	gtgaagttcgagggcgggcgcggggactctgacaccctggtgaac
116/117_R	gttcaccagggtgtcagagtccccgcgcccgccctcgaacttcac

140/141_F	catcctggggcacaaggggcgcggggactctctggagtacaactaca
140/141_R	tgtagttgtactccagagagtccccgcgccccttgtgccccaggatg
134/135_F	ttcaaggaggacggcgggcgcggggactctaacatcctggggcac
134/135_R	gtgccccaggatgttagagtccccgcgcccgccgtcctccttgaa
157/158_F	catggccgacaagcaggggcgcggggactctaagaacggcatcaagg
157/158_R	ccttgatgccgttcttagagtccccgcgcccctgcttgtcggccatg
172/173_F	gccacaacatcgaggggcgcggggactctgacggcagcgtgca
172/173_R	tgcacgctgccgtcagagtccccgcgcccctcgatgttgtggc
194/195_F	ggccccgtgctggggcgcggggactctctgcccgacaac
194/195_R	gttgtcgggcagagagtccccgcgccccagcacggggcc
189/190_F	cccccatcggcgggcgcggggactctgacggccccgt
189/190_R	acggggccgtcagagtccccgcgcccgccgatggggg
213/214_F	caaagaccccaacgaggggcgcggggactctaagcgcgatcacatgg
213/214_R	ccatgtgatcgcgcttagagtccccgcgcccctcgttggggtctttg
Primers to exchange original mCitrine sequence to GR*G*DS sequence	
49/53 FE	gaccctgaagttcatctgcggcaggggcgattcgcccgtgccctggcccacc
49/53 RE	ggtgggccagggcacgggcgaatcgcccctgccgcagatgaacttcagggtc
78/82 FE	ttcgcccgctaccccgaccacgggagggggggacagcttcttcaagtccgccatgcc
78/82 RE	ggcatggcggacttgaagaagctgtccccccctcccgtggtcggggtagcgggcgaa
116/121 FE	gcgccgaggtgaagttcgagggccgcggcgattcgaaccgcatcgagctgaagggca
116/121 RE	tgcccttcagctcgatgcggttcgaatcgccgcggccctcgaacttcacctcggcgc
140/144 FE	cggcaacatcctggggcacgggcgggggggacagctacaacagccacaacgtc
140/144 FR	gacgttgtggctgttgtagctgtcccccccgcccgtgccccaggatgttgccg
135/138 FE	aagggcatcgacttcaaggaggacggcaggggcgatagccacaagctggagtacaactacaacagcc
135/138 FR	ggctgttgtagttgtactccagcttgtggctatcgcccctgccgtcctccttgaagtcgatgccctt
156/160 FE	ccacaacgtctatatcatggccgacgggcggggggacagcatcaaggtgaacttcaagatccg
156/160 RE	cggatcttgaagttcaccttgatgctgtccccccgcccgtcggccatgatatagacgttgtgg
171/174 FE	cttcaagatccgccacaacggcaggggcgacagcgtgcagctcgccgac
171/174 RE	gtcggcgagctgcacgctgtcgcccctgccgttgtggcggatcttgaag

194/198 FE	gcgacggccccgtggggcggggcgacagccactacctgagct
194/198 RE	agctcaggtagtggctgtcgccccgccccacggggccgtcgc
190/194 FE	gaacacccccatcggccgcggcgactcgctgctgcccgacaac
190/194 RE	gttgtcgggcagcagcgagtcgccgcggccgatgggggtgttc
211/215 FE	ccagtccgccctgagcaaagacggcagggggggatagcgatcacatggtcctgctgga
211/215 RE	tccagcaggaccatgtgatcgctatccccccctgccgtctttgctcagggcggactgg
Primers to exchange the original mCitrine sequence to a GRADS sequence	
140/141GRADS_ F	catcctggggcacaaggggcgcgcggactctctggagtacaactaca
140/141GRADS_R	tgtagttgtactccagagagtccgcgcgccccttgtgccccaggatg
194/195GRADS_ F	ggccccgtgctggggcgcgcggactctctgcccgacaac
194/195GRADS_R	gttgtcgggcagagagtccgcgcgccccagcacggggcc
Primer for mCitrine negative control	
C48V_F	gcaagctgaccctgaagttcatcgtgaccaccggcaagctg
C48V_R	cagcttgccggtggtcacgatgaacttcagggtcagcttgc

Amino acid sequence of His-mCitrine-Strep:

-mCitrine: bold

-GRGDS insertion positions: underlined

- GRGDS exchange positions: green box

M H H H H H H S S G L V P R G S **V S K G E E L F T G V V P I L V E L D G D V N G H K F S V S G E G E G D A T Y G K L T L K F I C T T G K L P V P W P T L V T T F G Y G L M C F A R Y P D H M K Q H D F F K S A M P E G Y V Q E R T I F F K D D G N Y K T R A E V K F E G D T L V N R I E L K G I D F K E D G N I L G H K L E Y N Y N S H N V Y I M A D K Q K N G I K V N F K I R H N I E D G S V Q L A D H Y Q Q N T P I G D G P V L L P D N H Y L S Y Q S A L S K D P N E K R D H M V L L E F V T A A G I** S R V D V A W S H P Q F E K Stop

Expression of His-mCitrine-Strep:

The different constructs were transformed in *E.coli* BL21 (DE3) cells. Clones containing the respective plasmid were inoculated in 20 ml Luria Bertani (LB) culture containing 50 µg / ml ampicillin. The culture was grown in a shaking incubator at 37°C overnight. On the next day, 10 ml of the overnight culture were used to inoculate 1 liter LB media containing 50 µg / ml ampicillin. The culture was placed on a shaking incubator at 37°C for about 2.5 hours. At an optical density of A_{600} = 0.9, the expression was induced with IPTG, final concentration 1 mM. The culture was placed back into the shaking incubator and incubated at 25°C overnight. Subsequently, the culture was harvested and pelleted by centrifugation at 10000 g, 10 min, at 4°C. The cell pellet was stored at -80°C.

Purification of His-mCitrine-Strep:

The cell pellet was thawed at room temperature and re-suspended in 5-10 ml Bugbuster containing 1 µl / ml benzonase. To lyse the cells, the mixture was incubated 30 min at 4°C on a shaker and centrifuged at 10000 g for 10 min. The soluble fraction was collected and loaded onto 5 ml Ni-NTA resin equilibrated with 5

column volumes binding buffer (20 mM Tris, 500 mM NaCl, pH 7.8). Subsequently the resin was washed with 5 column volumes wash buffer (binding buffer, 40 mM imidazole) and eluted with 5 column volumes elution buffer (binding buffer, 300 mM imidazole). All obtained fractions (loading, washing, eluting) were analyzed by SDS-PAGE gel electrophoresis and LC-MS. The eluent fraction was dialyzed 4 times against PBS and concentrated via Amicon filters (cut-off 10 kDa) to a final volume of 0.5 – 2 ml.

SDS-PAGE:

SDS-PAGE electrophoresis was performed on a Mini-PROTEAN 3 electrophoresis system (Biorad, Hercules, California). The gel consisted of a 5% stacking gel and a 12% or 15% separating gel. The running buffer contained 25mM Tris-HCl, 250mM glycine, and 0.1% (w/v) SDS in H_2O. Prior loading onto the SDS-gel, all protein samples have been a) mixed with sample buffer (100mM Tris-HCl, 20% (v/v) glycerol, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, pH 6.8 in Millipore H_2O) in a ratio 1:3, b) reduced by addition of β-mercaptoethanol and c) denatured by heating up the sample to 95 °C for 10 min. The electrophoresis was run at room temperature at 80 V for 20 min. and then at 140-160V for 60 min. The protein bands were stained with Coomassie Brilliant Blue.

Microtiter-plate based integrin binding assay:

MaxiSorb plates were coated with 100 µl integrin (0.5 µg / ml dissolved in: 50 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.4) on a shaker for 24 h at room temperature. Subsequently each well was washed three times 10 min with 100 µl blocking buffer (50 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.4, 0.1% milk powder (w/v)). The plates were incubated 24 h at 4°C in the dark with 100 µl His-mCitrine-Strep (150 µg / ml diluted in blocking buffer) and washed three times 10 min with 100 µl wash buffer (50 mM Tris, 150 mM NaCl, 1 mM $MgCl_2$, 2 mM $MnCl_2$, pH 7.4, 0.05% Tween 20 (w/v)). The washed plate was blocked three times 10 min with 100 µl blocking buffer and subsequently incubated with 100 µl Goat anti-6-His antibody (1:1000 diluted in blocking buffer). After an incubation time of 24 h at 4°C in the dark, the antibody was removed and the plate washed three times for 10min with 100 µl wash buffer as well as 100 µl blocking buffer. After blocking, 100 µl of the second antibody (Donkey Anti-Goat IgG (HRPO) 1:10000 diluted in blocking buffer) was added and the plate was incubated 24 h at 4°C in the dark. Subsequently, the second antibody was removed and the plates washed three times for 10 min with 100 µl wash buffer as well as 100 µl blocking buffer. 100 µl TMB substrate was added and the plate was incubated for 15 min at 37°C in the dark. The enzymatic substrate conversion was stopped by the addition of 100 µl stopper solution and the absorption was read out at 450 nm.

Fluorescent spectroscopy:

The fluorescence spectra (λ_{ex} = 515 nm, λ_{em} = 520 – 650 nm) of all His-mCitrine-Strep mutants were measured in Varian Cary Eclipse fluorescence equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. All measurements were perform in quartz cuvettes (10 mm light path), 2 ml minimal volume, at 20°C, concentration 1 µM in PBS.

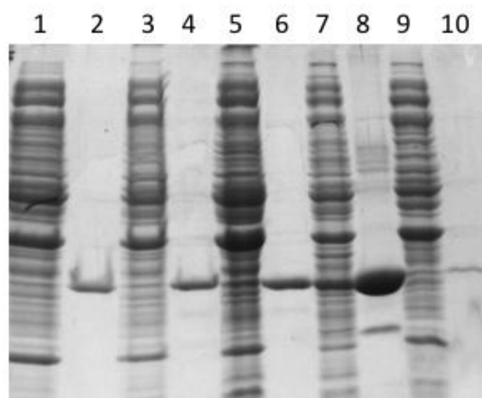
Absorbance spectroscopy:

The UV-VIS absorbance spectra of all His-mCitrine-Strep mutants were recorded in a Jasco V-650 spectrophotometer equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. All measurements were perform in quartz cuvettes (10 mm light path), 2 ml minimal volume, at 20°C, concentration 1 µM in PBS.

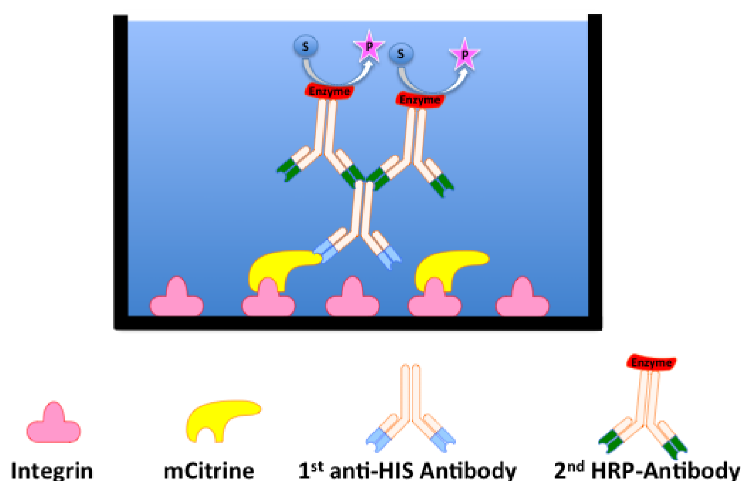
Supporting Figures and Tables

Supporting Table 1: Amino acid positions for GRGDS or cysteine insertion / exchange. Underlined amino acids: inserted / exchanged amino acids. *Mutation that could not be obtained.

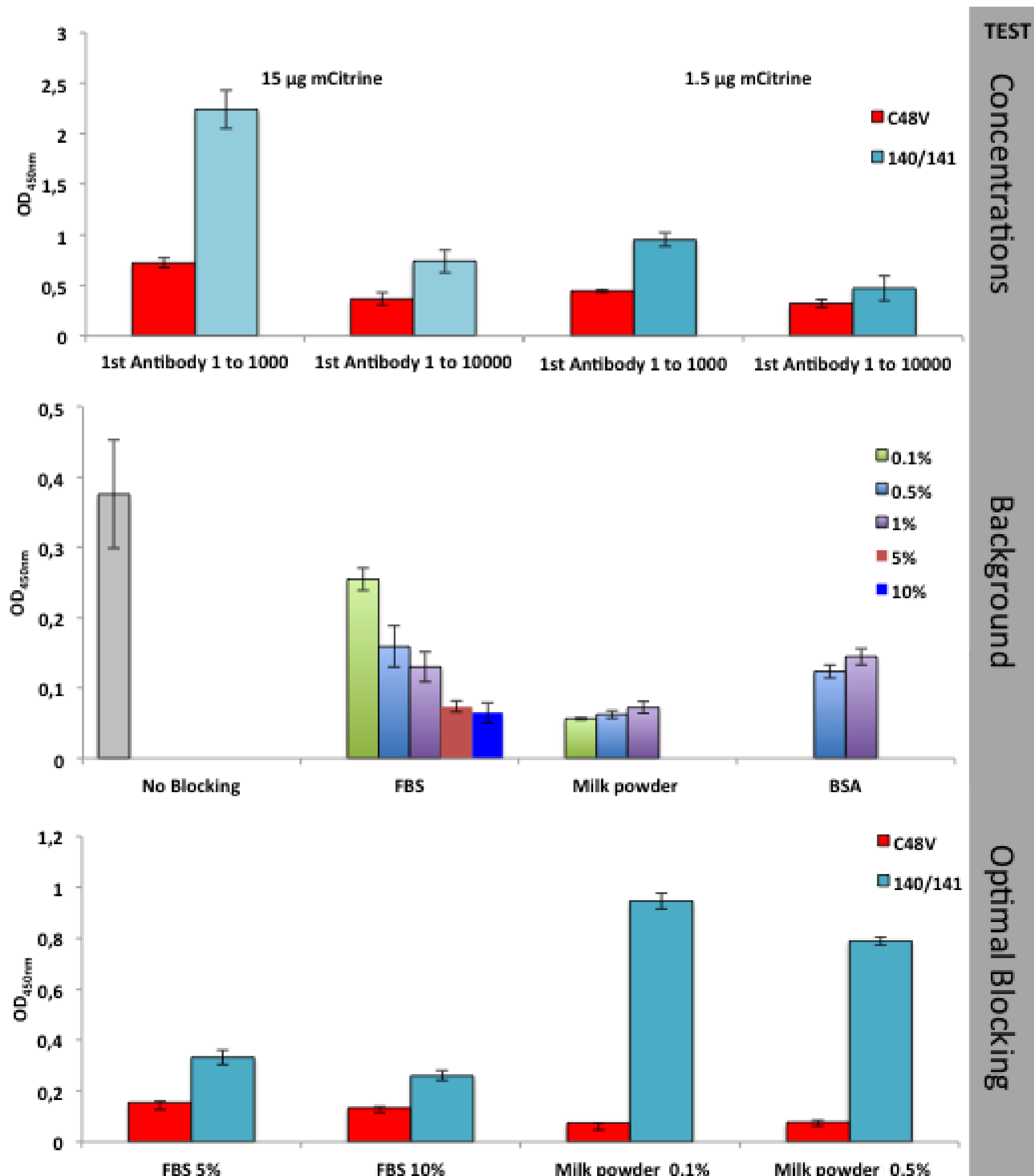
	Before mutagenesis	After mutagenesis
GRGDS inserted		
0/1	RGSVSK	RGSGRGDSVSK
49/50	FICTTG	FICGRGDSTTG
78/79	DHMKQH	DHMGRGDSKQH
116/117	FEGDTL	FEGGRGDSDTL
134/135	EDGNIL	EDGGRGDSNIL
140/141	GHKLEY	GHKGRGDSLEY
157/158	DKQKNG	DKQGRGDSKNG
172/173	NIEDGS	NIEGRGDSDGS
189/190	PIGDGP	PIGGRGDSDGP
194/195	PVLLPD	PVLGRGDSLPD
213/214	PNEKRD	PNEGRGDSKRD
240/241	PQFEK	PQFEKGRGDS
GRGDS exchanged		
49/53	TTGKL	GRGDS
78/82	MKQHD	GRGDS
116/121	GDTLV	GRGDS
135/138	GNILG	GRGDS
140/144	KLEYN	GRGDS
156/160	KQKNG	GRGDS
171/174	IEDGS	GRGDS
190/194	DGPVG	DGPVG*
194/198	LLPDN	GRGDS
211/215	PNEKR	GRGDS
Control		
C48V	KFICTTG	KFIVTTG



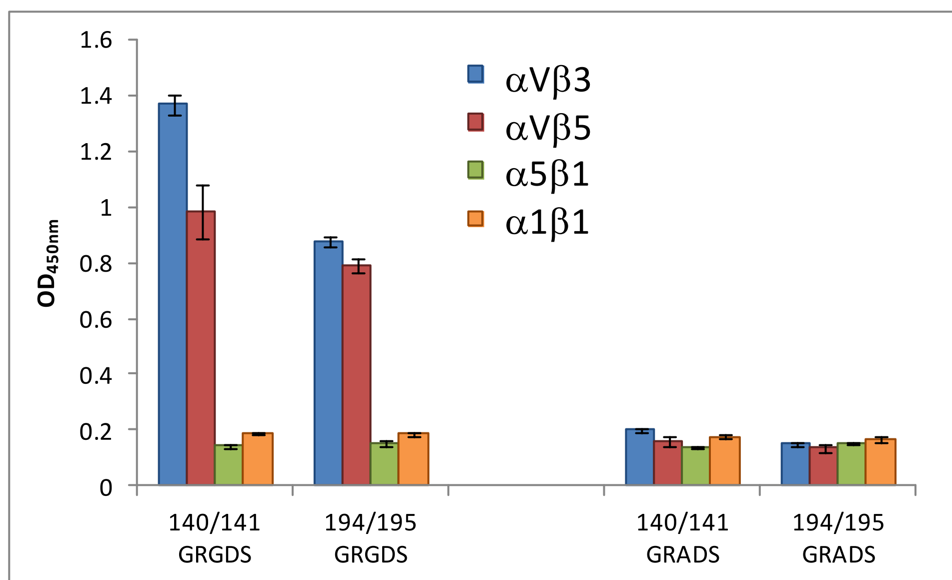
Supporting Figure 1. Representative SDS-PAGE of selective protein constructs comparing different mCitrine-RGD constructs before and after Ni-NTA purification. The gel is representative for all mCitrine-RGD/cysteine constructs. Lane 1/2: 49/50, lane 3/4: 78/79, lane 5/6: 116/117, lane 7/8: 140/141, lane 9/10: 134/135.



Supporting Figure 2. Schematic representation of the microtiter-plate based integrin binding assay. Integrins receptors were immobilized on the surface of multi-well plates and incubated with RGD-mCitrine. Binding of RGD-mCitrine (containing a His-tag) was detected using primary anti-His antibodies. The signal was amplified using a secondary antibody conjugated to horseradish peroxidase (HRPO). Following TMB substrate conversion by HRPO, the binding was quantified at 450 nm.



Supporting Figure 3. Determination of optimal conditions for the microtiter-plate based integrin binding assay. <u>Top:</u> Comparison of different mCitrine and primary antibody concentration on plates coated with human integrin receptor αVβ3 (0.05 µg / well in 96 well-plates), blocking reagent 0.5% BSA, <u>Middle:</u> Comparison of different blocking reagents, primary antibody 1:1000 diluted, well-plates not coated. <u>Bottom:</u> Comparison of the blocking reagents FBS and milk powder on plates coated with human integrin receptor: αVβ3 (0.05 µg / well using 96 well-plates), primary antibody 1:1000 diluted. mCitrine-RGD 140/141 was used as positive control, mCitrine C48V was used as negative control. Primary antibody: goat-anti-His, secondary antibody: anti-goat HRPO. All antibodies were diluted in 0.1% blocking reagent, the secondary antibody was diluted 1:10000. <u>Optimal determined concentrations:</u> mCitrine: 15 µg / well, primary antibody: 1:1000, secondary antibody: 1:10000, Blocking reagent: milk powder 0.1%.



Supporting Figure 4. Screening overview of mCitrine-RGD mutants in comparison to the respective mCitrine-RAD mutants. All mutants were screened against four human integrin receptors: αVβ3, αVβ5, α5β1 and α1β1.

Supporting References

[1] T. Phan, H. D. Nguyen, H. Göksel, S. Möcklinghoff, L. Brunsveld Phage display selection of miniprotein binders of the Estrogen Receptor Chem. Commun., 2010,46, 8207-8209.