Bispecific designed ankyrin repeat proteins (DARPins) targeting the epidermal growth factor receptor inhibit A431 cell proliferation and receptor recycling

Ykelien L. Boersma[1], Ginger Chao[2,3], Daniel Steiner[1,4], K. Dane Wittrup[2], and Andreas Plückthun[1*]

SUPPLEMENTAL DATA

[1]From the Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland

[2]The Department of Chemical Engineering and Bioengineering, Massachusetts Institute of Technology, 77 Massachusetts Avenue, Cambridge MA, 02139, USA.

[3]Present address: Adnexus, a Bristol-Myers Squibb R&D Company, 100 Beaver Street, Waltham MA, 02453, USA.

[4]Present address: Molecular Partners AG, Wagistrasse 14, 8952 Zurich-Schlieren, Switzerland

[*]Running title: *Bispecific EGFR-DARPins inhibit A431 cell proliferation*

To whom correspondence should be addressed: Andreas Plückthun, Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, 8057 Zurich, Switzerland. Tel: +41-44-635 5570. Fax: +41-44-635 5712. Email: plueckthun@bioc.uzh.ch

Keywords: Designed ankyrin repeat proteins; EGFR; tumor targeting; protein engineering; epitope mapping.

EXPERIMENTAL PROCEDURES

Competition experiments with bispecific DARPins – DARPins E01_LZ3_E69 and E69_LZ3_E01 were incubated with 10^6 A431 cells for 30 min on ice. The DARPin concentration was adjusted to result in a 1:1 molar ratio with EGFR, assuming $3 \cdot 10^6$ receptors / cell (1). Then, 100 nM of either E01_Alexa Fluor 488 or 100 nM of E69_Alexa Fluor 488 was added and incubated for another 20 min. Cells were washed twice with PBS and subsequently analyzed by flow cytometry. The change in fluorescence was compared to that of samples incubated only with the labeled monovalent DARPin.

REFERENCES

1. Haigler, H., Ash, J. F., Singer, S. J., and Cohen, S. (1978) *Proc. Natl. Acad. Sci. U. S. A.* **75**, 3317-3321

SUPPLEMENTARY TABLES

SUPPL. TABLE SI. Binding of DARPin E69 to yeast-surface displayed EGFR.

EGFR fragment	E69
1-124	-
1-176	~
1-294	+
273-621	-
294-543	-
475-621	-
1-621 (404SG)	+

+, binding; -, no binding; ~, very slight binding.

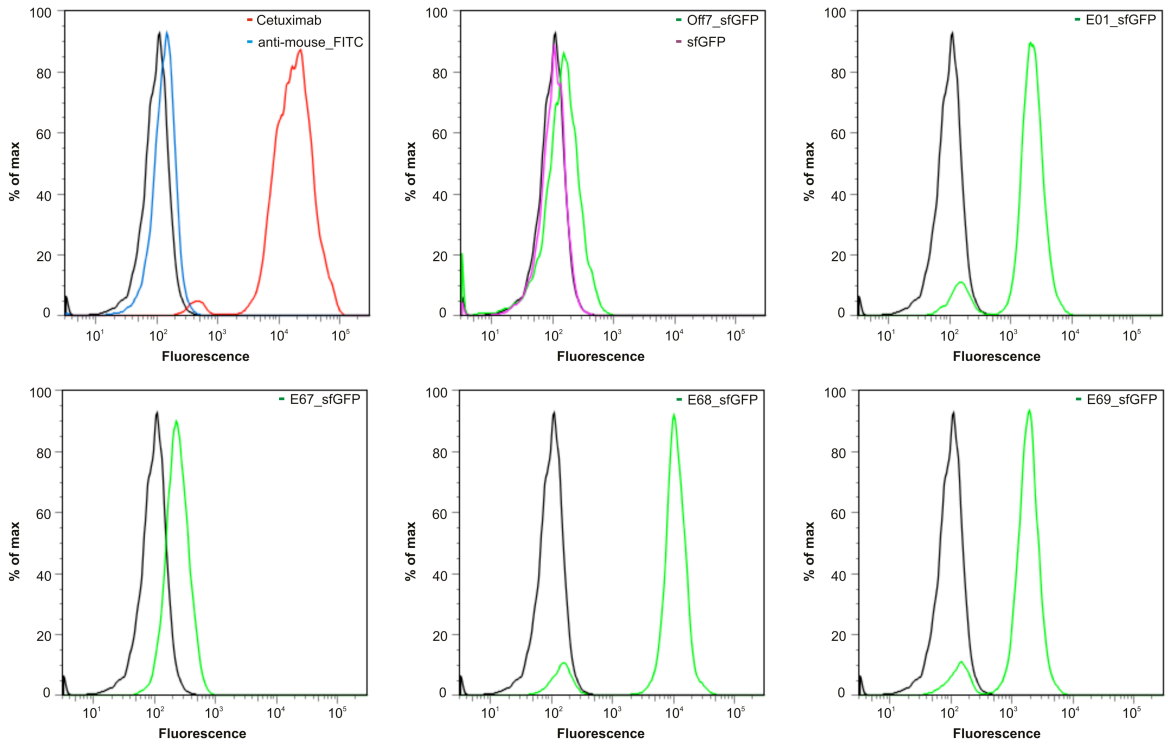
SUPPL. TABLE SII. Mutations leading to a loss of binding to sEGFR for DARPins E01 and E68

404SG wild-type residue	E01 mutations	E68 mutations
L381	V	V
Q384		H
Q408	H	
H409	D, P, Q, Y	D, P, Q
Q411	K, R	R
F412		V
A415	T, V	E, T, V
V417	A	A
G418	D	D
I438	K	
G441	E, R	E, R
K463		E, I, N, T
K465	E, I, N	N
I467	M	T
S468	G, R	G, R
N469	H	H, D
G471	D	

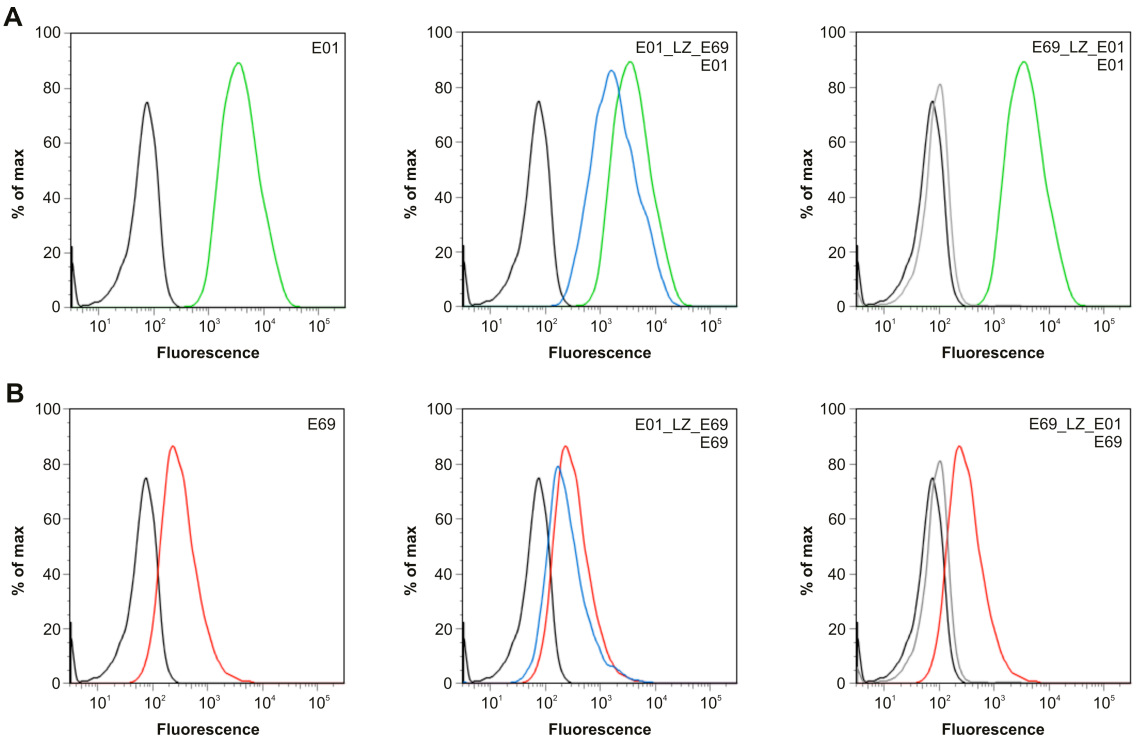
SUPPL. TABLE SIII. Mutations leading to a loss of binding to sEGFR for E69 and retention of binding for mAb EGFR1.

404SG wild-type residue	E69 mutation
Y101	H
D102	E
A103	P
N104	I
K105	E, I, N
T106	P
P130	H, L, S
H159	Y
L160	P
G161	D

SUPPLEMENTARY FIGURE



SUPPL. FIG. S1. Binding of DARPins to EGFR expressed on A431 cells, determined by flow cytometry. Autofluorescence by A431 cells is shown in black. As for cetuximab (red, secondary antibody in blue), DARPins E01, E67, E68 and E69 (green) all bind to A431 cells, whereas negative controls Off7_sfGFP (green) and sfGFP itself (violet) do not show a shift in fluorescence.



SUPPL. FIG. S2. Binding of E01_LZ3_E69 and E69_LZ3_E01 to EGFR expressed on A431 cells. Bispecific DARPins were incubated in a 1:1 molar ratio with EGFR at 4°C for 30 min on the A431 cellular surface, which should not induce significant internalization. Residual free binding places were detected by monovalent DARPins coupled to Alexa Fluor 488. (*A*) Measurement of fluorescence derived from E01_Alexa Fluor 488 (E01_488, green). A431 cells were either incubated with E01_Alexa Fluor 488 alone (left), or cells were preincubated with E01_LZ3_E69 (middle, blue) or with E69_LZ3_E01 (right, grey). After preincubation with E69_LZ3_E01, hardly any epitopes are available for E01_Alexa Fluor 488: the fluorescence signal is at the same level as the autofluorescence (black). However, for E01_LZ3_E69 residual fluorescence is measured, indicating that this construct does not completely block the E01 epitope on EGFR under these conditions. (*B*) Measurement of fluorescence derived from E69_Alexa Fluor 488 (E69_488, red). A431 cells were either incubated with E01_Alexa Fluor 488 alone (left), or cells were preincubated with E01_LZ3_E69 (middle, blue) or with E69_LZ3_E01 (right, grey). After preincubation with E69_LZ3_E01, hardly any epitopes are available for E69_Alexa Fluor 488 to bind to: the fluorescence signal is at the same level as the autofluorescence (black). In contrast, for E01_LZ3_E69 residual fluorescence is measured, indicating that this construct does not completely block the E69 epitope on EGFR under these conditions.