Supplementary information for:

Half-life extension of efficiently produced DARPin-FcRn serum albumin fusions as a function of FcRn affinity and recycling

Hannes Merten[a], Fabian Brandl[a,b], Martina Zimmermann[a], Jonas V. Schaefer[a], Linda Irpinio[a], Kine M. K. Sand[c,d], Jeannette Nilsen[c,d], Jan Terje Andersen[c,d], Uwe Zangemeister-Wittke[a,b,*], Andreas Plückthun[a,*]

[a]Department of Biochemistry, University of Zurich, 8057 Zurich, Switzerland
[b]Institute of Pharmacology, University of Bern, Inselspital INO-F, 3010 Bern, Switzerland
[c]Department of Immunology, University of Oslo and Oslo University Hospital Rikshospitalet, N-0372 Oslo, Norway
[d]Institute of Clinical Medicine, Department of Pharmacology, University of Oslo, N-0318 Oslo, Norway

Corresponding authors:

*Andreas Plückthun, Department of Biochemistry, University of Zurich, Winterthurerstrasse 190, CH-8057 Zürich, Switzerland, E-mail: plueckthun@bioc.uzh.ch, Phone: +41-44-635-5570, Fax: +41-44-635-5712

*Uwe Zangemeister-Wittke, Department of Biochemistry, University of Zürich, Winterthurerstrasse 190, 8057 Zürich, Switzerland and Institute of Pharmacology, University of Bern, Inselspital INO-F, 3010 Bern, Switzerland. E-mail: uwe.zangemeister@pki.unibe.ch, Phone: +41-31-632 3290, Fax: +41-31-632 4992

a

HSA-FLAG:

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHT
LFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLK
KYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQ
KFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS
SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALL
VRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKC
CTESLVNRRPCFSALEVDETYVPKEFNAETFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAVMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLDYKDDDDK

HSA7-FLAG:

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHT
LFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLK
KYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQ
KFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS
SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALL
VRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKC
CTESLVNRRPCFSALEVDETYVPKEFNAGTFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAAMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLDYKDDDDK

Ec1-HSA7-FLAG:

GSDLGKKLLEAARAGQDDEVRILVANGADVNAYFGTTPLHLAAAHGRLEIVEVLLKNGADVNAQDVWG
ITPLHLAAYNGHLEIVEVLLKYGADVNAHDTRGWTPLHLAAINGHLEIVEVLLKNVADVNAQDRSGKT
PFDLAIDNGNEDIAEVLQKAAKLGGSGGSGGSGGSGGDAHKSEVAHRFKDLGEENFKALVLIAFAQYL
QQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHTLFGDKLCTVATLRETYGEMADCCAKQEPERN
ECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLKKYLYEIARRHPYFYAPELLFFAKRYKAAFTE
CCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQKFGERAFKAWAVARLSQRFPKAEFAEVSKLV
TDLTKVHTECCHGDLLECADDRADLAKYICENQDSISSKLKECCEKPLLEKSHCIAEVENDEMPADLP
SLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPDYSVVLLLRLAKTYETTLEKCCAAADPHECYA
KVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALLVRYTKKVPQVSTPTLVEVSRNLGKVGSKCCK
HPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKCCTESLVNRRPCFSALEVDETYVPKEFNAGTF
TFHADICTLSEKERQIKKQTALVELVKHKPKATKEQLKAAMDDFAAFVEKCCKADDKETCFAEEGKKL
VAASQAALGLDYKDDDDK

HSA7-Ec1-FLAG:

DAHKSEVAHRFKDLGEENFKALVLIAFAQYLQQCPFEDHVKLVNEVTEFAKTCVADESAENCDKSLHT
LFGDKLCTVATLRETYGEMADCCAKQEPERNECFLQHKDDNPNLPRLVRPEVDVMCTAFHDNEETFLK
KYLYEIARRHPYFYAPELLFFAKRYKAAFTECCQAADKAACLLPKLDELRDEGKASSAKQRLKCASLQ
KFGERAFKAWAVARLSQRFPKAEFAEVSKLVTDLTKVHTECCHGDLLECADDRADLAKYICENQDSIS
SKLKECCEKPLLEKSHCIAEVENDEMPADLPSLAADFVESKDVCKNYAEAKDVFLGMFLYEYARRHPD
YSVVLLLRLAKTYETTLEKCCAAADPHECYAKVFDEFKPLVEEPQNLIKQNCELFEQLGEYKFQNALL
VRYTKKVPQVSTPTLVEVSRNLGKVGSKCCKHPEAKRMPCAEDYLSVVLNQLCVLHEKTPVSDRVTKC
CTESLVNRRPCFSALEVDETYVPKEFNAGTFTFHADICTLSEKERQIKKQTALVELVKHKPKATKEQL
KAAMDDFAAFVEKCCKADDKETCFAEEGKKLVAASQAALGLGGSGGSGGSGGSGGGSDLGKKLLEAAR
AGQDDEVRILVANGADVNAYFGTTPLHLAAAHGRLEIVEVLLKNGADVNAQDVWGITPLHLAAYNGHL
EIVEVLLKYGADVNAHDTRGWTPLHLAAINGHLEIVEVLLKNVADVNAQDRSGKTPFDLAIDNGNEDI
AEVLQKAAKLDYKDDDDK

b

end of AOX promoter
GGTCTCAAGCTATTGATTTTAACGACTTTTAACGACAACTTGAGAAGATCAAAAAACAAC

rbs pre-sequence
TAATTATTCGAAACGATGAGATTCCCATCCATCTTCACTGCTGTTTTGTTCGCTGCTTCT
M R F P S I F T A V L F A A S

TCCGCTTTGGCTGCTCCAGTCAATACTACTACTGAGGACGAGACTGCTCAGATTCCAGCT
S A L A | A P V N T T T E D E T A Q I P A
Signal peptidase

GAAGCTGTTATTGGTTACTCCGACTTGGAAGGTGACTTCGACGTTGCTGTTTTGCCATTC
E A V I G Y S D L E G D F D V A V L P F

pro-sequence
TCCAACTCCACTAACAACGGTTTGTTGTTCATCAACACTACTATCGCTTCCATTGCTGCT
S N S T N N G L L F I N T T I A S I A A

AAAGAAGAGGGAGTTTCCTTGGAGAAGAGAGAAGCTGAAGCT | Mature_ORF
K E E G V S L E K R | E A | E A | Mature_ORF

Kex2 DPAPase A DPAPase A

Figure S1: HSA and HSA fusion protein expression constructs. (**a**) Mature constructs, as they are secreted by *Pichia pastoris*. The HSA protein is in *black*, the point mutations of HSA7 are highlighted in *red*. The FLAG tag is shown in *blue*. DARPin Ec1 is shown in *green*, and the linker in *orange*. (**b**) Upstream and precursor region of the expression construct. The end of the AOX promoter and the ribosome binding site are shown. The pre-sequence of the α-mating factor (grey) is followed by the pro-sequence (green). The precursor protein is processed by signal peptidase, and then proteases Kex2 and dipeptidyl aminopeptidase A (DPAPase A), to release the mature ORF into the medium.

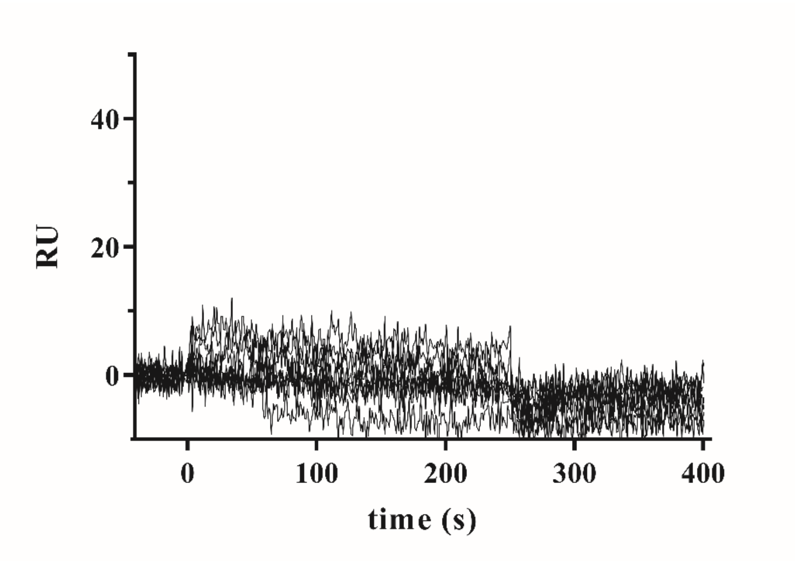

Figure S2: SPR sensorgram overlay of eight HSA (Albumedix) injections at 0.5 to 40 µM over immobilized murine FcRn (703 RU) at pH 6.0.

Table S1: Gene copy number determination in KM71H and SMDI1163

Strain/Gene	DNA amount (ng)	Absolute copy number actin (× 10^5)	Interpolated log value	Absolute copy number (× 10^5)	Relative copy number
KM71H/HSA7	10/30	9.86/29.6	6.18/6.67	15.0/47.1	1.5 ± 0.5
KM71H/Sh Ble	10/30	9.86/29.6	6.22/6.79	16.6/62.0	
SMD1163/HSA7	10/30	9.86/29.6	6.74/7.20	55.3/160	5.4 ± 0.2
SMD1163/Sh Ble	10/30	9.86/29.6	6.75/7.22	55.9/166	

Actin was used as a reference gene since only one copy is present in the *P. pastoris* genome. The log values of the absolute copy numbers of the actin gene at different genomic DNA amounts were plotted over C_t. From a linear regression the log values of the absolute copy numbers of the HSA7 and *Sh Ble* genes (phleomycin/bleomycin binding protein, present on the same plasmid, and thus expected to be present with the same copy number) at 10 or 30 ng genomic DNA were interpolated using the respective C_t value. The thereby calculated absolute copy numbers of the HSA7 and *Sh Ble* genes were divided by the respective absolute copy number of the actin gene to calculate the relative copy number. The averaged relative copy number of the two genes at two different DNA amounts is listed with the standard deviation.

Supplemental method

qPCR analysis

Gene copy number determination was conducted in triplicates on the same plate using a Brilliant II Ultra-Fast SYBR® Green QPCR Mastermix (Agilent Technologies) and a Mx3005P qPCR System (Agilent Technologies), following the guidelines of Marx et al. [1]. All DNA concentrations were measured with a Nanodrop spectrophotometer at 260 nm.

Primer pairs that result in amplicons with lengths between 130-150 bp and below 50% GC-content were used for the HSA7 gene (fw: 5'-AACTTGGGTAAGGTTGGTTCCAAGTGT-3'; bw: 5'-TCTGTCGGAAACTGGAGTCTTTTCGTG-3'), the Zeocin™ resistance gene *Sh Ble* (fw: 5'-CCCGGGACTTCGTGGAGGAC-3'; bw: 5'-ACCACTCGGCGTACAGCTCGTC-3'), and the actin reference gene (fw: 5'-CCTGAGGCTTTGTTCCACCCATCT-3'; bw: 5'-GGAACATAGTAGTACCACCGGACATAACGA-3'). *Sh Ble* is located on the same plasmid as the HSA7 gene and, hence, should result in the same copy number after the insertion into the yeast genome.

Genomic DNA was isolated from a 1 mL overnight culture of a selected *P. pastoris* clone (YPD medium, Formedium) that was centrifuged at 1500 × g for 5 min. The cell pellet was resuspended in 1 mL PBS and 750 µL glass beads of 0.5 mm diameter were added. The cells were lysed by shaking for 20 min with a frequency of 30 sec^{-1} using a tissue-lyser (Retsch). After centrifugation of the lysate (20,000 × g, 5 min, 4°C), 150 µL SDS-TE-buffer (2% (w/v) SDS, 100 mM Tris, 10 mM EDTA) were added and the solution incubated at 65°C for 5 min. Afterwards, 150 µL 5 M potassium acetate were added, and the mix was incubated on ice for 30 min. The solution was centrifuged (20,000 × g, 5 min, 4°C) and 200 µL sodium acetate (3 M, pH 6.0) and 1 mL isopropanol added to the supernatant. The mixture was incubated at –80°C for 10 min. Following centrifugation (20,000 × g, 10 min, 4°C), the pellet was dried for 20 min at room temperature. The pellet was resuspended by adding 200 µL isopropanol, 90 µL UHP water and 10 µL sodium acetate (3 M, pH 6.0). For the two following wash steps, 1 ml 70% ethanol was used, and the centrifugation steps were performed as described above. Finally, the genomic DNA pellet was dried at 37°C for 30 min, dissolved in 100 µL UHP water and stored at –20°C.

PCR was performed in a 10 µL reaction mix containing 5 µL Mastermix, 500 nM of forward and reverse primer and 10 or 30 ng genomic DNA. The amplification reaction profile included an initial denaturation at 95°C for 3 min followed by 40 cycles of 15 s at 95°C and

20 s at 60°C. The fluorescent signal was measured at the end of each extension step at 60°C (ex.: 492 nm; em.: 516 nm) and the fluorescent threshold value (C_t) was determined. The copy number of actin was calculated using the formula: copy number = (6.02 × 10^{23} [copy/mol] × template amount [g]) / (9.4 [Mbp] × 660 [g/mol×bp]).

A dilution series was generated and the logarithm of the absolute actin gene copy numbers plotted over C_t. The PCR efficiency was calculated from the slope of the generated standard line using the formula: efficiency = $10^{(-1/slope)}$-1. An efficiency between 0.9 and 1.1 was ensured. The absolute gene copy numbers of the HSA7 and *Sh Ble* genes were determined from the generated actin standard line by interpolating the logarithm of the gene copy numbers corresponding to the determined C_t values at 10 and 30 ng genomic DNA. The resulting absolute copy numbers were divided by the absolute copy number of the actin gene reference (one copy per yeast genome) to calculate the relative copy number. The relative copy numbers of *Sh Ble* and HSA7 were averaged for each strain.

To confirm the specificity of the PCR reactions, a melting curve analysis (peak of negative derivative) and agarose gel electrophoresis analysis were performed.

References

[1] Marx, H., Mecklenbrauker, A., Gasser, B., Sauer, M., Mattanovich, D., Directed gene copy number amplification in Pichia pastoris by vector integration into the ribosomal DNA locus, FEMS Yeast Res. 9 (2009) 1260-1270.