Supplementary Material for

Isolation of Intracellular Proteinase Inhibitors Derived from Designed Ankyrin Repeat Proteins by Genetic Screening

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Running Title**:** Designed Ankyrin Repeat Protein Libraries as Source for Intracellular Inhibitors

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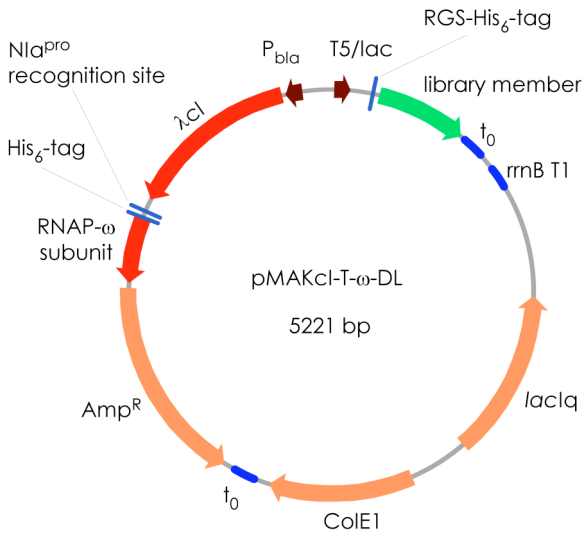

Supplemental Figure S1 - Vector map of the plasmid vector pMAKcI-T-ω-DL. ORFs are depicted as arrows, terminator sequences are indicated by a blue line, the NIa^{pro} recognition sequence and the His-tags are depicted as perpendicular blue lines and promoter regions are indicated by brown arrows.

Materials and Methods

Plasmids - Plasmids used in this study are listed in Table 1 in the main text. The sequences of all inserts in plasmids that were generated by PCR were confirmed by DNA sequencing.

The vector for the expression of NIa^{pro} proteinase in all *in vivo* experiments, pZA55-TEV, was constructed by inserting the PCR amplified *araC* gene plus P_{BAD} promoter sequence into pZA21-TEV, thereby replacing its *Xho*I/*Hind*III fragment. The *araC* gene-P_{BAD} promoter sequence was amplified from pBADGFPAC2 with oligonucleotides MAK_#34F (5'-ATCCGCTCGAGGCATAATGTGCCTGTC-3') and MAK_#34R (5'-ATCCCAAGCTTTCTCCCATATGTATATC

TCC-3'), and the product was cloned with the restriction enzymes *Xho*I and *Hind*III into pZA21-TEV. In turn, pZA21-TEV was constructed by inserting the PCR amplified gene of the catalytic domain of NIa^{pro} proteinase into pZA21, thereby replacing its *Kpn*I/*Bam*HI fragment. The gene of the catalytic domain of NIa^{pro} proteinase was amplified from pRK793 with oligonucleotides MAK_#28F (5'-ATTATGGTACCATGGGAGAAAGCTTGT TTAAGG-3') and MAK_28R (5'-GCAAGGCGATTAAGTTGGGTAACGC-3') and the product was inserted with the restriction enzymes *Kpn*I and *Bam*HI into pZA21, thus generating pZA21-TEV). The *Sac*I/*Aat*II fragment of this cloning intermediate was replaced by inserting the PCR amplified Tc^R restistance gene. The Tc^R restistance gene was amplified from pTRG with oligonucleotides MAK_#40F (5'-TATACGAGCT CCATTCAGGT CGAGGTGG-3') and MAK_#40R (5'-TATAGGACGT CTATTTTTTT GATGGGAAGG C-3') and the product was cut with the restriction enzymes *Sac*I and *Aat*II and ligated, thus generating pZA55-TEV.

pBRcI-T-ω is a derivative of pBRcI-ω containing a NIa^{pro} cleavage site. It was constructed by ligating insert 1 and insert 2 (see below) simultaneously into pBRcI-ω, thereby replacing its *Not*I/*Sal*I fragment. Insert 1 is a synthetic oligonucleotide duplex (5'-ATAAGAATGC GGCCGCTGGC TCGAGCGGTT CAGGCACTGA AAATCTTTAT TTTCAATCAG GTGCTAGCTA G-3') cut with the restriction enzymes *Not*I and *Nhe*I. Insert 2 was a PCR amplicon of the RNAP-ω gene from pBRcI-ω with oligonucleotides MAK_#29F (5'-TCTAGCTAGC CATCATCATC ATCATCATGG CTCTGCACGC GTAACTGTTC AGG-3') and MAK_#29R (5'- ATATGTCGAC TTAACGACGA CCTTCAGC -3') and the product was cut with the restriction enzymes *Nhe*I and *Sal*I and ligated together with insert 1, thus generating pBRcI-T-ω.

pMAKcI-T-ω-pD is a derivative of pQI-pD and constitutively expresses the λcI-T-ω fusion protein under control of the β-lactamase promoter P_{bla}. It was constructed by inserting the PCR amplified λcI-T-ω gene into pQI cut by *Ssp*I. The λcI-T-ω gene was amplified from pBRcI-T-ω with oligonucleotides MAK_#41F (5'- GCTGTTAATA TTGAAAAAGG AAGAGTATGA GCACAAAAAA GAAACC -3') and MAK_#41R (5'- GGTAGCAATA TTTTAACGAC GACCTTCAGC -3'), and the product was digested blunt ended with the restriction enzymes *Ssp*I and ligated into pQI.

pMAKcI-T-ω-DL is a derivative of pMAKcI-T-ω-pD and contains the pool of DARPins enriched by ribosome display, binding NIa^{pro} proteinase. It was used in all *in vivo* screening experiments. The ORFs of the ribosome-display-selected DARPin proteins were digested with *Nco*I and *Hind*III and ligated into pMAKcI-T-ω-pD, yielding the selection plasmid ready for *in vivo* screening.

pMAKcI-T-ω-DL-tag is a derivative of pMAKcI-T-ω-DL and was used for the DARPins 9_1s, 13_1b, 20_2b and E2_5 in the western blot experiments. In this vector the DARPin genes lack their N-terminal RGS-His_6 tag. Each was constructed by cutting out the DARPin gene from pMAKcI-T-ω-DL with restriction enzymes *BamH*I and *Hind*III and inserting this framgment into pQE60, thus replacing its *BamH*I/*Hind*III fragment. This intermediate cloning construct was cut by restriction enzymes *EcoR*I and *Hind*III, and the fragment containing the DARPin gene was placed back into pMAKcI-T- ω-DL, thus replacing its *EcoR*I/*Hind*III fragment.

pAT223-TEV is a derivative of pAT223 and was used for the expression of His-tagged biotinylated and non-biotinylated gpD- NIa^{pro} proteinase fusion protein. It was constructed by inserting the PCR amplified NIa^{pro} proteinase gene into pAT223, thereby replacing its *BamH*I/*Hind*III fragment. The NIa^{pro} proteinase gene was amplified from pRK793 with oligonucleotides MAK_#31F (5'- CGCGGATCCA TGGGAGAGAG CTTGTTTAAG GGG -3') and MAK_#31R (5'- ATCCCAAGCT TTTATTAGCG ACGGCG -3') and the product was cloned with the restriction enzymes *BamH*I and *Hind*III into pAT223.