Biotech Method

A simplified procedure for antibody engineering by yeast surface display: coupling display levels and target binding by ribosomal skipping

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Abbreviations: tGFP, an improved variant of green fluorescent protein (t=turbo); IgNAR, Immunoglobulin Novel Antigen Receptor; vNAR, variable domain of an IgNAR antibody; EpCAM, epithelial cell adhesion molecule; CDR, complementary determining region; SAPC, streptavidin allophycocyanin, a labeling reagent

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for biotinylated proteins; PBS, phosphate-buffered saline; BSA; bovine serum albumine; IgG, immunoglobulin; Fc, crystallizable fragment of an immunoglobulin; TEV, tobacco etch virus; K_D, dissociation constant; DAPI, 4',6-diamidin-2-phenylindole, a fluorescent stain that interacts with DNA

Abstract

Yeast surface display is a valuable, widely used method for protein engineering. However, current yeast display applications rely on the staining of epitope tags in order to verify full-length presentation of the protein of interest on the cell surface. We aimed at developing a modified yeast display approach that relies on ribosomal skipping, thereby enabling the translation of two proteins from one open reading frame and, in that manner, generating an intracellular fluorescence signal. This improved setup is based on a 2A sequence that is encoded between the protein to be displayed and a gene for green fluorescent protein (GFP). The intracellular GFP fluorescence signal of yeast cells correlates with full-length protein presentation and omits the need for the immunofluorescence detection of epitope tags. For method validation, shark-derived IgNAR variable domains (vNAR) were subjected to affinity maturation using the 2A-GFP system. Yeast library screening of full-length vNAR variants which were detected via GFP expression yielded the same high-affinity binder that had previously been isolated by our group using the conventional epitope tag-based display format. The presented method obviates the need for additional immunofluorescence cell staining, offering an easy and cost-friendly alternative to conventional epitope tag detections.

1 Introduction

High-throughput screening of large protein libraries using phage or yeast display as platform technology plays a pivotal role in modern biotechnology [1–3]. Although library sizes are generally several orders of magnitude smaller than the sizes achieved using phage display, yeast display is a straightforward alternative, particularly for the display of multidomain proteins containing several disulfide bonds [4]. In addition, yeast cells comprise the advantageous feature of a eukaryotic posttranslational modification machinery. This machinery offers an intrinsic quality control mechanism and involves enzymes such as foldases and chaperones, enabling the display of more complex proteins on the surface of these cells [5]. The yeast

surface display technique was first described by Boder and Wittrup almost 20 years ago and still represents one of the most commonly used methods for yeast library screenings [6]. The original assembly of proteins on the yeast surface, namely the fusion of a protein of interest to the a-agglutinin protein Aga2p that anchors the protein to the yeast cell wall, is the gold standard in most laboratories. In order to ensure accurate and full-length display of the Aga2p fusion protein, peptide tags such as the HA or myc tag have been included at either the *N*- or *C*-terminus, depending on the orientation of the Aga2p fusion (Figure 1A). In this manner, false-positive results caused by non-specific sticking of potentially truncated protein variants can be circumvented by two-dimensional immunofluorescence detection of surface display and target binding. However, such protein staining procedures can be time-consuming and cost-intensive, depending on the reagents that are utilized for detection, and also influence target binding.

Herein, we describe a novel method for yeast surface display that omits the necessity of detecting full-length surface presentation by cell labeling, while maintaining adequate coupling of display and target-binding levels. Our approach is based on the picornaviral 2A peptide that has been widely used for ribosomal skipping in various biotechnological applications as it allows the generation of multiple proteins from one mRNA transcript [7–9]. Most importantly and in comparison to functionally similar sequences such as internal ribosome entry sites (IRES), skipping of the ribosome and therefore the lack of formation of a peptide bond between the separated proteins results in the translation of equal amounts of each protein [9]. Overall, a variety of 2A peptides differing in sequence, length and cleavage efficiencies have been reported so far, as exemplarily described by Chng and coworkers [10]. Since the 2A release site is always located after the last glycine residue of the peptide sequence, the majority of the peptide is attached to the first protein [9]. In contrast, the last proline residue of each 2A peptide is the first residue of the second protein that is being translated. We chose the T2A sequence derived from the *Thosea asigna* virus that comprises 18 amino acids. As described by Chng et al, the T2A sequence in comparison to other 2A peptide variants exhibited the most efficient cleavage as tested in view of the co-expression of heavy and light chains of a monoclonal antibody in CHO cells [10]. Compatibility experiments encompassing the 2A cleavage system in *Saccharomyces cerevisiae* cells in particular have been performed by de Felipe and coworkers who used a 19 amino acid 2A peptide derived from the foot-and-mouth disease virus [11]. Their paper elegantly demonstrated that this system can efficiently be utilized in fungi, such as yeast. A similar approach regarding

the display of two proteins on the surface of yeast upon ribosomal skipping was previously reported by Sun and coworkers [12]. Their work encompassed the immobilization of two variants of the enzyme *Candida antarctica* lipase B on the surface of the yeast *Pichia pastoris* by genetic fusion to the *Saccharomyces cerevisiae* cell wall proteins Sed1 and Sag1.

Shark-derived IgNAR antibodies are homodimeric molecules which engage their antigen with only one single variable domain, referred to as vNAR, rather than with a heterodimeric paratope as in case of monoclonal antibodies. The unique structural features of vNAR domains contribute to increased physicochemical stability as well as their ability of targeting demanding epitopes which are difficult to address for conventional antibodies [13, 14]. Engineering of vNAR domains using yeast surface display yielded high-affinity binders against a variety of therapeutically relevant target proteins [15, 16]. Moreover, it could be shown that pH-sensitive vNAR domains can be isolated from histidine-doped yeast libraries, rendering these domains suitable for biotechnological applications [17].

In practice, our proposed method replaces the *C*-terminal myc tag of vNAR variants in a yeast surface display format with the sequence for the T2A peptide and a gene encoding for TurboGFP (tGFP; Figure 1A). Upon translation of the corresponding mRNA transcript at the ribosome, ribosomal skipping is induced at the 2A sequence resulting in the formation of both, the Aga2p-vNAR fusion protein containing a signal sequence for secretion, and tGFP which remains inside the cell. Since the tGFP coding sequence follows the vNAR gene, full length translation of the antibody domain followed by T2A synthesis is required for tGFP formation. Ribosomal skipping leads to the expression of tGFP which remains inside the cell and allows functional, fluorescent read-out using a microscope or a flow cytometer.

As a proof-of-concept study, we performed affinity maturation of yeast-displayed, EpCAM-binding vNAR domains that were previously isolated and affinity matured by our group using conventional detection of the c-myc tag in order to ensure full-length antibody display [15]. The initial vNAR domains that were utilized as starting material for affinity maturation comprised moderate affinities for EpCAM. We were able to show that library screening relying on tGFP formation upon ribosomal skipping is at least as efficient as using the conventional yeast surface display and screening approach.

This method is a convenient and cost-efficient alternative for conventional display-related immunofluorescence stainings of yeast cells, obviating the need for epitope tags and allowing improved affinity discrimination due to expression normalization.

2 Materials and methods

2.1 Assembly of a pCT-5005-T2A-tGFP construct

The amino acid sequence of the T2A peptide that was used for ribosomoal skipping comprised the following 18 amino acids: EGRGSLLTCGDVEENPG↓P. The arrow indicates the separation site. For the initial proof-of-concept experiment, a pCT vector [6] encoding for the vNAR variant 5005, a myc tag and the T2A sequence was digested using the restriction enzymes *Bam*HI and *Xho*I (*New England Biolabs*). The gene for TurboGFP (tGFP; *Evrogen*) was PCR-amplified from a pET32a vector, utilizing primers which attached sequence overhangs for the T2A sequence as well as the pCT vector. Since the utilized restriction sites for the digestion of the pCT plasmid cleaved in-between the vNAR sequence, vNAR 5005 was amplified separately from a different pCT template, adding overhangs for gap repair with the pCT vector at the 5' end and an overlapping sequence of the T2A peptide at the 3' end. Subsequently, an overlap extension PCR was performed in order to assemble the final 5005-T2A-tGFP insert, which was then purified and transformed into yeast cells. Electrocompetent *Saccharomyces cerevisiae* EBY100 cells were prepared according to a previously published protocol by Benatuil and coworkers [18]. Successful transformants were sequence-verified (*SeqLab Microsynth*, Göttingen, Germany).

2.2 Construction of a CDR1-randomized, yeast-displayed vNAR library

The pCT plasmids of two previously isolated single clones, H3 and H5, were utilized as templates for library generation [15]. Both single clones had been isolated from a CDR3-randomized vNAR library that was screened against EpCAM. For the purpose of CDR1 affinity maturation, three PCR reactions were carried out in order to assemble the final insert for transformation of *Saccharomyces cerevisiae* EBY100 cells. PCR reactions were essentially carried out as described [15]. However, in our case the third PCR attached overhangs for gap repair in yeast, comprising homologous sequences to the pCT plasmid (5' end) as well as

the T2A peptide (3' end). The pCT plasmid encoding for the pCT 5005-myc-T2A-tGFP construct was digested using the *Nhe*I, *Sac*II and *Bam*HI restriction sites, ensuring complete removal of the *C*-terminal myc tag. Transformation of *Saccharomyces cerevisiae* EBY100 cells was performed according to a protocol of Benatuil and coworkers [18]. Cultivation of yeast cells was carried out as described [15–17]

2.3 Library screening

Library screening for the isolation of antigen-specific vNAR variants was performed on a BD Influx™ cell sorter. Data were recorded and analyzed using the BD FACS™ Sortware v1.0. For the first sorting round, 1.5×10^8 cells were labeled with 10 nM biotinylated EpCAM (*ACROBiosystems*). Incubation steps were performed in PBS 0.1% BSA on ice for 20 min. The following rounds were also performed using 10 nM biotinylated EpCAM and upon staining at least ten times the number of yeast cells sorted in the previous round in order to ensure adequate library coverage. For the detection of biotinylated EpCAM, streptavidin conjugated with R-phycoerythrin (SPE, *affymetrix eBioscience*) or allophycocyanin (SAPC, *affymetrix eBioscience*), respectively, was employed. HA tag stainings were performed upon using an anti-HA tag antibody (*affymetrix eBioscience*), an anti-rabbit-biotin antibody (*Sigma-Aldrich*) and either SPE or SAPC.

2.4 Confocal microscopy

In general, 3×10^7 yeast cells displaying vNAR domain SH1 were incubated with 20 nM biotinylated EpCAM. Subsequently, cells were washed with PBS 0.1% BSA and incubated with SAPC. All incubation steps were performed on ice for 20 minutes. A negative control which was only incubated with SAPC was prepared in parallel. After immunofluorescence staining, the cells were washed thrice with PBS 0.1% BSA and resuspended in a final volume of 25 µL. In order to fix the cells for confocal microscopy analysis, 4 µL of cell suspension were spotted onto microscope slides and spread slightly using a pipette tip. Afterwards, the spotted yeast cells were air-dried for approximately 20 minutes and fixed using 1 drop of ProLong Diamond Antifade Mountant with DAPI (*LifeTechnologies*). After covering each fixed spot with a cover slide, the prepared samples were stored at 4°C. Analyses were performed on a Leica TCS SP5 confocal microscope equipped with a 100× objective (*Leica Microsystems*).

2.5 Subcloning, expression, purification and analyses of vNAR variants

Subcloning for soluble production of vNAR variant SH2 via fusion to IgG1 Fc, expression in Expi293F cells and subsequent purification via protein A chromatography was performed essentially as described [17].

3 Results and discussion

3.1 Intracellular GFP levels generated upon ribosomal skipping correlate with surface presentation

We first aimed at ensuring the functionality of the proposed method upon analyzing the EpCAM-binding vNAR single clone 5005 [15], which comprised the T2A as well as the tGFP sequence, on the surface of yeast. We performed immunofluorescence staining of the myc tag located downstream of the vNAR domain and correlated its labeling intensity with the tGFP signal (Figure 1B). As expected, the myc tag as well as the tGFP fluorescence signal intensities correlate, indicating co-expression of both proteins. Likewise, cells that refrained from displaying an antibody fragment also did not show tGFP fluorescence.

3.2 Screening of a vNAR yeast library using the 2A-GFP system

In the following steps, we used the EpCAM-binding vNAR domains H3 and H5 which were previously isolated from a semi-synthetic, CDR3-randomized vNAR library displayed on the surface of yeast [15]. Both single clones had an apparent dissociation constant (K_D) of 1301 and 1030 nM, respectively, as determined by affinity titrations on the yeast surface as well as biolayer interferometry measurements [15]. As described by Zielonka and coworkers, affinity maturation of a pool of vNAR variants after sorting round three (including H3 and H5) via randomization of the CDR1 loop yielded vNAR variant 5005, which had a significantly improved K_D of 14 nM [15]. In order to test the new ribosomal skipping display methodology, we performed CDR1 randomization of a mixture of vNAR variants H3 and H5, aiming at investigating whether it was possible to reproduce these results using the proposed method for affinity maturation of weak binders. After randomization and subcloning of the generated vNAR constructs, transformation of yeast cells was performed. After three days, the library size was determined to be 1.3×10^9 transformants. Successful

randomization of the CDR1 loop was confirmed upon analyzing single clones directly after library generation (data not shown). Next, we analyzed the library in terms of the correlation between HA-tag labeling intensity and tGFP expression levels. As depicted in Figure 1C, three cell populations are distinguishable, namely cells that display neither HA-tag nor tGFP expression, cells displaying both and cells showing HA-epitope staining but no tGFP expression. The latter fraction of cells supposedly represents vNAR variants which exhibit frameshift or stop mutations downstream of the HA tag, leading to the secretion of truncated and potentially misfolded artifacts that are not prone to ribosomal skipping and tGFP synthesis. We isolated these cells using fluorescence-activated cell sorting (FACS) and could confirm the aforementioned hypothesis upon performing sequence analysis of isolated single clones (Figure S2). However, it should be mentioned that the GFP signal only serves as a metric for translation rather than surface display of the protein of interest. Although good correlation is achieved in case of single clones (Figure 1B), Figure 1C demonstrates significant inter-clonal variability. Nevertheless, we started screening the affinity matured library upon isolating the cell population that displayed EpCAM binding as well as tGFP fluorescence signal. Double-positive cells were selected as depicted in Figure 2A. Overall, three rounds of sorting were performed. Sequence analysis of single clones after the third sorting round identified two dominant vNAR domains, in the following termed SH1 and SH2, which were overrepresented in the pool and possessed the same CDR1 sequence as the previously isolated vNAR domain 5005. SH1 comprised an amino acid sequence completely identical to vNAR variant 5005 [15], while SH2 only differed in three framework amino acid residues (Figure 3). A dot plot diagram depicting tGFP fluorescence intensity and EpCAM labeling intensity of identified single clone SH1 is shown in Figure 2A. As vNAR variants SH1 and 5005 are identical and thorough characterization of this clone has already been performed [15], we recombinantly expressed SH2 as Fc fusion in Expi293F cells and analyzed it using biolayer interferometry (Figure S3). The determined K_D value was 12.47 ± 0.15 nM and is consistent with the value obtained for SH1/5005 [15].

3.3 Confocal microscopy verifies ribosomal skipping through intracellular GFP fluorescence

In addition to flow cytometric analyses, we employed confocal microscopy in order to show that there is efficient coupling of display levels and target binding (Figure 2B). Yeast cells displaying vNAR variant SH1 on their surface were labeled consecutively with 20 nM biotinylated EpCAM and SAPC and subsequently fixed on microscope slides. Figure 2B shows cell images recorded in DAPI, tGFP and SAPC fluorescence channels and the corresponding overlays. It is observable that almost every yeast cell exhibiting tGFP fluorescence also exhibits EpCAM binding. In addition, yeast cells with weaker tGFP fluorescence also show EpCAM binding on their surface to a smaller extent. A negative control employing cells which were not incubated with EpCAM but with SAPC only do not comprise any fluorescence signal in the corresponding channel.

Our results demonstrate that the proposed method is a valuable and competitive alternative to the conventional yeast surface display format. In contrast to directly fusing a GFP moiety to the protein of interest, intracellular expression of the fluorophore reduces steric hindrances that might occur upon covalently fusing it to the *C*-terminus of the protein to be displayed. Additionally, the proposed method is most likely independent of the anchor protein which is utilized for surface display[19] as well as the yeast strain. Although the utilization of the T2A peptide sequence attaches overall 17 amino acids to the *C*-terminus of the Aga2p-vNAR fusion protein (in comparison, the myc epitope tag comprises 10 amino acids) as well as a proline residue to the *N*-terminus of tGFP, functional levels of expression and fluorescence are achieved.

4 Concluding remarks

We describe a novel method for the screening of yeast surface display libraries that takes advantage of the T2A peptide, a sequence that leads to skipping of the ribosome upon translation of the corresponding mRNA. Using this sequence, it is possible to obtain several proteins from one open reading frame. For yeast library screening, the protein to be displayed on the cell surface is *C*-terminally fused to the T2A sequence

and a gene encoding for tGFP. Therefore, upon translation of the mRNA transcript, the protein of interest is being formed and secreted while tGFP remains inside the cell, allowing for detection of yeast cells that display a full-length protein on their surface. Successful affinity maturation of shark-derived IgNAR variable domains demonstrates the applicability of the developed methodology for yeast surface display library screening. Since no labeling steps are required for the detection of epitope tags in order to verify full-length antibody display on yeast, this strategy is cost-efficient, rapid and facilitates screening of large antibody libraries.

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The authors declare no commercial or financial conflict of interest.

5 References

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Figure Legends

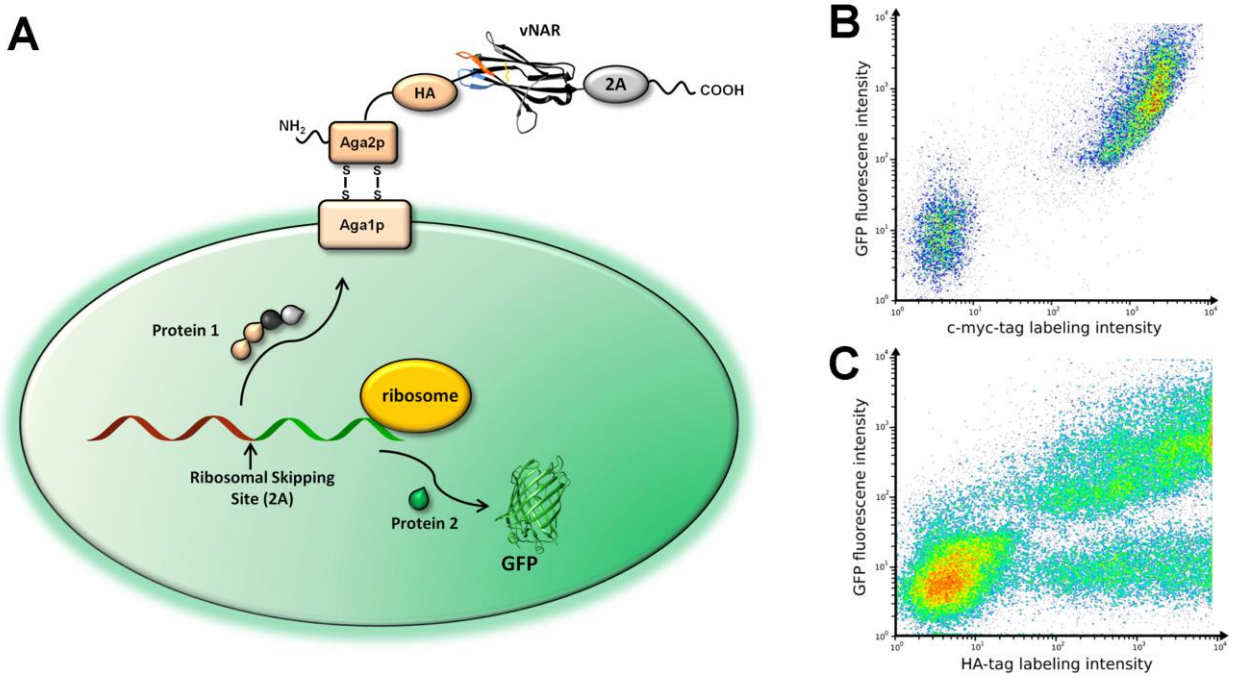

Figure 1. (A) Schematic depiction of the ribosomal skipping approach presented in this paper. Upon translation of the mRNA transcript encoding for the Aga2p-vNAR-T2A-tGFP fusion protein, ribosomal skipping is induced at the 2A sequence, leading to release of the Aga2p-vNAR protein from the ribosome and secretion to the yeast surface. Afterwards, translation of the remaining stretch of the mRNA transcript encoding for tGFP takes place, leading to accumulation of the protein inside the cell (GFP modified from PDB-ID 1EMA, vNAR modified from 4HGK using UCSF Chimera) (B) In order to validate the functionality of our method, the EpCAM-binding vNAR single clone 5005 was genetically fused to the T2A-tGFP sequence and transformed into yeast cells. The dot plot diagram depicts induced yeast cells which were labeled for myc tag presentation. The tGFP fluorescence intensity is depicted on the y-axis, myc tag labeling intensity is plotted on the x-axis (C) Dot plot depicting the fluorescence labeling intensities of the HA tag as well as tGFP fluorescence of a CDR1-randomized vNAR library prior to sorting.

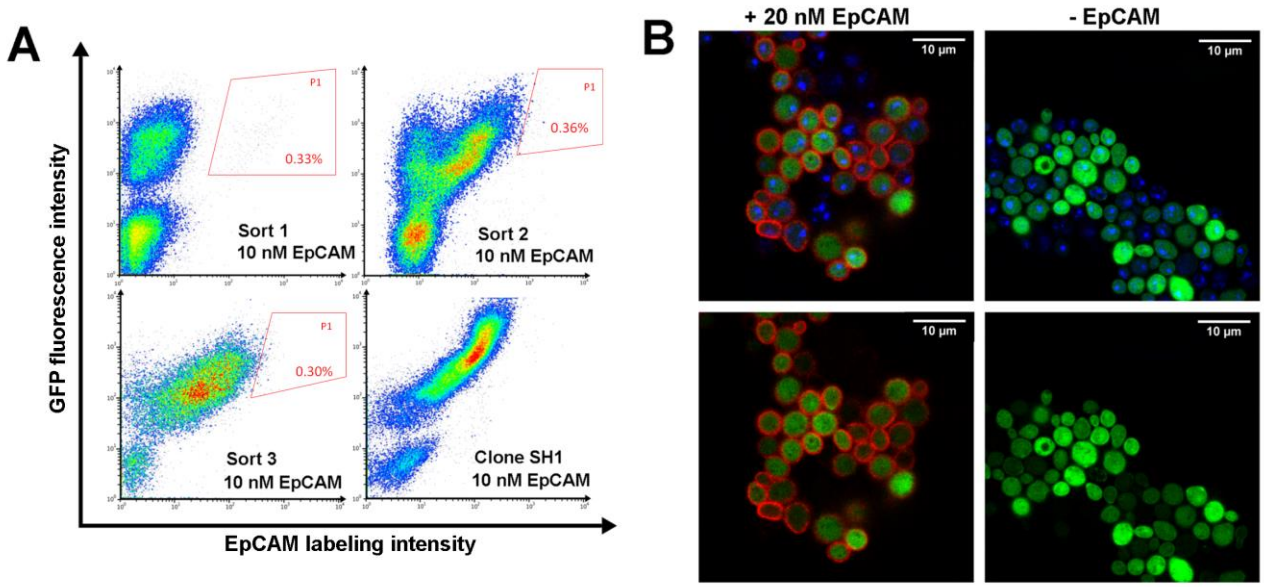

Figure 2. (A) Upper panel and lower left: Library screenings of the affinity maturation library towards EpCAM employing the detection of full-length antibody presentation upon analyzing tGFP fluorescence intensity. Overall, three rounds of selection using 10 nM biotinylated EpCAM were performed. Dot plot diagrams depicting tGFP fluorescence of yeast cells as well as EpCAM labeling intensities are shown. Cells in the corresponding gates were selected and further cultivated for subsequent rounds of sorting or analysis of single clones. Lower right: Dot plot depicting tGFP fluorescence as well as EpCAM labeling intensity of the isolated single clone SH1. SH1 comprised the same CDR1 sequence as vNAR variant 5005, which was selected from affinity maturation libraries upon immunofluorescence staining of the *C*-terminal myc tag [15] (B) Confocal microscopy images of yeast cells presenting vNAR variant SH1 on their surface. Cells were labeled using 20 nM biotinylated EpCAM and SAPC as secondary labeling reagent. Cells of the corresponding negative control were exclusively labeled with SAPC. Upper panel: Merged images depicting DAPI (blue), tGFP (green) and SAPC (red) fluorescence. Lower panel: Merged images depicting tGFP and SAPC fluorescence.

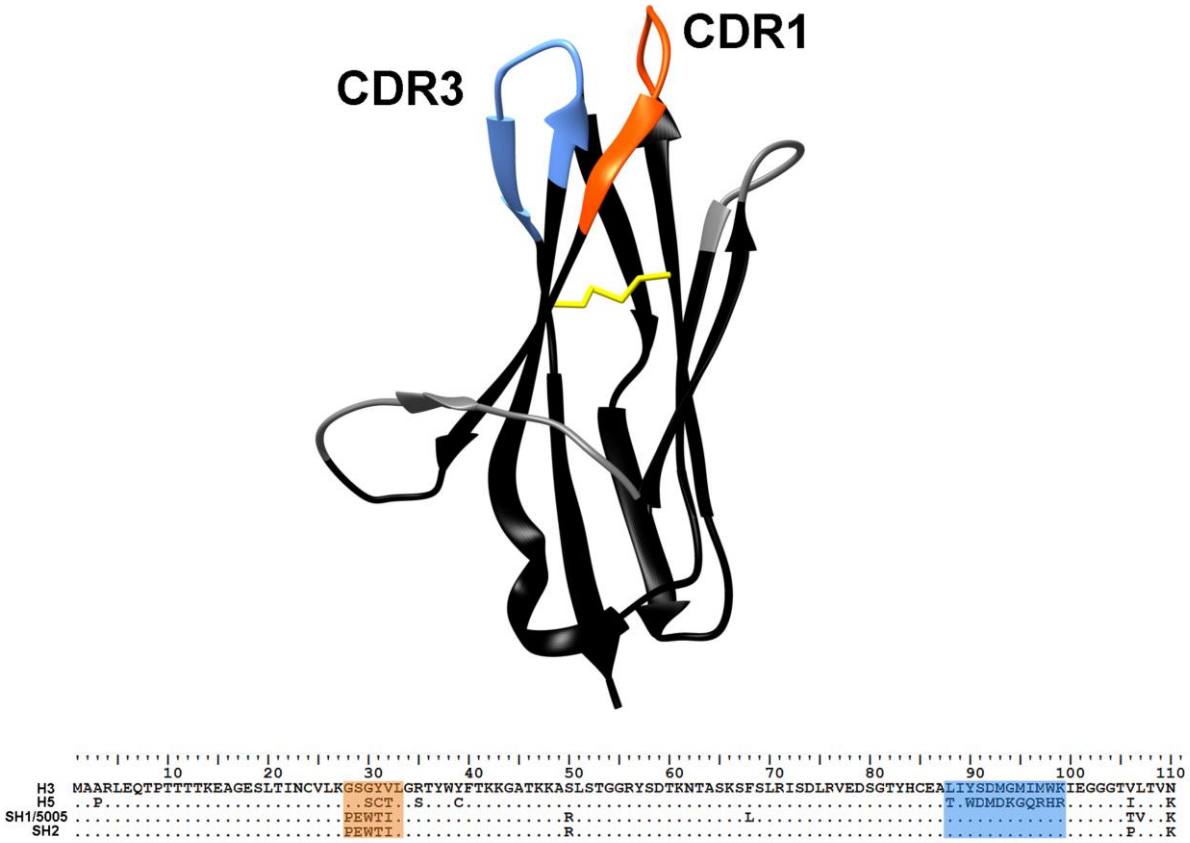

Figure 3. Protein structure of an IgNAR variable domain (vNAR; modified after PDB ID 4HGK using UCSF Chimera). CDR1 and CDR3 binding sites are depicted in orange and blue, respectively. Additionally, a sequence alignment of initial single clones H3 and H5 as well as the affinity matured single clones 5005/SH1 and SH2, which were obtained after yeast library screening, is depicted.