

Optimization of a Nanobody Targeting the Tyrosine Kinase Domain of Human EGFR via Hitchhiker-mediated Survival Selection

Chanason Lardarpajid¹, Apisitt Thaiprayoon¹, Onapa Punpeng², Aiyaret Kraivisootsup², Duangtip Moonmangmee², Lueacha Tabtimmai³, Kiattawee Choowongkamon⁴, and Dujduan Waraho-Zhmayev^{1,*}

¹ Biological Engineering program, Faculty of Engineering, King Mongkut's University of Technology Thonburi, Bangkok, Thailand

² Department of Microbiology, Faculty of Science, King Mongkut's University of Technology Thonburi, Bangkok, Thailand

³ Department of Biotechnology, Faculty of Applied Science, King Mongkut's University of Technology North Bangkok, Bangkok, Thailand

⁴ Department of Biochemistry, Faculty of Science, Kasetsart University, Bangkok, Thailand

Email: champ.chanason1994@gmail.com (C.L.); Nick_mic4@hotmail.com (A.T.); onapa.punp@mail.kmutt.ac.th (O.P.); aiyaret.krai@mail.kmutt.ac.th (A.K.); duangtip.moo@kmutt.ac.th (D.M.); Lueacha.t@sci.kmutnb.ac.th (L.T.); fsciktc@ku.ac.th (K.C.); dujduan.war@mail.kmutt.ac.th (D.W.-Z.)

*Corresponding author

Abstract—The Epidermal Growth Factor Receptor (EGFR) is essential for cell growth, differentiation, and migration, and its overexpression is closely linked to tumor progression and drug resistance. Although conventional EGFR-targeting antibodies and small-molecule inhibitors are effective, they often face limitations such as poor tissue penetration and acquired resistance. Nanobodies offer advantages including small size, high stability, and strong binding specificity. This study aimed to optimize a previously reported anti-EGFR tyrosine kinase domain nanobody, VHH35, by isolating improved variants using the Functional Ligand-binding Identification by Tat-based Recognition of Associating Proteins (FLI-TRAP) system. A VHH35 library of approximately 7.68×10^5 clones was generated, leading to the isolation of two mutants, M.5 and M.6, which exhibited enhanced solubility while maintaining specific binding to EGFR-TK. Dot blot analysis confirmed strong EGFR reactivity with minimal nonspecific binding. This work demonstrates a practical strategy for enhancing intracellular nanobody performance using FLI-TRAP, yielding promising candidates for cancer therapeutics.

Keywords—antibody engineering, Epidermal Growth Factor Receptor (EGFR), *Escherichia coli* (*E. coli*), Functional Ligand-binding Identification by Tat-based Recognition of Associating Proteins (FLI-TRAP), protein isolation strategy, single-domain antibody

I. INTRODUCTION

The Epidermal Growth Factor Receptor (EGFR), also known as HER1, is a member of the receptor Tyrosine Kinase (TK) superfamily that regulates essential cellular processes, including proliferation, differentiation,

survival, cytoskeletal rearrangement, and migration [1]. The overexpression of EGFR has been linked to multiple cancers, particularly non-small cell lung cancer (NSCLC), where it contributes to tumor growth, metastasis, and angiogenesis [2, 3]. Two main therapeutic strategies currently target EGFR, including small-molecule Tyrosine Kinase Inhibitors (TKIs) and monoclonal Antibodies (mAbs). TKIs inhibit ATP binding at the receptor's catalytic site, suppressing downstream signaling pathways. However, they often exhibit off-target effects due to interactions with other kinases sharing a common ATP substrate and can cause systemic toxicity, particularly hepatic and intestinal epithelial toxicity [4–6]. Meanwhile, mAbs block the receptor's extracellular domain to prevent ligand binding and dimerization, but long-term use frequently results in resistant EGFR mutations, and their large size limits tissue penetration and reduces efficacy [7–9].

Intracellular antibodies or intrabodies are antibody molecules or fragments that are expressed or delivered inside cells, where they recognize and bind specific intracellular antigens via their Complementarity-Determining Regions (CDRs). Since their first demonstration in the early 1990s, the field has expanded significantly, enabling applications in target validation and the modulation of intracellular pathways. These engineered antibodies can be fused to functional moieties that replace the Fc region to confer specific intracellular activities. Depending on the fusion partner, intracellular antibodies can inhibit protein–protein or protein–DNA interactions, relocalize target proteins to specific compartments such as the nucleus or endoplasmic reticulum, or trigger programmed cell death and protein degradation [10].

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The development of single-chain variable Fragments (scFvs), which combine heavy (VH) and light (VL) variable regions into a single polypeptide, demonstrated that antibody fragments can fold correctly and remain functional inside cells even without disulfide bonds [11]. Structural analyses further confirmed that these cysteine residues are not essential for proper folding [12]. Subsequent work revealed that single variable domains alone—such as human-derived intracellular single-domain antibody fragments (iDAb)s [13, 14], camelid single-domain VHH (nanobodies) [15, 16], or shark-derived VNAR heavy chains [17]—are the smallest functional antibody units capable of specific antigen binding. These single-domain antibodies have become powerful tools for engineering intracellular antibodies with enhanced stability, solubility, and functional versatility, representing a new generation of molecular tools for studying and manipulating cellular processes [10].

Nanobodies (Nbs), derived from the variable domains of camelid heavy-chain antibodies, represent a novel class of antibody fragments with unique structural and biochemical advantages. Their small size (~15 kDa), strong stability, and high specificity allow them to access epitopes that are otherwise difficult for conventional antibodies to reach. Nbs emerge as a promising alternative to conventional mAbs, addressing limitations related to tissue penetration and drug resistance [9]. In 2019, Tabtimmai *et al.* [18] developed VHH35 as a cell-penetrable Nb that inhibits the Tyrosine Kinase (TK) activity of EGFR. This inhibition is achieved through the Nb's CDR3, which interacts with EGFR-TK residues near the catalytic site, likely sterically hindering ATP binding to the TK. By suppressing these key signaling pathways, VHH35 diminished the motility and survival of human lung adenocarcinoma cells. These findings indicate significant potential for the Nb's further clinical development.

To enhance intrabody discovery and screening, the Functional Ligand-binding Identification by Tat-based Recognition of Associating Proteins (FLI-TRAP) system provides an *in vivo* selection method that links intrabodies–antigen interactions to bacterial survival. Specifically, when a functional complex is formed between the target antigen fused to β -lactamase, Bla, and the antibody fragment fused to the Twin-arginine translocation (Tat) signal sequence of the *Escherichia coli* (*E. coli*) trimethylamine N-oxide reductase, ssTorA, the complex is transported to the periplasm, where the active Bla confers β -lactam ring antibiotic resistance. This survival-based mechanism allows for the direct screening and maturation of antibody fragments, yielding variants with enhanced solubility and/or binding affinity proportional to the achieved antibiotic resistance levels. This system enables the identification of high-affinity binders directly within living cells, ensuring proper protein folding and functional expression inside the reducing environment of the cell's cytoplasm, where disulfide bonds cannot form [19].

FLI-TRAP platform was extended to enable rapid *in vivo* selection of scFv intrabodies with enhanced stability and affinity. Remarkably, a single round of enrichment resulted in intrabody variants with approximately tenfold yield enhancement and eightfold affinity improvement, highlighting FLI-TRAP as a powerful strategy for functional maturation of intrabodies without antigen purification or immobilization [20].

This study aimed to apply the FLI-TRAP platform for the selection of improved anti-EGFR-TK VHH35 variants from an error-prone mutant library. Using this approach, we successfully isolated two VHH35 mutants that conferred higher antibiotic resistance than the wild-type (wt) VHH35, suggesting enhanced performance within the FLI-TRAP selection system. These clones were subsequently characterized *in vitro* using Western Blot analysis to assess their solubility and dot blot assays to verify their binding activity and specificity toward EGFR-TK.

II. MATERIALS AND METHODS

A. Plasmid and Library Construction

The DNA sequences of wt-VHH35 and EGFR-TK were PCR-amplified and cloned into pDD18-Cm ssTorA-Ref8-FLAG and pDD322-Kan CAT-Bla [21], generating pDD18-Cm ssTorA-wt-VHH35-FLAG and pDD322-Kan EGFR-TK-Bla, respectively. Error-prone mutations were introduced into the wt-VHH35 gene using PCR with the GeneMorph II Random Mutagenesis Kit (Agilent Technologies). The resulting PCR products were cloned into pDD18-Cm ssTorA-Ref8-FLAG to construct the pDD18-Cm ssTorA-mu-VHH35 library-FLAG. Ligation reactions were transformed into *E. coli* NEB10 β (New England Biolabs) by electroporation. The transformed cells were combined in a 250-mL flask and incubated at 37°C with shaking at 200 rpm for 1 h. To estimate library size, 100- μ L aliquots were serially diluted and plated on LB agar supplemented with 100 μ g/mL chloramphenicol (Cm), followed by overnight incubation at 37°C. The remaining culture was supplemented with 100 mL of fresh LB containing 100 μ g/mL Cm and grown overnight at 37°C, 200 rpm. Then, the pDD18-Cm ssTorA-mu-VHH35 library-FLAG plasmids were isolated by miniprep and transformed into *E. coli* BL21(DE3). The resulting BL21(DE3) library culture was rendered electrocompetent for co-transformation with pDD322-Kan EGFR-TK-Bla.

B. Enhanced VHH35 Mutant Selection Using the FLI-TRAP Assay

The background growth of *E. coli* when ssTorA-wt-VHH35-FLAG or ssTorA-Ref8-FLAG (negative control) was co-expressed with EGFR-TK-Bla in the FLI-TRAP system was assessed using a spot-plate assay. Luria-Bertani (LB) agar plates were prepared with varying Carbenicillin (CB) concentrations 0, 12.5, 25, 50, 100, and 200 μ g/mL and 1% (w/v) arabinose. Cultures were adjusted to an initial OD₆₀₀ of 2.5 and serially diluted from 10⁻¹ to 10⁻⁶. A 5 μ L aliquot from each

dilution was spotted onto the plates, which were then incubated at 25°C and 30°C for 48 h.

For enhanced mutant selection, conditions were chosen to prevent the wt-VHH35 from growing on CB-containing plates, ensuring selective pressure that favored identification of enhanced VHH35 mutants that still bind to EGFR-TK. The *E. coli* culture harboring the mutant VHH35 error-prone library was grown overnight in 100 mL LB supplemented with 100 µg/mL Cm and kanamycin (Kan) at 37 °C with 200 rpm shaking. In parallel, *E. coli* containing the FLI-TRAP construct with the wt-VHH35 was cultured under identical conditions in 10 mL LB. Both the library and reference cultures were standardized to OD₆₀₀ = 2.5, serially diluted, and plated on LB agar containing 1% (w/v) arabinose and the defined CB concentrations. Plates were incubated for 48 h. Selected colonies were subjected to Sanger sequencing (U2Bio Co., Ltd., Korea).

C. Protein Expression and Whole-Cell Lysate Preparation

Overnight *E. coli* cultures containing both pDD18-Cm and pDD322-Kan were sub-cultured into 100 mL LB medium until the OD₆₀₀ reached 0.6–0.7, then induced with 1% (w/v) arabinose for Nb expression from pDD18-Cm in NEB10β or BL21(DE3), allowing protein expression at 25°C or 30°C. For EGFR-TK expression, no inducer was required. Samples were collected at 4 or 16 h post-induction. Whole-cell lysates were prepared by sonicating cells normalized to OD₆₀₀ = 75 in 1× PBS, followed by centrifugation to remove insoluble material.

D. Western Blot Analysis

Twenty microliters of denatured samples (normalized by cell number) were separated on 10% polyacrylamide gels prepared using TGX FastCast Acrylamide Solutions (Bio-Rad). Western blotting was performed using standard protocols. PVDF membranes were probed with either mouse anti-FLAG M2–HRP (1:3,000, Abcam) to detect FLAG-tagged Nbs or rabbit polyclonal anti-EGFR (1:500, Abcam) followed by goat anti-rabbit IgG–HRP (1:20,000, Sino Biological Inc.) to detect EGFR-TK-Bla. Images were captured using the Gel Doc EZ imaging system (Bio-Rad).

E. Dot Blot Analysis

Purified EGFR-TK expressed in *E. coli* and suspended in 1×PBS was provided by our collaborator [18]. Vascular Endothelial Growth Factor (VEGF) was purchased from Sino Biological Inc., and Bovine Serum Albumin (BSA) was purchased from New England Biolabs. VEGF and BSA were used as negative controls. For native protein preparation, samples were diluted to the desired concentrations in 1× PBS. To generate denatured EGFR-TK, the protein was heated at 95°C for 10 minutes.

For the dot blot assay, 5 µL of each sample was spotted onto a nitrocellulose membrane and allowed to dry. The membrane was then blocked overnight with 5% (w/v) skim milk. After that, it was washed twice with 1% (v/v) TBST and incubated for 1 h with whole-cell lysates

containing ssTorA-wt-VHH35-FLAG or ssTorA-mu-VHH35-M.5 or -M.6-FLAG. After three washes with 1% (v/v) TBST, the membrane was incubated with anti-FLAG–HRP (1:3,000, Abcam) for detection. Images were captured using the Gel Doc EZ imaging system (Bio-Rad).

III. RESULT AND DISCUSSION

A. Determining Optimal Expression Conditions for Soluble EGFR-TK-Bla in the FLI-TRAP System

For FLI-TRAP selection to be effective, it was essential to confirm that both components—ssTorA-VHH35-FLAG and EGFR-TK-Bla—were expressed as soluble proteins in the cytoplasm of *E. coli*. In a previous study, two FLI-TRAP configurations were developed: a bicistronic FLI-TRAP system, in which both components are co-expressed under the arabinose-inducible *pBAD* promoter, and a monocistronic FLI-TRAP system, in which ssTorA-scFv-FLAG is controlled by *pBAD* while the antigen-Bla fusion is expressed from the constitutive *TatABC* promoter. Therefore, identifying the optimal system for producing soluble EGFR-TK-Bla was necessary.

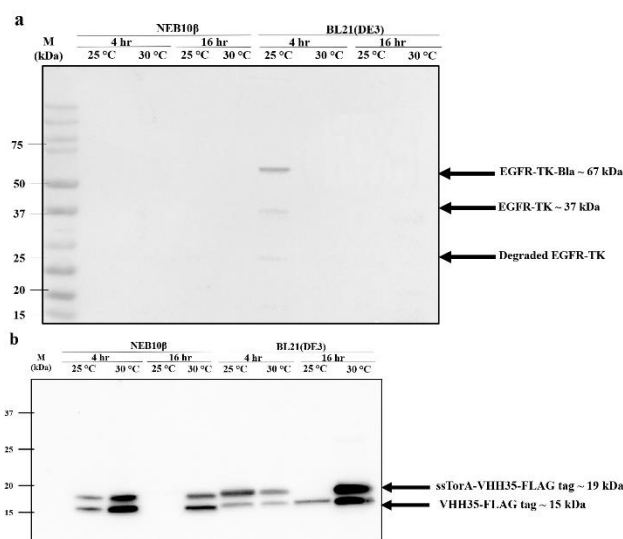


Fig. 1. Expressions of (a) EGFR-TK-Bla from pDD322-Kan EGFR-TK-Bla and (b) ssTorA-VHH35-FLAG fusion from pDD18-Cm ssTorA-wt-VHH35-FLAG in *E. coli* strains NEB10β and BL21(DE3). The expression was conducted at 25°C and 30°C with 0.2% (w/v) arabinose. Cells were collected at 4 and 16 hours after induction.

In our initial attempt using the bicistronic FLI-TRAP system, soluble EGFR-TK-Bla was undetectable by Western blot analysis. EGFR-TK is a human-derived protein. Its overexpression in the bicistronic system may hinder proper folding, leading to insoluble aggregates or inclusion body formation. We subsequently cloned the FLI-TRAP components into the monocistronic FLI-TRAP system and tested expression in various *E. coli* strains. As shown in Fig. 1(a), soluble EGFR-TK-Bla expression was detectable only in *E. coli* BL21(DE3) grown at 25°C for 4 h. Fig. 1(b) shows that anti-FLAG immunoblotting revealed two bands corresponding to

ssTorA-VHH35-FLAG and the cleaved VHH35-FLAG, resulting from ssTorA removal after periplasmic export. Since VHH35 expression was successful in both strains, *E. coli* BL21(DE3) at 25°C was selected for subsequent FLI-TRAP screening, as it allowed proper expression of the cognate antigen EGFR-TK.

B. Error-Prone PCR Library Generation and Optimization of FLI-TRAP Selection Conditions

A mutant VHH35 library targeting EGFR-TK was generated by error-prone PCR and cloned into the monocistronic FLI-TRAP system, yielding approximately 7.68×10^5 CFU. This library size indicates adequate diversity for downstream selection while remaining within the practical screening limit of FLI-TRAP, which is restricted to fewer than 10^7 clones due to background growth associated with the β -lactamase reporter. When excessive numbers of cells are plated on β -lactam-antibiotic-containing agar, lysed cells can release active Bla, leading to unintended colony formation and reduced selection stringency.

Prior to library screening, spot-plating assays were performed using cells co-expressing ssTorA-wt-VHH35-FLAG and EGFR-TK-Bla to determine the dilution range at which wt-VHH35 fails to confer survival. Selection for improved VHH35 variants must be carried out at this threshold to ensure that only enhanced mutants grow. As a reference control, the non-specific Nb Ref8 [21] was included to evaluate whether background survival in the VHH35/EGFR-TK pair exceeded that of a random Nb interaction.

As shown in Fig. 2, ssTorA-wt-VHH35-FLAG co-expressed with EGFR-TK-Bla exhibited slightly higher background growth than ssTorA-Ref8-FLAG, particularly on plates containing 25 μ g/mL CB at both temperatures tested. This experiment served two purposes: (i) to use the spot-plating assay of wt-VHH35 to identify the appropriate selection conditions, and (ii) to confirm that FLI-TRAP is suitable for isolating improved VHH35 mutants, as the wt-VHH35/EGFR-TK interaction provides measurable, though modest, CB resistance when compared with non-specific Nb/EGFR-TK.

Based on these results, mutant VHH35 clones were selected by plating 10^{-3} and 10^{-4} dilutions on selective plates containing 1% (w/v) arabinose and 50 μ g/mL CB at 25°C and 30°C, and 10^{-2} dilutions on plates containing 1% (w/v) arabinose and 100, 150, or 200 μ g/mL CB at 25°C.

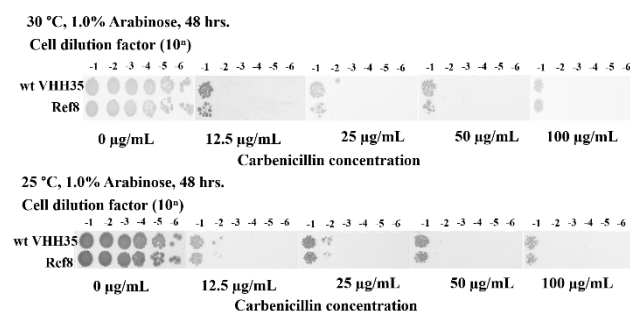


Fig. 2. Spot plating assay used to identify conditions for Nb selection using the FLI-TRAP system.

C. Isolation and Validation of Enhanced VHH35 Mutants

At high CB concentrations, plating the 10^{-2} dilution yielded 40–160 colonies, whereas only 2–5 colonies were obtained when 10^{-3} – 10^{-4} dilutions were plated on 50 μ g/mL CB. A total of 32 colonies were selected for sequencing, resulting in 10 unique full-length Nb sequences containing 1–4 mutations distributed across both framework regions and CDRs (Fig. 3). Only clones with complete Nb coding sequences were re-transformed into fresh *E. coli* BL21(DE3) cells to verify that CB resistance originated from plasmid-encoded ssTorA-Nb-FLAG and EGFR-TK-Bla expression rather than spontaneous chromosomal mutations. The resulting transformants were spot-plated to confirm their resistance profiles (Fig. 4).

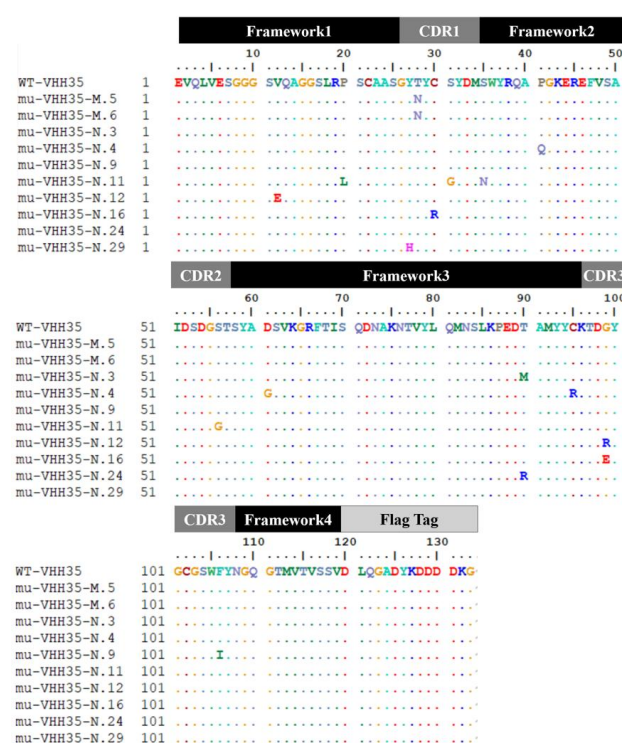


Fig. 3. Alignment of amino acid sequences between wt-VHH35 and its mutants isolated using FLI-TRAP.

Although most candidates exhibited increased CB resistance relative to wt-VHH35, only two mutants—mu-VHH35-M.5 and mu-VHH35-M.6, both isolated at 50 μ g/mL CB and 25°C—demonstrated higher resistance than all variants obtained from selections at higher antibiotic concentrations. This result is not unexpected; when large numbers of cells are plated, the probability of false positives increases due to background Bla release from lysed cells, making it difficult to extrapolate high-density spot-plating data with full accuracy.

To verify that these two mutants genuinely confer greater resistance than wt-VHH35, an additional spot-plating assay was performed using CB concentrations lower than those applied during their selection. As shown in Fig. 5, both mu-VHH35-M.5 and mu-VHH35-M.6 displayed markedly enhanced resistance

at 12.5 $\mu\text{g/mL}$ CB, confirming that their improved phenotype is attributable to the mutant Nb variants.

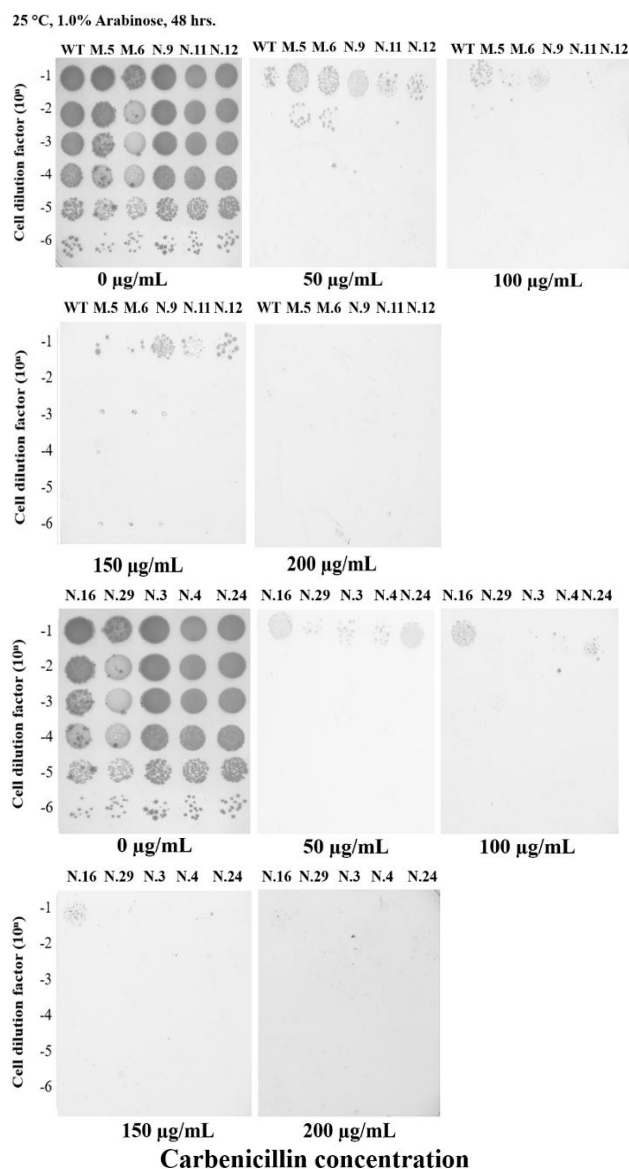


Fig. 4. Spot plating of serially diluted re-transformants comparing VHH35 mutants with wt-VHH35 (ssTorA-fusion) co-expressed with EGFR-TK-Bla under indicated conditions.

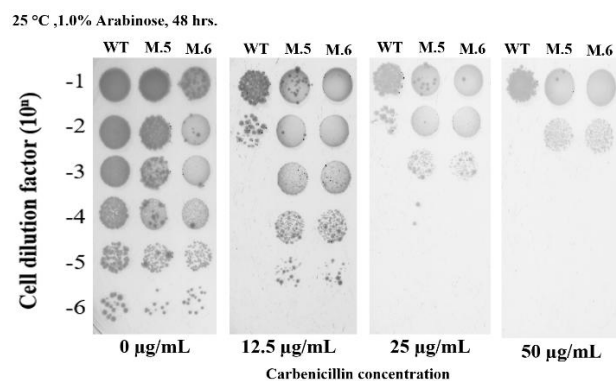


Fig. 5. Spot plating of serially diluted re-transformants comparing mu-VHH35-M.5, and mu-VHH35-M.6 with wt-VHH35 (ssTorA-fusion) co-expressed with EGFR-TK-Bla under indicated conditions.

D. Enhanced Solubility and Periplasmic Translocation Explain Improved Mu-VHH35 CB Resistance

To investigate the basis of the increased CB resistance, we first examined the solubility of mu-VHH35-M.5 and mu-VHH35-M.6 using Western blot analysis. As shown in Fig. 6, both variants produced markedly higher levels of soluble Nb compared with wt-VHH35 when expressed from the pDD18-Cm ssTorA-Nb-FLAG plasmid in *E. coli* BL21 (DE3). Interestingly, in whole-cell lysates, wt-VHH35 appeared primarily as a 19-kDa species corresponding to the signal peptide-containing form (ssTorA-VHH35-FLAG), whereas both mutant Nbs migrated at ~15 kDa, consistent with the periplasmic, signal peptide-cleaved form (VHH35-FLAG). This is expected because the Tat pathway transports fully folded proteins to the periplasm, where the signal peptide is removed by a specific peptidase immediately upon—or shortly after—translocation across the inner membrane.

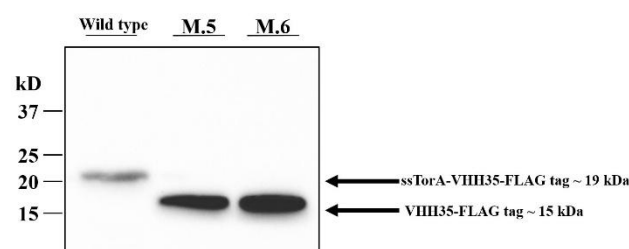


Fig. 6. Expression of wt- and mu-VHH35 in *E. coli* BL21(DE3) harboring pDD18-Cm. Induction was performed with 1% (w/v) arabinose at 25 °C for 4 h.

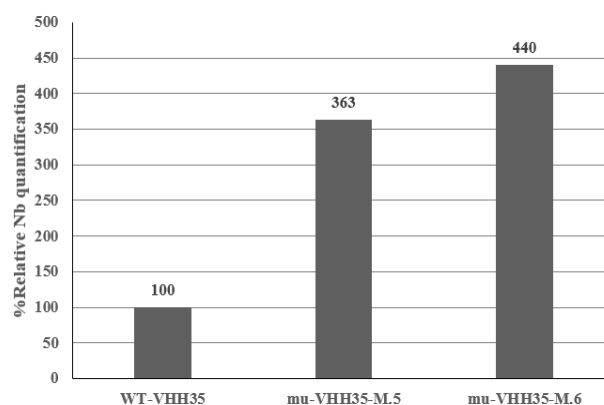


Fig. 7. Relative quantification of wt- and mu-VHH35 expression, based on Western blot analysis shown in Fig. 6. Band intensities were quantified using the Bio-Rad ChemiDoc™ MP Imaging System.

These findings suggest that mu-VHH35-M.5 and mu-VHH35-M.6 can fold efficiently in the cytoplasm without reliance on disulfide bond formation, enabling successful Tat-dependent export to the periplasm. In contrast, wt-VHH35 appears to accumulate predominantly in the cytoplasm, likely due to inefficient folding that prevents export. Additionally, the stronger band intensities observed for both mutants indicate improved solubility and/or expression stability under identical induction conditions. To quantify these differences, densitometry analysis was performed for all three VHH35 variants (Fig. 7). The results showed that

mu-VHH35-M.5 exhibited approximately 263% higher expression than wt-VHH35, while mu-VHH35-M.6 displayed approximately 340% higher expression relative to the wt-Nb. The increased intracellular concentration of ssTorA-mu-VHH35 is expected to drive enhanced complex formation with EGFR-TK-Bla. Consequently, a greater quantity of the EGFR-TK-Bla fusion protein will be transported to the periplasm.

E. Functional Validation of mu-VHH35 Mutants by Dot Blot Analysis

Protein stability and function are often constrained by an inherent trade-off. Mutations that enhance solubility can inadvertently compromise a protein's native activity, a phenomenon commonly referred to as the solubility–function trade-off [22]. This challenge frequently emerges in protein engineering, where improving one property—such as solubility—may reduce another, including catalytic efficiency or binding affinity.

To evaluate whether mu-VHH35-M.5 and mu-VHH35-M.6 retained their binding activity and specificity toward EGFR-TK after acquiring mutations that enhance cytoplasmic solubility, we performed a dot blot assay against both native and denatured EGFR-TK, using native VEGF and BSA as negative controls (Fig. 8). Both mutants bound robustly to native and denatured EGFR-TK with intensities comparable to wt-VHH35, indicating that the T28N substitutions in CDR1 of mu-VHH35-M.5 and mu-VHH35-M.6 (Fig. 3), improve solubility in the reducing cytoplasmic environment without impairing antigen recognition. As expected, neither mutant showed detectable binding to BSA or VEGF, confirming that antigen specificity was preserved.

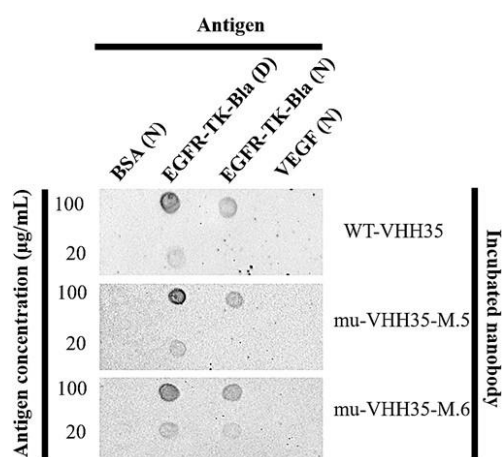


Fig. 8. Dot blot assay demonstrating anti-EGFR Nb reactivity and specificity using denatured and native EGFR-TK (positive controls) and VEGF/BSA (negative controls). ‘D’ denotes denatured antigen, and ‘N’ denotes native antigen.

IV. CONCLUSION

In this study, we demonstrated that the FLI-TRAP system is an effective platform for isolating Nbs targeting EGFR-TK. By integrating in vivo screening with antibiotic-based functional selection, we identified two

variants—mu-VHH35-M.5 and mu-VHH35-M.6—that exhibit markedly improved cytoplasmic solubility while preserving both binding activity and target specificity. These variants may be particularly useful as intrabodies to target the tyrosine kinase domain of EGFR and modulate its function. Previous applications of FLI-TRAP have shown the successful redirection of a SARS-CoV-2 neutralizing nanobody to recognize the Omicron BA.5 variant [23], highlighting the system’s versatility in reshaping nanobody–antigen interactions. Collectively, our findings highlight the potential of FLI-TRAP as a practical approach for engineering Nbs against challenging or clinically relevant targets. Its use to redirect VHH35 specificity toward drug-resistant EGFR mutants represents a promising direction for future development [9].

CONFLICT OF INTEREST

The authors declare no conflict of interest.

AUTHOR CONTRIBUTIONS

C.L., A.T., O.P., and A.K. conducted the research. C.L., A.T., L.T., D.M., and D.W.-Z. analyzed the data; C.L., A.T., L.T., D.M., K.C., and D.W.-Z. validated the data and performed formal analysis; C.L., A.T., and D.W.-Z. prepared the original draft; C.L., L.T., D.M., K.C., and D.W.-Z. reviewed and edited the manuscript; D.W.-Z. supervised the research and acquired funding; all authors had approved the final version.

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