Engineering an autonomous VH domain to modulate intracellular pathways and to interrogate the eIF4F complex

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An attractive approach to target intracellular macromolecular interfaces and to model putative drug interactions is to design small high-affinity proteins. Variable domains of the immunoglobulin heavy chain (VH domains) are ideal miniproteins, but their development has been restricted by poor intracellular stability and expression. Here we show that an autonomous and disulfide-free VH domain is suitable for intracellular studies and use it to construct a high-diversity phage display library. Using this library and affinity maturation techniques we identify VH domains with picomolar affinity against eIF4E, a protein commonly hyper-activated in cancer. We demonstrate that these molecules interact with eIF4E at the eIF4G binding site via a distinct structural pose. Intracellular overexpression of these miniproteins reduce cellular proliferation and expression of malignancy-related proteins in cancer cell lines. The linkage of high-diversity in vitro libraries with an intracellularly expressible miniprotein scaffold will facilitate the discovery of VH domains suitable for intracellular applications.

Many intracellular macromolecular interfaces exist in human cancers that are highly desirable anti-cancer therapeutic targets, such as Myc:Max, KRAS:RAF and eIF4E:4G. Some of these interactions present significant hurdles to small molecule development due to the nature of their molecular surfaces. eIF4E is a particularly attractive oncogenic target as it forms part of the eIF4F complex, which performs a critical role in mediating cap-dependent protein synthesis and whose components (eIF4E, eIF4A and eIF4G) are frequently mis-regulated in many tumours and often associated with poor prognosis in patients and chemoresistance. eIF4E interacts with the m’G cap structure at the 5’ end of mRNAs and recruits the eIF4F complex via eIF4G, which in turn initiates translation. This process regulates the translation of a specific subset of sensitive mRNAs (e.g., c-Myc, CCND1), which are significantly involved in cellular transformation and oncogenesis.

1p53 Laboratory (A*STAR), 8A Biomedical Grove, #06-04/05, Neuros/Immunos 138648, Singapore. 2Disease Intervention Technology Laboratory (DITL), Institute of Molecular and Cell Biology, A*STAR, Singapore 138673, Singapore. 3Insilico Medicine Taiwan Ltd., Taipei City 112308, Taiwan. 4Department of Pharmacy, National Yang Ming Chiao Tung University, Taipei City 112304, Taiwan. 5DotBio Pte. Ltd., 1 Research Link, Singapore 117604, Singapore. 6School of Biological Sciences, Nanyang Technological University, 60 Nanyang Drive, 637551 Singapore, Singapore. 7Department of Oncology and Pathology, Karolinska Institutet, Stockholm 17177, Sweden. 8Department of Microbiology, Tumor and Cell Biology, Karolinska Institutet, Biomedical Quarter 78-C Solnavägen 9, 17165 Solna, Sweden. 9Institute of Sustainability for Chemicals, Energy and Environment (ISCE2), A*STAR, 8A Biomedical Grove, #07-01 Neuros Building, 138665 Singapore, Singapore. 10Bioinformatics Institute (A*STAR), 30 Biopolis Street, #07-01 Matrix, 138671 Singapore, Singapore. 11Department of Biological Sciences, National University of Singapore, 14 Science Drive 4, 117543 Singapore, Singapore. 12These authors contributed equally: Yuri Frosi, Yen-Chu Lin, Jiang Shimin. e-mail: ignacio.asial@dotbiopharma.com; cjbrown@imcb.a-star.edu.sg
One approach to circumvent the issues associated with small molecule discovery is to design small high-affinity proteins (approx. 60 to 100 amino acids in size) to target these interfaces, termed miniproteins\(^1,3\). Miniproteins are of special interest as they can easily be expressed within mammalian cells and engineered into transgenic mice, where they can be induced in a temporal and tissue-specific manner. In the absence of pharmacological specificity, reagents they offer a precise tool for the interrogation of fundamental biology and target validation\(^6\). Miniproteins unlike traditional knock-down and knock out strategies to validate candidate proteins for therapeutic development, more closely model the fundamental interactions of drugs with their targets\(^2\).

Variable domains of the human immunoglobulin heavy chain (VH domains) are ideal candidates for use as miniproteins. They possess three binding loops of variable length (CDR-H1, CDR-H2 and CDR-H3) that are naturally randomised to generate a wide repertoire of binders for antigen recognition by the immune system. The development of these scaffolds as single domain binding reagents for intracellular studies has been restricted by poor stability, due to the loss of stabilizing interactions with the variable light-chain domain in the intact antibody\(^1,14\). However, single chain variable fragments (ScFv) termed “intra-bodies” consisting of a VH and a VL domain connected by a flexible peptide linker have been used to probe intracellular targets. Unfortunately, ScFvs are deleteriously affected by the reducing conditions of the cell, which prevent the formation of the VH and VL intra-domain disulfide bonds and consequently hinders their proper folding leading to ScFvs that are non-functional, poorly expressed with short half-lives and poor solubilities\(^1\).

Several approaches have been devised to overcome these liabilities and to increase the discovery rate of intra-bodies, such as screening intra-body-antigen interactions within cells and the use of predetermined frameworks and consensus sequences with improved solubility and expression properties in vivo\(^1\). Single domain antibody fragments generated from VH and VL domains have also been reported to express within cells and to retain their functionality\(^2\). Consensus sequences derived from these intra-bodies were unaffected by substitution of the residues responsible for the VH domain intra-domain disulfide bond. The dispensability of the intra-domain disulfide bond is also supported by crystallographic data showing that its absence does not perturb VH and VL domain structures\(^1\). An alternative antibody-type modality capable of intracellular expression are Nanobodies (VHH)\(^12,22\). These are variable domains derived from the heavy-chain only camelid antibodies that are monomeric and soluble that contain 1 to 2 intra-domain disulfide bonds. However, most Nanobodies yielded from conventional screening approaches are non-functional within living cells\(^24\). To overcome these issues, different groups have reported approaches to improve selection of intracellularly functional binders from synthetic or immunized libraries\(^23,31,33\) and efforts to identify cysteine-free, non-immunoglobulin based miniproteins that are suitable for intracellular expression have also been reported\(^1\).

Motivated by these observations and by data demonstrating that engineered human VH domain scaffolds can exist monomerically in solution\(^1,27\), we endeavour in this work to address the liabilities associated with intracellular expression of ScFvs and other miniproteins by engineering and optimising an autonomous and disulfide-free human VH domain for intracellular expression studies through the use of CoFi\(^13\) and Hot-CoFi\(^17\) directed evolution techniques. We then exploit the optimised VH framework sequence to generate a phage display library with randomised CDRI, 2 and 3 loops and use it to identify several VH domain binders against elf4E. The isolated VH domains are shown to inhibit the elf4E:4G interaction and are then further evolved by affinity maturation to generate a picomolar binder against elf4E (termed VH-36) suitable for activity modulation studies in mammalian cells (Fig. S1). Failure to regulate the elf4E complex frequently occurs in cancers when the 4E-binding proteins (4E-BP1,2 and 3) are hyperphosphorylated by mTOR\(^10,11\), and fail to displace elf4G from elf4E. Both proteins possess a common primary binding motif (XYXXXXL\(\Phi\), \(\Phi\) = any amino acid and \(\Phi\) = hydrophobic amino acid) to elf4E\(^14\). The high-affinity VH domain interactor shares an overlapping interaction site with peptides derived from this binding motif but interacts though an alternative binding mode. The interface between elf4G and elf4E is a potential site of therapeutic development and has been targeted with chemical small molecule and peptide strategies\(^12,33\). However, many of these molecules are poorly active and have not been clinically approved and the alternative interaction pose formed by VH-S4 with elf4E represents a distinct and unexplored avenue for therapeutic development strategies.

The autonomous VH domain technology presented here offers a rapid and efficient pipeline to discover new binding poses for therapeutic lead development, as well as the identification of modalities that can be used to therapeutically model and validate these sites for drug development in biological systems. This methodology also enables the advantageous linkage of high diversity in vitro libraries (\(10^7\)–\(10^9\)) with an intracellularly expressible miniprotein scaffold, in contrast to in vivo selection techniques that use smaller libraries (\(10^3\)–\(10^7\)) to increase the discovery rate of VH domains suitable for intracellular applications.

**Results**

**Generation of a stable, disulfide-free, autonomous antibody variable domain for intracellular expression**

The 4D5 VH domain was chosen as the template for stabilization, as its parental IgG trastuzumab is an approved FDA drug with a favourable immunogenic and stability profile\(^1\). The 4D5 VH domain was fused to an N-terminal signal sequence for periplasmic expression in E. coli and a random mutagenesis library of 4D5 created (Table S1). This was used to initiate 2 rounds of selection using Hot-CoFi\(^17\) described in Fig. 1a, resulting in the isolation of 53 stabilized VH domain variants (Fig. 1b). Hot-CoFi is an attractive system for the selection of thermally stable VH variants as high-yield bacterial expression has been shown to correlate well with ScFv expression in the cytoplasm of mammalian cells\(^10\). Among the identified variants, VH-33 and VH-36 were the most thermally stable, with TC\(_{50\%}\) (midpoint of thermal cellular aggregation curves, see materials and methods) of 72.9 and 73.4 \(^\circ\)C, respectively, an increase of over 20 \(^\circ\)C in comparison to the 4D5 clone (Fig. 1c and Table S1). The VH-36 variant contained the following mutations, H33D, S93G and W103R. Both H33D and W103R are located at the former VH and VL domain interface and mainly improve the thermal stability of the VH variant by decreasing its hydrophobicity (Fig. S2).

VH-36 was selected as the starting template for evolution of a disulfide-free VH domain. Three residues in VH-36, C22 and C92, as well as the core residue A24, were then randomised to generate a library suitable for screening. A24 was included to facilitate optimal packing of residues in the hydrophobic core of the protein due to its proximity to C22 and C92. The signalling peptide, stII, was also removed from the VH-36 expression construct (termed VH-36i) to ensure cytoplasmic expression and allow screening in a cellular reducing environment. The 4D5 VH domain was chosen as the template for stabilization, as its parental IgG trastuzumab is an approved FDA drug with a favourable immunogenic and stability profile. The 4D5 VH domain was fused to an N-terminal signal sequence for periplasmic expression in E. coli and a random mutagenesis library of 4D5 created (Table S1). This was used to initiate 2 rounds of selection using Hot-CoFi described in Fig. 1a, resulting in the isolation of 53 stabilized VH domain variants (Fig. 1b). Hot-CoFi is a promising system for the selection of thermally stable VH variants as high-yield bacterial expression has been shown to correlate well with ScFv expression in the cytoplasm of mammalian cells. Among the identified variants, VH-33 and VH-36 were the most thermally stable, with TC\(_{50\%}\) (midpoint of thermal cellular aggregation curves, see materials and methods) of 72.9 and 73.4 \(^\circ\)C, respectively, an increase of over 20 \(^\circ\)C in comparison to the 4D5 clone (Fig. 1c and Table S1). The VH-36 variant contained the following mutations, H33D, S93G and W103R. Both H33D and W103R are located at the former VH and VL domain interface and mainly improve the thermal stability of the VH variant by decreasing its hydrophobicity (Fig. S2).

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**Optimization of a disulfide-free autonomous antibody variable domain for phage display**

The VH-36i.1 clone and its parent templates (VH-36 and VH-36i) all contained the mutation A100bP (Ala to Pro mutation at position 100b, according to Kabat numbering within the CDR-H3 loop). An undesirable feature that restricts the conformational and diversity space open to randomized CDR-H3 loop regions and in turn decreases their probability of successful target interaction. Therefore, the CDR-H3 region (W95 to P100b) was removed and replaced by the sequence SSA, creating the new construct VH-37i (Table S1), which had significantly increased the number of unique binding motifs.
combined to generate VH38i with an improved Tm thus stabilising the VH framework also. These mutations were comprised of A78V in VH-H3 loop (Fig.1d and Table S1). The introduction of A78V most likely leads to improved packing interactions in the hydrophobic core of the protein around C22S and C92T, whilst the S93V substitution likely leads to improved packing interactions in the hydrophobic core of VH-37i.1 bearing a A78V mutation in the core of the VH domain. By probing the nitrocellulose membrane with antibodies, colonies expressing soluble proteins can be identified. If performing the Hot CoFi method, the induction plate is incubated at the desired temperature above the wild-type protein melting temperature at step iv), before transferring the filter membrane onto the nitrocellulose membrane in native lysis buffer to release the VH domain clones from the bacterial colonies. By probing the nitrocellulose membrane with antibodies, colonies expressing soluble proteins can be identified. If performing the Hot CoFi method, the induction plate is incubated at the desired temperature above the wild-type protein melting temperature at step iv), before transferring the filter membrane onto the nitrocellulose membrane in native lysis buffer to release the VH domain clones from the bacterial colonies.

A library of VH domains were then displayed on the pIII protein of M13 phage, where the CDR1 and CDR2 of the VH-38i.1 template were randomized conservatively, whilst CDR3 was randomized with sequences on different lengths ranging from 10 to 24 residues and biased towards residues serine and tyrosine with ~20% frequency, respectively (Fig. S4), resulting in a library with 2.87 × 10^9 unique clones (see methods and materials). The valine introduced at position 93 in VH-37i.1 and retained in VH-38i (blue) and CDR3 loop replacement (red). Magenta shows location of mutations on former VH:VL interface (Fig. S2). Figure 1a is reprinted by permission from Springer Nature: Nature Methods, Colony filtration blot: a new screening method for soluble protein expression in Escherichia coli, Cornvik T., Dahlroth S.L., Magnusdottir A., Herman M.D., Knaust R., Ekberg M. and Nordlund P., Copyright © 2005.

Fig. 1 | Engineering and optimising an autonomous and disulphide-free human VH domain for phage display and intracellular mammalian expression. a Schematic of the CoFi selection procedure. (i) Cells transformed with the randomly mutagenized library are plated and grown on LB plates. (ii) The filter membrane is overlaid on top of the colonies, (iii) which adhere to the membrane and can be removed from the LB agar. (iv) Protein expression is induced in the transformed bacterial colonies by placing the filter on IPTG containing LB plates. (v) The colonies are lysed by and soluble proteins diffuse through the filter membrane and are captured on the nitrocellulose membrane while inclusion bodies are retained on the filter membrane. Cell lysis is performed by placing the filter and the nitrocellulose membranes in native lysis buffer to release the VH domain clones from the bacterial colonies. By probing the nitrocellulose membrane with antibodies, colonies expressing soluble proteins can be identified. If performing the Hot CoFi method, the induction plate is incubated at the desired temperature above the wild-type protein melting temperature at step iv), before transferring the filter membrane onto the nitrocellulose membrane in native lysis buffer to release the VH domain clones from the bacterial colonies.

To remove the final cysteine at position 24, it was either mutated to the de-free human full-length eIF4E, followed by screening of 95 individually picked phage clones by ELISA led to the identification of nine unique VH domains (Table S2). VH-1A2 and VH-1C5 were validated as the highest affinity binders with Kds of 115.2 ± 4.4 nM and 154.3 ± 70.3 nM, respectively (Fig. 1d). The amino-acid sequence of each VH variant is available in Table S1, and their corresponding stability assessment curves in Fig. S3.
**Fig. 2** | Structural characterization studies delineating eIF4E VH domain binding sites. 

**a** Locations of the cap-binding and eIF4G interaction sites on eIF4E. Surface plasmon resonance sensograms showing 2-fold titration series of VH-1C5 and VH-1A2 from 2.5 µM to 25 nM against eIF4E immobilised via amine coupling on CM5 chips, respectively. Derived Kd values are shown on the graphs (mean ± standard error) and are calculated from n = 2 independent experiments.

**b** Comparison of naturally occurring eIF4E interacting sequences (4E-BP1 and eIF4G1) with the selected VH domain library sequences (1A2 and 1C5). Graphs show representative titrations of VH domains 1C5 and 1A2 against eIF4E in fluorescent polarization competition experiments using a FAM labelled eIF4G1 peptide (Ac-KKRYSRDFL-LALQK-(FAM)). Derived IC50s are shown in the graph (mean ± standard error) and were calculated from n = 2 independent experiments. Source data is provided in the Source Data File.

**c** Crystal structure of eIF4E complexed with VH-1C5. The phage library selected CDR3 loop insert that interacts with eIF4E is highlighted in red, whilst the main fold of the VH domain is shown in cyan. Highlighted in orange are close contacts between the VH domain and eIF4E that lie outside the library insert region of the CDR3 loop.

**d** Overlay of the CDR3 region (red) with the eIF4E interacting sequence from 4E-BP1 (cyan). Both sequences adopt very different secondary structural folds to each other and have overlapping interaction sites with eIF4E. The VH-1C5 CDR3 loop forms additional contacts with a hydrophobic region on the surface of eIF4E that the 4E-BP1 peptide does not, whilst the positively charged N-terminal extended strand of 4E-BP1 instead interacts with a negatively charged patch on eIF4E.

**e** Residues A101 and D104 of VH-1C5 form contacts with the hydrophobic region of eIF4E, whilst L105 recapitulates the interactions of the M60 residue from 4E-BP1. Y117 from the CDR3 loop of VH-1C5 forms a hydrogen bond with E132, replacing the electrostatic interaction observed with R56 in 4E-BP1.

**f** The F120 residue found in VH-1C5 mimics the hydrophobic contacts made by L59 and Y54 of 4E-BP1 against eIF4E. In addition, the conserved hydrogen bond formed between the carbonyl backbone of L59 with the indole side chain of W73 is repeated by the carbonyl backbone of F220.

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**eIF4E Interacting Sequences**

- eIF4G1: LEEEKKRDREFLLGOFQIFA
- 4E-BP1: GGTRIIYDRLKLMECRNSPV
- 1C5: VAKALNSSPSFVVNTYSFFG
- 1A2: MAYSYPSSFSASSFDLYYA
and L59/629 of 4E-BP1 and eIF4G1, respectively). The conserved Y and L residues of the eIF4E interaction motif (Y54/624 and L105) of W73 on eIF4E (Fig. 2f), which replicated the interactions formed by the eIF4E:4G interaction
eral hydrophobic contacts with the eIF4E residues L39, V69 and I138 revealed eIF4E:4G interaction –shaped conformers formed by 4E-BP1 and eIF4G derived linear peptides when in complex with eIF4E (Fig. 2c, d)34. The random coil section (residues 101–104) of the CDR3 loop orients the VH domain with a mid-picomolar binding affinity to interact with and shield hydrophobic residues from the solvent (Fig. S6)22,39,40. The sensitivity of VH-1C5 binding to eIF4E when this cluster is mutated confirms that these residues ensure the correct folding of the CDR3 loop. A salt bridge further rigidifies the CDR3 loop that forms between residues R52 and D37 at the CDR3 loop and the VH framework interface, which orients R52 to form electrostatic interactions with the CDR3 backbone carboxyls of S107 and S108 (Fig. 3e).

Table 1 | Binding and kinetics parameters of various VH domains against eIF4E

| Domain        | $K_d$ (nM) | $K_m$ (S$^{-1}$ M$^{-1}$) | $k_{on}$ (S$^{-1}$) |
|---------------|------------|---------------------------|---------------------|
| VH-1A2        | 115.2 ± 4.4 | 3.43 ± 10$^{-1} \times 10^{7}$ | 3.78 ± 10$^{-2} \times 10^{7}$ |
| VH-1C5        | 154.3 ± 70.3 | 2.85 ± 10$^{-3} \times 10^{5}$ | 4.11 ± 10$^{-2} \times 10^{5}$ |
| VH-1C5D104A   | 4.8 ± 1.7   | 2.46×10$^{-2} \times 10^{6}$ | 1.18 ± 10$^{-2} \times 10^{6}$ |
| VH-1C5D104A/S108R/F120I (VH-S4) | 0.94 ± 0.024 | 2.63 ± 10$^{-3} \times 10^{6}$ | 2.48 ± 10$^{-3} \times 10^{6}$ |
| VH-1C5D104A/S108R/F120I (VH-S4) | 0.64 ± 0.06 | 1.18 ± 10$^{-4} \times 10^{5}$ | 7.64 ± 10$^{-4} \times 10^{5}$ |
| VH-1C5D104A/S108R/F120I (VH-S4) | 0.057 ± 0.004 | 5.18 ± 10$^{-5} \times 10^{2}$ | 2.96 ± 10$^{-6} \times 10^{3}$ |
| 4E-BP14ALA (1:1) | 0.60 ± 0.14 | 1.05 ± 10$^{-1} \times 10^{2}$ | 5.93 ± 0.56 |
| 4E-BP14ALA (2 state) | 0.68 ± 0.13 | 8.35 ± 10$^{-1} \times 10^{2}$ | 3.19 ± 10$^{-1} \times 10^{2}$ |
| 4E-BP14ALA (2 state) | 1.70 ± 10$^{-1} \times 10^{2}$ | 1.70 ± 10$^{-1} \times 10^{2}$ | 1.84 ± 10$^{-1} \times 10^{3}$ |

$K_d$, $K_m$, and $k_{on}$ values were derived from SPR experiments using either single cycle or multi cycle injection experiments against eIF4E amine coupled to a CM5 sensorchip. Parameters were derived using a 1:1 site binding model. However, with regards to 4E-BP14ALA, binding parameters were initially determined using a 1:1 site binding model, which generated a poor fit and physically irrelevant $K_d$ and $K_m$ values beyond the detection limit of the machine. This analysis was superseded by a 2-state analysis, which generated more meaningful kinetic parameters. The use of a 2-state model is supported by the following reasons: (1) 4E-BP14ALA undergoes a disorder to order transition upon binding eIF4E and, (2) it interacts at eIF4E through 2 binding sites. Binding and kinetic data values (mean ± standard error) shown in the table were calculated from $n = 2$ independent experiments. Data shown for 4E-BP14ALA was calculated from $n = 3$ independent experiments. Source data is provided in the Source Data File.

VH-1A2 and VH-1C5 domains shared little similarity to the shared interacting motif of eIF4E binding proteins such as eIF4G1 and the 4-E-BP family (YXXXXXLΦ) (Fig 2b)34. Competitive-based fluorescence anisotropy experiments were performed that mapped the binding of VH-1A2 and VH-1C5 to the eIF4E interaction site (Fig. 2b).

The crystal complex of eIF4E and VH-1C5 (Fig. S5) revealed that the interaction interface are critical for binding Alphal scanning mutagenesis reveals VH-1C5 residues distal to the interaction interface are critical for binding Alphal scanning mutagenesis scanning of the CDR3 loop revealed two classes of mutants that adversely affected the binding of VH-1C5 to eIF4E (Fig. 3a, b). The first class of mutants describe residues identified that are in direct contact with eIF4E (K102, A103, D104, L105, T116, V117 and F120). Replacement of D104 with alanine (Fig. 3a) induced an ~7-fold improvement in affinity (Fig. 3c and Table 1) with a $K_d$ of 4.8 ± 1.7 nM in the VH-1C5 variant (VH-M4). The second class of mutations describe the alanine substitution of residues V101, P110, V113, V114 and F122 that abolished eIF4E binding but were not located at the VH-1C5 interface. These residues form a distinct hydrophobic cluster that interact with several hydrophobic residues located on the β-sheet face of the VH domain (V39, L47 and W49) on the former VL interface (Fig. 3d). This is highly similar to CDR-H3 loop interactions that occur in a variety of Nanobodies, where an extended CDR3 loop folds back on to the protein to interact with and shield hydrophobic residues from the solvent (Fig. S6)22,39,40. The sensitivity of VH-1C5 binding to eIF4E when this cluster is mutated confirms that these residues ensure the correct folding of the CDR3 loop. A salt bridge further rigidifies the CDR3 loop that forms between residues R52 and D37 at the CDR3 loop and the VH framework interface, which orients R52 to form electrostatic interactions with the CDR3 backbone carboxyls of S107 and S108 (Fig. 3e).

Evolution of an ultra high-affinity miniprotein inhibitor of the eIF4E:4G interaction

The VH-M4 binder ($K_d = 4.8 \pm 1.7$ nM) was used as a template sequence to generate a randomly mutagenized library for affinity maturation to identify improved binders against eIF4E using yeast display (Fig. 4a). Three rounds of kinetic selection were performed with increasing incubations times of 8, 60 and 110 min to increase the competitive pressure of unlabelled eIF4E against the VH domain library complexed to fluorescent eIF4E (Fig. 4b). The final round of kinetic selection resulted in the identification of only three eIF4E binders: VH-1C5D104A/S108R/F120I and VH-1C5D104A/S108R/F120I (VH-S4) clone (termed VH-S4) dominated the final round with a frequency > 90%, which bound eIF4E with a mid-picomolar $K_d$ of 0.057 ± 0.004 nM (Fig. 4c and Table S3). The two substitutions identified in the VH-S4 sequence were then individually introduced into the VH-1C5-M4 clone to generate VH-1C5D104A/S108R/F120I and VH-1C5D104A/S108R/F120I (termed VH-S2). These bound eIF4E with $K_d$ of 0.94 ± 0.04 nM and 0.64 ± 0.06 nM, respectively (Fig. 4c and Table 1).

The crystal structure of eIF4E:VH-1C5D104A/S108R/F120I (VH-S4) complex revealed that the FI20I mutation improved the hydrophobic contacts between the two proteins (Fig. 4d and Fig. 5). The structure also showed that the S108R mutation does not interact directly with the surface of eIF4E, but that it engages a structured water network that forms an H-bond to the backbone carbonyl of R128 on eIF4E (Fig. 4e and Fig. 5). In addition, S108R formed a cation-π interaction with the indole side chain of W73 on eIF4E (Fig. 2f), which replicated the interactions formed by the conserved Y and L residues of the eIF4E interaction motif (V54/624 and L59/629 of 4E-BP1 and eIF4G1, respectively).

VH-1C5D104A/S108R/F120I (VH-S4) complex formation and cap-dependent translation in vitro

VH-S4 immuno-precipitated higher amounts of endogenous eIF4E than the more weakly binding VH domain variants (VH-1C5, VH-M4 and VH-1C5D104A/S108R/F120I (VH-S2)) (Fig. 3a). A VH-1C5 negative control (VH-1C5D104A), where the CDR3 loop residues were scrambled, also failed to pull-down eIF4E

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demonstrating the specificity of the VH domains for eIF4E (Fig. 5a). VH-S4’s ability to interact with eIF4E was also compared to a constitutively active form of the repressor protein 4E-BP1 (termed 4E-BP1^{4AlA}), where the phosphorylation sites (Thr37, Thr46, Ser65, Thr70) responsible for modulating its binding with eIF4E were mutated to alanine. The $K_d$ of 4E-BP1^{4AlA} was determined to be $4.8 \pm 1.7 \text{nM}$, ~10-fold weaker than the VH-S4:eIF4E interaction, with a substantially weaker off-rate (Table 1 and Fig. S8). Both constructs exhibited comparable levels of activity in immuno-precipitating eIF4E, whilst the 4E-BP1^{4AlA} negative binding control (termed 4E-BP1YLM) had negligible effects. VH-S4 also more efficiently disrupted the eIF4G-eIF4E interaction in cells than the weaker affinity VH domain variants (1C5, S2 and M4) using the NanoBit eIF4E:eIF4G606–646 cell-based assay (Fig. 5b). 4E-BP1^{4AlA} was also assessed and again was similar in activity to VH-S4.
the VH domains except the control were then shown to inhibit cap-dependent translation using a bicistronic reporter assay, with the magnitude of cap-independent inhibition again correlating to the affinity of the eIF4E interacting VH domains (Fig. 5c and Fig. S9). It is well established that disruption of the eIF4F complex prevents eIF4G mediated phosphorylation of eIF4E by Mnk1. Therefore, in parallel, lysates were co-prepared from the cells used in the NanoBit assay and the levels of phosphorylated eIF4E detected (Fig. 5c). eIF4E phosphorylation as expected closely followed the levels of eIF4F complex disruption measured in the NanoBit eIF4E:eIF4G606–646 system. 4E-BP1ΔAda also specifically decreased eIF4F complex formation and phosphorylation levels in a similar manner to the VH-S4 domain (Fig. 5c).

The effects of eIF4F complex disruption directly upon cap-dependent protein translation were monitored by measuring Cyclin D1 protein levels. Increasing amounts of VH-M4 and VH-S4 expression plasmids were transfected into mammalian cells with a concomitant inhibitory effect on eIF4E phosphorylation and cyclin D1 protein levels (Fig. 5d). This effect of eIF4F disruption upon

**Fig. 4 | VH-1C5 D104A domain affinity maturation using yeast surface display (YSD).** a) Dissociation rate of VH-1C5 D104A was compared to the error prone library, which was generated using VH-1C5 D104A as template, to determine optimal competition time for dissociation rate engineering. Optimal competition time was approx. 8 min. b) High affinity VH-1C5 D104A variants were selected via three rounds of kinetic selection. Initial selectants were isolated using flow cytometry after competition with excess non-fluorescently labelled ligand after 8 mins. Selectants were then amplified and then kinetically selected after 60 mins. This was then repeated a third time with an incubation period of 110 mins elf4E (see Fig. S15 for sorting strategy, % of cells selected for each round indicated). c) Single representative injection cycle surface plasmon resonance sensograms of VH1C5 D104A/S108R/F120I, VH-1C5 D104A/F120I, and VH-1C5 D104A/S108R being titrated across eIF4E immobilised via amine coupling on CM5 chips, using threefold concentration series from 0.37 nM to 30 nM (see "Methods"). Binding and kinetic data (mean ± standard error) are shown on the graphs and were calculated from n = 2 independent experiments. d) Mutation of F120I that occurs in the high-affinity VH-1C5 D104A/S108R/F120I domain results in improved surface recognition of elf4E. e) The arginine that replaces S108 results in several new interactions. It engages a structured water network that forms hydrogen bonds with the backbone carbonyl of R128 and with both E132 and Y117. In addition, it stabilises the fold of the CDR3 loop further by forming a cation–π interaction with F112. f) Overlay of VH-1C5:elf4E (cyan) and VH-1C5 D104A/S108R/F120I:elf4E (magenta) complex crystal structures showing the conformation change induced in Y76 of elf4E upon mutation of D104A. Associated structured water network also migrates with change in Y76 conformation.
CCND1 translation and eIF4E phosphorylation was also reflected by transfection experiments with 4E-BP14 Ala (Fig. 5d). m7GTP mediated pull down experiments verified that all the VH domains (S4, M4, S2 and 1CS) and 4E-BP14 Ala, except for the controls, were able to competitively displace eIF4G from eIF4E and decrease the amount of endogenous eIF4F complex detected (Fig. 5e). In addition, the specificity of VH-S4 for eIF4E was demonstrated through anti-FLAG mediated IP of VH-S4 from whole cell lysate, where no other members of the eIF4F complex were detected apart from eIF4E (Fig. S10).

**VH-S4 modulates eIF4F mediated signalling pathways specifically**
eIF4E inhibition reduces the expression of malignancy-related proteins (e.g., cyclin D1, Mcl-1, and BCL-xL). In addition, eIF4E inhibition causes significant reductions in cellular proliferation and induction of
apoptosis. To examine the effects of VH-S4 and 4E-BP14ALA mediated inhibition of eIF4E, stably transfected inducible expression systems were constructed for both proteins in A375 melanoma and MBA-MD-321 breast carcinoma cells. VH-S4 and 4E-BP14 were both induced with doxycycline for 24 h in both cell lines and were shown to decrease eIF4F complex formation (Fig. S11A) in comparison to mock control cells. Both proteins’ expression was induced for over 7 days and also caused significant decreases in the cellular proliferation and viability (Fig. 6a, b, Fig. S1C and D). The most dramatic effects were seen in A375 cells, where both 4E-BP14ALA and VH-S4 decreased cellular proliferation and viability to the same extent (Fig. 6a, b). In contrast, VH-S4 was less efficacious than 4E-BP14ALA in MD-MB-231 cells, despite disrupting eIF4F complex formation to the same extent (Fig. S11B).

Both VH-S4 and 4E-BP14ALA when induced for 24 h resulted in significant decreases in the protein expression levels of cyclin D144 and of MCL-145, but had little effect on Bcl-xl46 and 4E-BP1/2 levels. Additionally, neither the miniprotein nor the small molecules tested affected ERK phosphorylation.

The effects of small molecules upon the eIF4F complex were extended to a wider set of compounds (including the eIF4E inhibitor, 4E-G1 and the Mnk Kinase inhibitor, CPG-57380) and assessed with PP242 and staurosporine-induced apoptosis at 72 h (Fig. 6c). The effects of PP242 and staurosporine on protein expression levels of Mcl-1 at 24 h (Fig. 6c) were alleviated by 72 h (Fig. 6e), whilst maintained with respect to cyclin-D1, 4E-BP1 and 4E-BP2. CPG-57380 as expected resulted in dephosphorylation of eIF4E at 72 h (Fig. 6e) with negligible effects on cyclin-D1 or 4E-BP1/2 protein levels. Additionally, PP242 and staurosporine-induced apoptosis at 72 h as indicated by PARP cleavage (Fig. 6e and Fig. S1Z) in contrast to the miniproteins demonstrating that specific inhibition of the eIF4E-G interface does not lead to apoptosis and that this is the result of other modes of action by either small molecule. Interestingly 4E-G1, a molecule that allosterically disrupts binding of eIF4E to eIF4G, also elicits PARP cleavage and promotes eIF4E phosphorylation in contrast to VH-S4 and 4E-BP14ALA.

The presence of C22 and C92 in VH-S4 is not detrimental to function or intracellular expression

With the VH-S4 domain demonstrating sufficient mammalian cellular expression to perturb eIF4F function, we assessed the effects of reintroducing C22 and C92 into VH-S4 upon its performance, whereupon negligible differences in cellular expression, proteosomal stability and in the amounts of eIF4E immune-precipitated were observed between the two variants (Fig. 7a, b). This prompted further examination of the differences in mammalian expression levels of the various VH domain mutants evolved at different stages of the directed evolution process (Fig. 7c) with or without C22 and C92. From these results, it was apparent that the 4DS template sequence with or without C22 and C92 was suboptimal for cellular expression and that improvements in the thermal stability of the VH variants generally correlated with increased soluble mammalian cellular expression (Fig. 7c and Fig. S3). Incorporation of C22 and C92 predominately increased the thermal stability of the purified VH domains by approx. 10 °C indicative of the formation of intra-domain disulfide bond formation in vitro (Fig. S1J).

However, the formation of this bond in the cytoplasm is difficult to establish but it is apparent that the other mutations in VH-36 and later
Fig. 6 | VH-S4 modulates eIF4E-mediated signalling pathways. a A375 Mock, 4E-BP1<sup>4A</sup>a and VH-S4 inducible cell line confluence was measured in presence or absence of doxycycline for the indicated time (hrs) using an Incucyte (EssenBiosciences). b A375 stable cell lines treated as in A) were assayed for viability over the indicated time (days). All values represent mean ± SD (n = 3). c Inducible A375 cells harbouring Mock, 4E-BP1<sup>4A</sup>a and VH-S4 were incubated for 24 hrs with or without 1 ng/ml of Doxycycline. Lysates were analysed by western blot using the antibodies indicated in the blot (for further details see materials and methods). β-Actin was used as a loading control. d A375 inducible stable cell lines were simultaneously incubated with or without doxycycline and where either treated with or without IFN-γ (50 ng/ml) for 48 h. After cell lysis, extracts were analysed by western blot and protein levels for STAT1, Phosho-eIF4E, eiF4E and FLAG tagged 4E-BP1<sup>4A</sup>a and VH-S4 were measured. e A375 cells were treated with the indicated compounds at the labelled concentrations for 72 h with a residual DMSO concentration of 1% (v/v). Lysates were analysed by western blot using the antibodies indicated in the blot (for further details see “Materials and methods”). All western blot analyses were repeated twice to verify their reproducibility. Representative blots are shown. Source data for a, b, c, d and e are provided in the Source Data file.
variants contributed to the soluble and stable expression of the VH domain constructs intracellularly in the absence of C22 and C92. The replacement of the previous CDR3 loop in VH-37i resulted in loss of cellular expression and thermal stability that was either compensated for by the re-introduction of C22 and C92, suggesting that these residues are forming stabilising interactions with each other inside the cell, or by the incorporation of S93V and A78V in VH-37i.1 and VH-37i.2, respectively, that both facilitate improved scaffold stability.

The **VH-S4 intra-domain disulfide bond is retained in a reducing environment**

The rescue of intracellular expression of VH-37i by the re-insertion of C22 and C92 raises the possibility of intra-disulfide bond formation.
occuring inside the cell, especially with both residues residing internally near each other in the VH domain and occluded from the reducing cellular environment. To establish the presence a disulfide, recombinant VH-S4(S22C-T92C) was analysed by liquid chromatography/mass spectrometry after treatment with defined redox conditions (Fig. 7d). Treatment of VH-S4(S22C-T92C) with the reducing agent DTT and the chemical denaturant guanidinium chloride led to a molecular weight shift of approximately 2 Da, consistent with the loss of an intradomain disulfide bond. As expected from literature precedent\textsuperscript{50}, disulfide reduction was accompanied by a shift in the spectral envelope toward higher charge states, providing a second line of evidence that a disulfide had been present before reducing/denaturing treatment. No significant shift in mass or spectral envelope was measured after treatment with DTT in non-denaturing conditions, suggesting the disulfide would be stable intracellularly. Together this data suggests that recombinant VH-S4(S22C-T92C) contains a disulfide bond that is preserved in the reducing environment of the cell. VH-S4(S22C-T92C) was also crystallised in complex with elf4E, where the presence of the intradomain disulfide bonds caused no conformational changes in the global fold of the VH domain compared to the disulfide-free domain (Fig. 3A4). Interestingly, the S108R sidechain is found forming a direct electrostatic interaction with EL32 and displacing the structured waters observed in the VH-S4 structure (Fig. 4d and Fig. 5A4).

**Discussion**

We have developed a repertoire of VH domain-based miniproteins that sample a wide range of Kd\textsubscript{a} (picomolar to micromolar), which disrupt the elf4F complex though a distinct binding pose and that are amenable to cellular expression. We have also established that the residues (C22-C92) required for intra-domain disulfide bond formation are not detrimental to the expression of the majority of VH variants with improved thermal stabilities (Fig. 7c) and that these residues form a disulfide bond when re-substituted into the VH-S4 domain in the presence of a reducing environment. The re-introduction of the intra-domain disulfide bond also has no effect on the VH domain structure or its interaction with elf4E (Fig. 7 and Fig. 5A4). Further, the re-substitution of C22 and C92 into VH-37I restored its cellular expression in the absence of other VH variant mutations (VH-37L1 and VH-37L2) that thermally stabilized the VH domain (Fig. 7c). These results suggest that if poorly expressing VH domains transiently form correctly folded structures upon protein synthesis, then occlusion of C22 and C92 from the reducing environment will enable disulfide formation and allow improved cellular expression. This suggests that in phage selections utilizing the disulfide-free VH scaffold, which fail to identify a clone amenable to cellular expression, re-substitution of C22 and C92 is a potential rescue strategy.

In contrast to PPIs such as Mdm2:p53\textsuperscript{35} and Bcl-2:Bax\textsuperscript{46}, the elf4E:4G interaction interface is relatively planar with no distinguishing clefts and is much more structurally similar to PPIs such as the interface found in the Notch transcription complex between ICN:CSL and MAML and β-catenin:TCF\textsuperscript{77}. The elf4E interacting VH-S4 domain has evolved an optimised CDR3 loop for recognizing planar PPI surfaces, and with randomization of the appropriate residues should serve as an ideal template for building libraries to explore these molecular surfaces. In addition, no clinically approved drugs exist that target the elf4E:4G interface directly, despite the description of several antagonistic small molecules that albeit interact weakly with elf4E and have little cellular efficacy\textsuperscript{5}. The VH-S4:IC5 structures offer two possible avenues for future lead molecule development: (1) the CDR3 loop could be used as a template to drive forward a macrocyclic discovery strategy and (2) the critical residues involved in binding elf4E could be used as a basis for small molecule design.

The applicability of VH domains for cellular pathway modulation and therapeutic modelling were demonstrated by comparing their mode of action to several small molecules and a phosphorylation deficient mutant of 4E-BP1 (4E-BP1\textsuperscript{4ALA}) known to modulate the elf4F complex. Both 4E-BP1\textsuperscript{4ALA} and VH-S4 exhibited similar potencies toward each other in terms of their bioactivity, which is unexpected as VH-S4 interacts more strongly with elf4E (Table 1). 4E-BP1\textsuperscript{4ALA} was also more effective in reducing cellular proliferation of MB-MDA-231 cells despite similar efficacies to VH-S4 in terms of elf4F complex disruption. These results suggest that other mechanisms apart from displacement of elf4G are putatively playing a role in the effects of 4E-BP1\textsuperscript{4ALA} mediated inhibition. Such effects could be post-translational modifications apart from phosphorylation (e.g., ubiquitination) or additional inhibition by endogenous 4E-BP1 through unappreciated feedback loops. Therefore, VH domains that are inert to cellular regulation pathways can be used to decipher their effects on naturally occurring repressors provided they interact at the same binding site.

Furthermore, the VH-S4 domain was compared to several well-known compounds used to study the role of the elf4F complex. For example, inhibitors of mTORC1, the multi-subunit protein responsible for phosphorylating 4E-BP1 and controlling its ability to inhibit elf4E, is also responsible for regulating the activity of other target proteins and their downstream pathways, such as the rS6 kinase\textsuperscript{51,52}. Here we demonstrated that VH-S4 disrupts elf4F complex disruption in the absence of effects on rS6 kinase phosphorylation (including 4EBP1 also) and is thus a more appropriate tool for understanding the impact of elf4E. Additionally, we also compared it to the small molecule inhibitor of the elf4E:4G interaction, 4EGI-135, where in contrast to the VH-S4 domain the induction of apoptosis was seen. A result shared with the in mTORC1 inhibitor that was used (PP242). These results further demonstrate how specific miniproteins can be used to refine our understanding of small molecule inhibition on cellular phenotypes, and to delineate whether desirable outcomes such as apoptosis are the result of direct on target engagement or indirect off-target effects. Tools that could be useful for therapeutic design and research.

**Methods**

**Library generation for stability improvement**

Random mutagenesis libraries were created by error-prone PCR using Genemorph II Mutagenesis Kit (Stratagene). The cloned wild-type
genes (for first library) or improved variants (for subsequent libraries) were used as templates, and the reactions were performed according to the manufacturer’s protocol. The primers used annealed to sequences flanking the open reading frame (ORF) to be mutated. An average of 50 ng of insert template was used, in a 30 cycles PCR reaction with phosphorylated primers, leading to an average of 2–3 amino-acid mutations per gene in the final plasmid library. The error-prone PCR product was gel purified using a Qiagen Gel purification Kit.

The plasmid library was created by Megaprimer PCR of Whole Plasmid (MEGAWHOP) reaction. Typically, 1–10 ng of plasmid containing the wild-type (WT) gene was used as template, and amplified using approximately 1 ng of insert per bp of insert length of purified error prone PCR product as the megaprimer (e.g., 500 ng for a 500 bp insert), in a PCR reaction with KOD Xtra polymerase (Merck). The buffer composition was the standard recommended by the manufacturer, with the addition of 1 mM NAD+, 40 U of Taq DNA ligase, and using 0.5 U of KOD Xtra polymerase. The PCR conditions were as follows: 94 °C for 2 min, (98 °C for 10 s, 65 °C for 30 s, 68 °C for 6 min) for 30 cycles, 4 °C on hold. After PCR, 20 U of DpnI (NEB) was added to the PCR reaction, and incubated for 3 h at 37 °C.

To generate a library that would allow the identification of a disulfide-free version of VH36, first the stIT signal sequence was removed by site-directed mutagenesis, generating clone VH36i. Then, a site-directed mutagenesis library was generated by MEGAWHOP, in which residue positions C22, A24 and C96 were randomized. A PCR using phosphorylated forward (5′-GGCTACTCCGTGTTCCTCNC-NK-CNKKTCGGCTACATTAAAGAC-3′) and reverse (5′-GCTCTCCA CGGCCMN ATATAAGACGGCAGTG-3′) primers was performed using VH-36i as template, and KOD HotStart polymerase (Merck), according to the manufacturer’s protocol. The PCR product was gel purified, and the MEGAWHOP reaction performed as described previously, after which DpnI treatment was performed. DH10B cells were electroporated with the DpnI-treated, purified MEGAWHOP product yielding libraries of ~10^5 unique members.

**Solubility and stability screening using colony filtration (CoFi)**

Rosetta2 cells were transformed with the mutagenesis libraries and plated on 24.5 cm diameter square LB-Agar plates supplemented with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol. These were termed the master plates. Colonies were transferred to a Durapore 0.45 μm filter membrane (Millipore), and placed on LB-Agar plates (colonies facing up) supplemented with antibiotics and 30 μg/mL Iso- propyl β-D-1-thiogalactopyranoside (IPTG). Induction was performed overnight at room temperature (RT) for protein expression.

After protein production, the induction plates were subjected to the desired temperature (room temperature for solubility screen (termed CoFi), or higher temperatures for the stability screen (termed Hot-CoFi)) for 30 min. The Durapore membrane was transferred to a lysis sandwich composed of a Whatman paper, soaked in CoFi lysis buffer [20 mM Tris, pH 8.0, 100 mM NaCl, 0.2 mg/mL lysozyme, 11.2 U/mL Benzonase Endonuclease (Merck) and 1:1000 dilution of Protease Inhibitor Cocktail Set III, EDTA-Free (Merck)], a nitrocellulose membrane (Millipore) and incubated at the screening temperature for another 30 min. Cell lysis was further improved by three freeze-thaw cycles at −80 °C and RT, respectively. The Durapore membrane and the Whatman paper were discarded, and the nitrocellulose membrane was incubated in blocking buffer [TBS-T buffer (20 mM Tris, pH 7.5, 500 mM NaCl, 0.05% (vol/vol) Tween 20) and 1% Bovine Serum Albumin (BSA)] for 1 h at RT.

After blocking, the nitrocellulose membrane was washed three times in TBS-T, 10 min at room temperature, with shaking at 70 revolutions per minute (rpm). The presence of soluble protein was detected either by incubating the membrane in TBS-T containing a 1:5,000 dilution of HisProbe-HRP (Thermo Scientific, Cat #: 15163), or with a 1:10,000 dilution of protein A-HRP probe (Life Technologies, Cat #: 200-103-077) in TBS-T with 1% BSA, for 1 h at RT, with shaking at 30 rpm. Three washing were then performed as previously described. The nitrocellulose membrane was developed using Super Signal West Dura chemiluminescence kit (Thermo Scientific) and imaged using a CCD camera (FujiFilm LAS-4000).

The sequence of the variants producing high CoFi or Hot-CoFi signal was determined by aligning the developed images with the master plates, and picking the colonies linked to the high signals obtained. The picked colonies were grown overnight at 37 °C in LB media with 50 μg/mL kanamycin and 34 μg/mL chloramphenicol, plasmids isolated and submitted to Sanger sequencing.

**Determination of temperature of cellular aggregation (Tcagg)**

Starter cultures composed of 1 mL Terrific Broth (TB) supplemented with antibiotics were inoculated from glycerol stocks of the clones to test and incubated overnight at 37 °C with shaking. The next day, 200 μL were used to inoculate 20 mL of TB supplemented with antibiotics and incubated at 37 °C with shaking until an OD_600 of approximately 2 was reached. The cultures were then induced with 30 μM IPTG and incubated overnight at 18 °C. The expression cultures were centrifuged at 4800 × g for 15 min at 4 °C, and the pellets were resuspended in 5 mL of CoFI lysis buffer. Cell lysis was performed through three freeze-thawing cycles at −80 °C and RT, respectively. The samples were then transferred to a PCR plate in 100 μL aliquots. Each aliquot was then subjected to a different temperature, using a temperature gradient in a PCR machine, for 15 min and then cooled down to 4 °C. The heat-treated samples were centrifuged at 3200 × g for 15 min at 4 °C. The supernatant was transferred to a 0.65 μm filter plate and filtered by centrifugation at 2000 × g for 15 min at 4 °C. 2 μL of the filtrate was dotted onto a nitrocellulose membrane and developed using HisProbe-HRP as described. After development, the average intensity of each dot was calculated using ImageJ software (NIH) and the midpoint of the transition determined using the Boltzmann equation on Prism 8.0 (GraphPad Software).

**Determination of melting temperature (T_m) using Differential Scanning Fluorimetry (DSF)**

To perform Differential Scanning Fluorimetry, 7.5 μg of protein (see VH domain expression and purification section) was diluted into a 25 μL PBS buffer solution containing 5x SYPRO Orange fluorescent dye (Bio-Rad). Proteins were tested in triplicates, the fluorescence was...
monitored using a 96-well Real-Time PCR detection system (iCycler iQ, Bio-Rad), from 25 to 95 °C, with a gradual temperature increase of 1 °C every 10 s. The melting temperature was determined using the Boltzmann equation on Prism 8.0 (GraphPad Software).

Phage display library generation

The open reading frame coding for VH-38i.1 was synthesized with a still signal sequence at the 5' end and the C-terminal domain of the M13 gene 3 at the 3' end. Positions 28-31, 33, 52a, 53-54, 56 and 58 (according to Kabat numbering) were mutated to Gly for flexibility, to form the VH template sequence used in the library creation. Sub-libraries were created by Kunkel mutagenesis with the VH template sequence used in the library creation.

| Primer name | Primer sequence |
|-------------|-----------------|
| H1          | CCTCTGAATTTCTGTCCTC [XYZ] NT [XYZ] ACT [XYZ] TATAGACTGGTGCTGCAAG |
| H2          | CTGGAATGAGGTTGAAAGATT [XYZ] ACT [XYZ] TATAGACTGGTGCTGCAAG |
| H3.6        | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.7        | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.8        | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.9        | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.10       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.11       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.12       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.13       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.14       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.15       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.16       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.17       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.18       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.19       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |
| H3.20       | GCCGTCTTATATTACGCGGC [XYZ] [XYZ] [XYZ] [XYZ] GSTNTKGACTACCGGGGTCAG |

Expression, refolding, and purification of eIF4E

Rosetta pLyS5 competent bacteria were transformed with the pET11d expression plasmid containing the full-length eIF4E clone. The cells expressing the full-length eIF4E construct were grown in LB medium at 37 °C to an OD600 of ~0.6 and eIF4E induction was started with 1 mM IPTG. The culture was immediately placed in a shaker-incubator for 3 h at 37 °C. Cells were harvested by centrifugation and the cell pellets were re-suspended in Tris/Triton buffer. After re-centrifugation, the remaining pellet was solubilised in 6 M guanidinium hydrochloride, 50 mM Hepes-KOH pH 7.6, 5 mM DTT. The protein concentration of the sample was then adjusted to 1 mg/mL. The denatured protein was refolded via a 1:10 dilution into refolding buffer consisting of 20 mM Hepes-KOH, 100 mM KCl and 1 mM DTT. The refolded protein was concentrated and desalted using a PD10 column into refolding buffer. The eIF4E protein sample was run over a monQ column and eluted with a 1 M KCl gradient. eIF4E eluted as a sharp peak at a ~0.3 M KCl.
**eIF4E biotinylation for use in phage selections**

Sulfo-NHS-LC-CL biotin was added in an equimolar ratio to a solution of eIF4E at a concentration of 100 μM and incubated at room temperature (ThermoFisher Scientific). After 1 h, unreacted biotin was removed by passing the solution over a fast-desalting column (equilibrated with phosphate-buffered saline) twice. Biotinylated eIF4E was stored at 4 °C for a maximum period of up till 1 week.

**eIF4E Phage display library screening**

500 μL of the phage display library (~2.5 × 10^13 pfu) was precipitated with PEG/NaCl buffer and resuspended in BSA block buffer (BBB); 4% BSA in PBS supplemented with 0.05% Tween20 (PBST). Immunotubes (Nunc) were coated with NeutrAvidin (ThermoFisher Scientific, 5 μg/mL of Neutravidin in PBS) and incubated at 4 °C overnight. The coated tubes were then washed with PBST and blocked with BBB for 1 h at RT. After washing the tube with PBST, 100 μL of biotinylated eIF4E (5 μg/mL in PBST) was then added and incubated in the tubes for 1 h at RT. A negative selection tube was also prepared as described above, but without adding eIF4E. The tubes were then washed with PBST (3×). The phage library was first incubated for 1 h at RT in the negative selection tube, then transferred to the eIF4E coated immunotube and incubated with the target protein for 1 h with rotation. The non-bound phage were then washed away with three washes of BBB, followed by three washes with PBST and then two washes with PBS. The bound phage were eluted with 1 mL of 1 mg/mL trypsin in trypsin buffer (TBS + 2 mM CaCl2). The eluted phages were used to infect 50 mL of TG1 culture (in 2YT media) in exponential growth phase (OD600 ~ 0.5), incubated 30 min at 37 °C. From round 2 onwards, 1.2 mL of infected TGI cells were stored with 20% glycerol at −80 °C, to be used for monoclonal screening (glycerol stocks for monoclonal screening).

**Plating and sequencing of selected phage**

To identify unique anti-EIF4e VH domains, the glycerol stocks for monoclonal screening were plated onto 2YT agar plates supplemented with 100 μg/mL carbenicillin and 50 μg/mL kanamycin. After overnight incubation at 30 °C, the bacterial-lawn obtained was resuspended into 25 mL TBS, and phage purified by precipitation with PEG/NaCl buffer. After two rounds of PEG/NaCl precipitation, the phage were then resuspended in PBS + 10% glycerol. From the second selection round onwards, the number of washes was increased to seven washes with BBB, seven washes with PBST, and two washes with PBS. The rest of the panning procedure was identical.

**Screening eIF4E binding by biolayer interferometry (BLI)**

Biotinylated eIF4E was immobilized on Streptavidin Biosensors (FortBio/Satorius), and the VH clones were assessed for binding for 120 s at a single concentration of 2.5 μM in Kinetic Buffer (PBS + 0.02% Tween20, 0.1% BSA, 0.05% sodium azide), followed by a 120 s dissociation in the same buffer without VH. BLI measurement was carried out with a Blitiz analysis system (FortBio/Satorius). The equilibrium constant (Kd) was calculated using Blitiz Pro software (FortBio/Satorius).

**eIF4E immobilisation for surface plasmon resonance (SPR) binding assays**

Pure eIF4E was immobilized on a CM5 sensor chip. The CM5 chip was conditioned with a 6 s injection of 100 mM HCL, followed by a 6 s injection of 0.1% SDS and completed with a 6 s injection of 50 mM NaOH at a flow rate of 100 μl/min. Activation of the sensor chip surface was performed with a mixture of NHS (115 mg/mL) and EDC (750 mg/mL) for 7 min at 10 μl/min. Purified eIF4E was diluted with 10 mM sodium acetate buffer (pH 5.0) to a final concentration of 0.5 μM with m7GTP present in a 2:1 ratio to stabilize eIF4E. The amount of eIF4E immobilized on the activated surface was controlled by altering the contact time of the protein solution and was approximately 250 RU. After the immobilization of the protein, a 7 min injection (at 10 μl/min) of 1 M ethanolamine (pH 8.5) was used to quench excess succinimidyl ester groups. Six buffer blanks were first injected to equilibrate the instrument fully. Surface Plasmon resonance experiments were performed on a Biacore T100 machine. Stock protein solutions were serially diluted into running buffer immediately prior to analysis. Running buffer consisted of 10 mM Hepes pH 7.6, 0.15 M NaCl, 1 mM DTT and 0.1% Tween20.
Surface plasmon resonance (SPR) multi-cycle injection experiments

Multi-cycle injection experiments were performed using a flow rate of 50 µl/min, compounds were injected for 60 s and dissociation was monitored for 180 s. Individual proteins were injected across the CMS chip in threefold dilution series to determine their respective binding constants. Each independent protein injection sampled one concentration only and was immediately followed by a similar injection of SPR buffer to enable the chip surface to be fully re-generated by dissociation. Any protein which possessed extremely slow off-rates, and thus making dissociation an unsuitable method for regenerating the CMS chip surface, were analysed using single-injection cycles. Responses from the target protein surface were transformed by: (i) subtracting the responses obtained from the reference surface that contained no immobilised protein, and (ii) subtracting the responses of the buffer injections from those of peptide injections. The last step is known as double referencing, which corrects the systematic artefacts. Kd was determined using a Biacore Evaluation software (Biacore) and calculated from both the response of the elf4E coated CMS chips at equilibrium and kinetically from the dissociation and association phase data for each of the peptides. Both the equilibrium and kinetic data were fitted to 1:1 binding model. Each individual peptide Kd was determined from three separate titrations. Within each titration at least two concentration points were duplicated to ensure stability and robustness of the chip surface. Data analysis performed with Biacore T100 evaluation software (v2.0.4).

Surface plasmon resonance (SPR) single injection experiments

Proteins that possessed extremely slow off-rates were analysed using a single-cycle kinetics approach. For each independent protein, samples were injected consecutively across the CMS chip surface at different concentrations with no intervening regeneration steps. Concentrations used sampled a threefold dilution series with the series proceeding from the lowest concentration to the highest. A complementary single injection was performed over a reference surface containing no immobilised elf4E. The response from the target protein surface was then transformed by: (i) subtracting the response the control surface and then (2) the response from of the buffer injections from those of the protein injections. The transformed response was analysed using Biacore T100 evaluation software (v2.0.4) to derive the binding and kinetic parameters using a 1:1 binding model.

Expression and purification of GST-fused elf4E and 4E-BP14ALA

elf4E and 4EBP14ALA mutants were cloned into the GST fusion expression vector pGEX-6P1 (GE Lifesciences). BL21 DE3 competent bacteria were then transformed with the GST-tagged fusion constructs. A single colony was picked and transformed cells were grown in LB medium at 37 °C to an OD600 of ~0.6 and induction was carried out overnight with 0.3 mM IPTG at 16 °C. Cells were harvested by centrifugation, and the cell pellets were re-suspended in 20 mls of lysis buffer (100 mM HEPES pH 7.5, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 0.5 mM TCEP, buffer). Hexahistidine tagged SrtA was then eluted with a 1 M imidazole linear gradient. Hexahistidine tagged SrtA was then eluted with 1 M imidazole linear gradient. The protein was further purified by size-exclusion chromatography (HiLoad 16/60 Superdex 75 prep grade, Cytiva Lifesciences) using a 20 mM HEPES pH 7.5, 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP, buffer. Protein concentration was determined using A280 and an extinction coefficient determined from the primary sequence of the construct determined through ProtParam (https://web.expasy.org/protparam).

N-terminal biotin labelling of elf4E mediated by SrtA4M

Sortase-mediated ligation was used to specifically label elf4E at the N-terminal with biotin. Cleavage of the GST-fused elf4E with thrombin leaves a single glycine at the N-terminal. The ligation was carried out with thrombin cleaved elf4E at 50 µM, SrtA4M at 1 µM, and biotin-KGGLPET-GG-OHse(Ac)-amide peptide at 200 µM in 200 µl of ligation buffer (50 mM Tris pH 8.0, 150 mM NaCl, 1 mM TCEP). SortaseA4–206/8 M contains mutations that increase ligation efficiency and make it calcium-independent. The ligation was incubated at room temperature for 4 h. SrtA4M which contains a C-terminal 6×His-tag was removed with Dynabeads His-Tag (cat# 10104D, Thermo Fisher). The biotinylated protein was then dialyzed at 4 °C using slide-A-Lyzer cassette (10k MWCO) against 2 L of an appropriate buffer. The buffer was changed after 4–5 h and the dialysis was repeated for overnight. The biotinylated protein was aliquoted, snap-frozen with liquid nitrogen, and stored at –80 °C.

Alanine scanning mutagenesis using yeast surface display (YSD)

Yeast display protocols were adapted from Wittup and co-workers. The VH-1C5 gene was ordered from Integrated DNA Technologies and the pCT-CON vector was digested using Sall, Nhel, and BamH restriction enzymes (NEB) to ensure complete linearization and absence of full-length insert therefore preventing transformation of yeast cells with parental plasmid. The VH-1C5 gene was then PCR
amplified using primers containing 50 base pairs of homology to the pCT-CON2 vector. 300 ng of VH-ICS gene and 1 µg of plasmid vector were combined with 50–100 µL of electrocompetent EBY100 yeast cells and electroporated at 0.54 kV and 25 µF. Homologous recombination of the linearized vector and VH-ICS insert yielded intact plasmid. Cells were grown in YPD (1% yeast extract, 2% peptone, 2% glucose) for 1 h at 30 °C, 250 rpm. The pCTCON2-VH-ICS plasmid was then isolated and purified from EBY100 yeast cells using a Zymoprep kit II and then cleaned using the Qiagen PCR Purification kit. The CDR3 loop of the VH-ICS gene was then sequentially mutated to alanine via site-directed mutagenesis using an in-fusion HD cloning Plus kit (Takara Bio), following the manufacturer’s instructions, to generate the following plasmids pCTCON2-VH-M1 to VH-M20. This corresponded to an alanine scan of the following range of amino acids in the VH-ICS scaffold: 100–120.

Individual transformed yeast clones were then grown in SD-CAA, pH 5.3 to 3.0 (0.07 M sodium citrate, pH 5.3, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L glucose, 0.1 g/L kanamycin, 100 µL/L penicillin, and 0.1 g/L streptomycin), at 30 °C, 250 rpm to logarithmic phase, pelleted, and resuspended to 1 x 10^7 cells/mL in SD-CAA, pH 6.0 (0.1 M sodium phosphate, pH 6.0, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L dextrose, 1 g/L glucose, 0.1 g/L kanamycin, 100 µL/L penicillin, and 0.1 g/L streptomycin). Cells were then pelleted by centrifugation (14,000 × g, 25 µL of electrocompetent EBY100 cells at 0.54 kV and 25 µF, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L glucose, 0.1 g/L kanamycin, 100 µL/L penicillin, and 0.1 g/L streptomycin) and incubated at room temperature for 8 minutes to enable dissociation of biotinylated eIF4E. Cells were washed and incubated with 200 nM of biotinylated eIF4E. Yeast were then washed and resuspended with PBS (0.1 M sodium phosphate, pH 7.4, 0.137 M sodium chloride, 1 g/L bovine serum albumin) resuspended in PBSA to a density of 1 x 10^6 cells/mL and then used for affinity maturation described in the paragraph below.

FACS selections of the mutated VH-M4 library was conducted with the kinetic competition. Three rounds of kinetic selection were performed. Yeast were washed and incubated with 200 nM of biotinylated eIF4E. Yeast were then pelleted, washed in 1 mL PBSA (0.1 M sodium phosphate, 2.0 mL PBSA, pH 6.0, 182 g/L sorbitol, and 0.1 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L glucose) and incubated at room temperature for 8 minutes to enable dissociation of biotinylated eIF4E. Cells were washed in PBSA, resuspended in PBSA with Anti-HA Ab Alexa Fluor 488 (ThermoFisher Scientific, catalogue #: A-11039, dilution used 1:200) and Streptavidin-phycocerythrin or neutravidin-phycocerythrin (ThermoFisher Scientific, Catalogue #: S866 and A26660, respectively) for 10 min, and incubated on ice. Working dilutions used for Streptavidin-phycocerythrin and neutravidin-phycocerythrin were 1:100 and 1:50, respectively. Labelled tubes were washed with 1 mL PBSA, resuspended in 0.5–2.0 µL PBSA and analysed by flow cytometry using an Aria (Becton Dickinson) cytometer in conjunction with the BD FACSDiva 6.1 software package. Cells positive for anti-HA and eIF4E were selected and sorted. FACS gating strategy shown in Fig. S5. Collected cells were grown in SD-CAA, pH 5.3, at 30 °C, 250 rpm and either induced in SG-CAA, pH 6.0, for further selection or used for plasmid recovery. Two further rounds of kinetic selection were performed as described above extending the periods of dissociation to 60 and 120 min, respectively. Yeast isolated from each round for plasmid recovery were serially diluted and plated on SD-CAA plates (0.1 M sodium phosphate, pH 6.0, 182 g/L sorbitol, 6.7 g/L yeast nitrogen base, 5 g/L casamino acids, 20 g/L glucose) and individual clones grown. Plasmid DNA was then isolated using the Zymoprep kit II, cleaned using the Qiagen PCR Purification kit, and transformed into DH5α (Invitrogen) cells. Purified plasmids were then sequenced using BigDye chemistry. Fifty clones were sequenced from each affinity maturation round of selection. The final round of kinetic selection resulted in the identification of only three eIF4E binders: VH-ICSD102AA/S108R/F120I, VH-ICSQ103DA/S108R/F120I and VH-ICSNQ103DA/S108R/F120I. The VH-ICSQ103DA/S108R/F120I clone (termed VH-S4) dominated the final round with a frequency >90% and was therefore selected for scale up and SPR analysis. The clone was then amplified using PCR and ligated into the pET22B bacterial protein expression vector for protein purification as outlined in “Bacterial Expression and Purification of VH Domains” section.

**Bacterial expression and purification of VH domain binders**

VH-ICS and VH-HA2 sequences were ordered as gene fragments from Integrated DNA Technologies (IDT). Both coding sequences were PCR amplified and cloned directly into the bacterial expression vector pET22b (+) with an in-frame C-terminal six-histidine tag. VH-M4 was directly PCR amplified from the pETCON2 plasmid used in the yeast alanine scanning experiments, whilst VH-S4 was amplified from the plasmid isolated through the affinity maturation selection. Both sequences were then cloned into pET22b (+) as described earlier. Using VH-M4 as a template sequence, the in-fusion mutagenesis kit (Takara) was used to generate the following mutants in the pET22b (+) backbone (VH-ICSQ103DA/S108R and VH-ICSNQ103DA/S108R/F120I). Each VH domain plasmid was separately transformed into E. coli BL21 (DE3) cells and used to inoculate 10 mL of LB broth (containing 100 µg/mL) started culture, which were incubated overnight before being used to seed
Proteins were purified the eluted proteins were subjected to gel filtration chromatography on a Superdex 75 column (Cytiva Lifesciences) using PBS buffer containing 0.1 M Sodium HEPES pH 7.5, 25% PEG 6000. For X-ray data collection at the Australian synchrotron (HX Beamline) using a CCD detector, and integrated and scaled using XDS. The initial phases of the VH-domain complexed crystals of eIF4E were solved by molecular replacement with the program PHASE 

using the human eIF4E structure (PDB accession code 1EJ4) and the VH domain structure (PDB accession code: STD8, chain B) as independent search models. The starting models were subjected to rigid body refinement and followed by iterative cycles of manual model building in Coot and restrained refinement in Refmac 6.0. Final models were analysed using PYMOL (Schrodinger). See Table S2 for data collection and refinement statistics. The eIF4E complex structures with VH-1CS, VH-S4 and VH-S4ss has been deposited in the PDB under the submission codes 7D6y, 7DB8 and 7XTp, respectively. For data collection and refinement parameters see Table S3.

**Plasmid and reagents for cell biology**

All plasmids were purchased from Addgene where not otherwise otherwise. 4D5 VH domain plasmid was provided by DotBio. Mutant VH domains were generated with In-fusion mutagenesis kit (Clontech). VH domain (VH-1CS, VH-1A2, VH-M4, VH-S2, VH-S4, VH-S4ss and VH-1C5<sup>90</sup>) nucleotide sequences are listed in Table S5. VH mutants were cloned into a pCDNA3.1 vector (Thermo Fisher Scientific) harbouring a C-terminal 3× FLAG tag via NheI/BamHI sites to allow mammalian cell overexpression. For bacterial expression, VH domain mutants were cloned into either a pHt22B or pCW57 plasmid using either BamHI/ Xhol or BamHI/AvrII cloning sites, respectively. pCW57 Luc-polRES-fluc (bicistronic reporter), eIF4E and eIF4G<sup>4ala</sup> were purchased from Addgene, whilst all other chemicals unless otherwise stated were purchased from Selleck Chemicals. Primer sequences used for construction of expression plasmids shown in Table 3.

**Mammalian cell culturing conditions**

All cell lines were cultured in DMEM cell media supplemented with 10% foetal calf serum (FBS) and penicillin/streptomycin. For A375 and MDA-MB 231 stable cell lines medium FBS was replaced with TET-system approved FBS (Thermo Fisher Scientific). Cells were maintained in a 37 °C humidified incubator with 5% CO2 atmosphere. A375, HEK293T cells was used to generate lentivirus for infection of target cells. Packaging cells were transfected using calcium phosphate transfection described by Trono laboratory (https://www.epfl.ch/labs/tronolab/). 6 μg of pCW57 plasmid (Addgene, USA) harbouring either 4EBP1<sup>4ala</sup> or VH-S4 or no insert were co-transfected into HEK293T cells with plasmids encoding pLVSVG (viral envelope), pLP1 (gag-pol) and pLP2 (rev), in a ratio of 2:1:2:2 to generate viral particles. 48 h later the conditioned medium harbouring viral particles from the transfected HEK293T cells was filtered and viral particles were concentrated by ultracentrifugation. A375 and MDA-MD-231 cell lines were seeded in 12-well plates and infected with viral particles over a 12 h period prior to cell media replacement with fresh medium. 72 h post infection, A375 and MDA-MB-231 cells were supplemented with 800 μg/ml of geneticin and selections for stably transfected cells were carried out for 2 weeks, replacing the antibiotic-containing media every 3 days. Polyclonal geneticin-resistant pools of cells were then obtained. These were then incubated with 1 μg/ml of doxycycline for 24 h, where upon GFP positive single clones were isolated by FACs into 96-well plates. Monoclonal stable cell lines were verified using western blot and then expanded for subsequent analysis.

| Table 3 | Primer sequences used for construction of expression plasmids |
|---|---|
| Primer name | Primer sequence |
| pCDNA3 Fw Nhel | CCAAGCTTGGCTAGCATGAGCGAAGTGCACTGGT |
| pCDNA3 Rv BamHI | CGGAAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| pCW57 Fw AvrII | AACCAGCTGCTAGAGCCACGCGCTGGT |
| pCW57 Rv BamHI | CGAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| peT22B Fw Xhol | GATGCGAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| pCDNA3 4EBP1 Fw Nhel | CCAAGCTTGGCTAGCATGAGCGAAGTGCACTGGT |
| pCDNA3 4EBP1 Rv BamHI | CGGAAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| pCW57 4EBP1 Fw AvrII | CCAAGCTTGGCTAGCATGAGCGAAGTGCACTGGT |
| pCW57 VH Fw Nhel | GATGCGAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| pCDNA3 VH Fw Xhol | GATGCGAATTCGCGTCGATTGCAATGAGCGAAGTGCACTGGT |
| pCDNA3 VH Rv BamHI | CCAAGCTTGGCTAGCATGAGCGAAGTGCACTGGT |
| pCW57 VH Rv BamHI | CCAAGCTTGGCTAGCATGAGCGAAGTGCACTGGT |

1000 mls of fresh LB broth. Bacterial cultures were grown at 37 °C and when they reached a OD<sub>600</sub> of 0.6–0.8, the cells were induced with a final concentration 0.5 mM of IPTG and incubated overnight at 25 °C. Cells were harvested by centrifugation at 17,000 × g for 10 min and pellets were resuspended in lysis buffer (25 mM HEPES pH 7.5, 300 mM NaCl, 20 mM imidazole, 1 mM DTT) and then sonicated for 5 min. Bacterial supernatants were then filtered through 1.2 µm syringe filters. Proteins were purified through a standard two-steps protocol: first, supernatant were loaded onto a 1 ml HisTrap column (Cytiva Life-sciences), which was pre-equilibrated then extensively washed with buffer A (25 mM HEPES pH 7.5, 300 mM NaCl, 1 mM DTT) and then eluted with buffer A that also contained 500 mM imidazole; second, the eluted proteins were subjected to gel filtration chromatography on a Superdex 75 column (Cytiva Life-sciences) using PBS buffer containing 1 mM DTT. Protein fractions were analysed by SDS page gel and concentrated. Protein concentration was determined using absorbance at 280 nm.

**Protein crystallization**

The eIF4E-VH-ICS and eIF4E-VH-VH-S4 complexes were crystallized by vapour diffusion using the sitting drop method. Crystallization drops contained eIF4E, m'GTP and VH-ICS at concentrations of 100, 300 and 100 μM respectively. Sitting drops were set up in 48 well Intelli-Plates (Hampton research) with 1 µl of the protein sample mixed with 1 µl of the mother-well solution. eIF4E-VH-ICS crystals grew over a period of time but in 0.01 M Tris pH 8.5. eIF4E-VH-4S4S crystals grew over a similar period of time but in 0.01 M Tri-sodium citrate and 16% PEG6000 (v/v). Crystals containing the m'GTP:eIF4E-VH-4S4S complex were isolated by FACs and then expanded for sub-milligram scale growth. Crystals were cryoprotected in 25% PEG6000 and 0.1 M Sodium HEPES pH 7.5, 25% PEG 6000. For X-ray data collection at 100 K, crystals for all sets of crystallization conditions were transferred to an equivalent mother liquor solution containing 25% (v/v) glycerol and then flash frozen in liquid nitrogen.

**Crystal data collection and refinement**

X-ray diffraction data were collected at the Australian synchrotron (MXI beamline) using a CCD detector, and integrated and scaled using XDS. The initial phases of the VH-domain complexed crystals of eIF4E were solved by molecular replacement with the program PHASE

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Immunoprecipitation and mGTP pull down experiments
Twenty-four hours prior to transfection or drugging, cells were seeded at a cell density of 1,000,000 (HEK293) or 250,000 (Hela) or 300,000 (A375) cells per well of a six-well plate (ThermoFisher Scientific). Transfections were performed using Lipofectamine 3000 (ThermoFisher Scientific) with either 1 μg or the indicated amount of plasmid vectors per well according to the manufacturer’s instructions. After a 48 h (or as indicated in the relevant figure) incubation period, the cell media was then removed and the cells washed with PBS saline. Cells were directly lysed in the wells with 300 μl of lysis buffer containing 20 mM Hepes pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM DTT with protease (Roche) and phosphatase (Sigma-Aldrich) inhibitor cocktail sets added as outlined by the manufacturer’s protocols. Cellular debris was removed by centrifugation, and the protein concentration was then determined using the BCA system (Pierce). m7GTP content was then determined using the BCA system (Pierce). m7GTP (Jena Bioscience) or anti-FLAG M2 antibody (Roche) immobilised agarose beads for 2 h at 4°C on a rotator. Beads were then washed four times with lysis buffer containing no protease or phosphatase inhibitors. This was then followed by the addition of Laemmli buffer (2×) and the beads boiled for 5 min at 95 °C. Samples were centrifuged and the supernatant removed for western blot analysis.

NanoBit® elf4E:elf4G604-646 complementation assay
Opaque 96-well plates were seeded with 30,000 HEK293 cells per well in DMEM and 10% FCS. Transfections in 96-well plate format were performed using FUGENE6 (Roche) with 30 ng of the bicistronic reporter (pcDNA3-rLuc-polRES-fluc) plasmid and 150 ng of the indicated plasmid. Forty-eight hours after transfection, Renilla and firefly luminescence activity was determined using the Dual Glo Luciferase Assay System (PRO-MEGA). A replicate plate following the experimental conditions above was also concurrently prepared, where the cells were instead lysed with 50 μl of passive lysis buffer (PerkinElmer) for 15 min and 10 μl of lystate from each well was transferred into a white bottom 384-well plate. GAPDH and pS209 eIF4E levels were then determined using the Alphascreen®Surefire® GAPDH and elf4E (p-Ser209) assays (PerkinElmer) as outlined in the manufacturer’s instructions. Luminescence readings were performed using an Envision Multi-plate reader (PerkinElmer).

Mammalian cell protein expression analysis
Transfected HEK293 cells (prepared as described in the NanoBit and Cap-dependent translation Experiments sections) were seeded with 30,000 cells per well in 96-well plates. After an incubation period indicated in the relevant figure, cells were washed with PBS and directly lysed in the wells of the plate with 50 μl of cell lysis buffer (20 mM Hepes pH 7.4, 100 mM NaCl, 5 mM MgCl₂, 0.5% NP-40, 1 mM dithiothreitol) with protease (Roche) and phosphatase (Sigma-Aldrich) inhibitor cocktail sets added as outlined by the manufacturer’s protocols. Cellular debris was separated by centrifugation, and without further quantification, samples were analysed by western blot.

Cap-dependent translation and AlphaScreen®Surefire® assays
Opaque 96-well plates were seeded with 30,000 HEK293 cells per well in DMEM and 10% FCS. Transfections were performed using FUGENE6 (Roche) with 30 ng of the bicistronic reporter (pcDNA3-rLuc-polRES-fluc) plasmid and 150 ng of the indicated plasmid. Forty-eight hours after transfection, Renilla and firefly luminescence activity was determined using the Dual Glo Luciferase Assay System (PRO-MEGA). A replicate plate following the experimental conditions above was also concurrently prepared, where the cells were instead lysed with 50 μl of passive lysis buffer (PerkinElmer) for 15 min and 10 μl of lystate from each well was transferred into a white bottom 384-well plate. GAPDH and pS209 eIF4E levels were then determined using the Alphascreen®Surefire® GAPDH and elf4E (p-Ser209) assays (PerkinElmer) as outlined in the manufacturer’s instructions. Luminescence readings were performed using an Envision Multi-plate reader (PerkinElmer).

Western Blot analysis
Samples were resolved on midi or mini Tris-Glycine 4–20% gradient gels (Bio-Rad) according to the manufacturer’s protocol. Western transfer was performed with an Immuno-blot PVDF or nitrocellulose membrane (Bio-Rad) using a Trans-Blot Turbo system (Bio-Rad). Western blots were then performed. B-actin levels were measured to ensure equal loading. For details of antibodies and experimental dilutions used see Table 4.

Cell proliferation assay
A375 cell lines were plated in 96-well clear bottom plates at a density of 4000 cells per well in 200 ul DMEM and 10% FCS medium. After 24 h,
cell media was replaced with 200 µl of medium containing doxycycline at 1 ng/ml. Cell confluence and cell growth was then measured continuously over 7 days using an IncuCyte FLR instrument (Essen Bioscience).

Cell viability assay
Opaque 96-well plates were seeded with A375 cells and cells treated with doxycycline as described in Cell Proliferation Assay section. At the indicated time points, Cell Titer-GLO 2.0 reagent (Promega) was added to each well and cellular viability determined according to the manufacturer’s instruction. Luminescence readings were performed using an Envision Multi-plate reader (PerkinElmer).

Global protein synthesis measurements
A375 cell lines were seeded in 12 well-plates at a cell density of 120,000 cells per well in 1 ml of DMEM and 10% FCS medium. The SUNSET assay was used to monitor the rate of protein synthesis (REF). Transfected cells were pulse labelled with the addition of 10 µg/ml of puromycin (ThermoFisher Scientific) to the cell media prior to cell lysis. As a control, cycloheximide (Sigma) was added at 10 µg/ml five minutes before puromycin addition, resulting in complete blockade of protein synthesis. All other drugs were added for the period indicated in the figures. Cell extracts were processed and analysed for western blotting using anti-puromycin antibody, as outlined in the western blot analysis section.

Mass spectrometry analysis of intra-domain disulfide formation
Mass spectra were acquired in positive mode using a Shimadzu LCMS-2020 single quadrupole liquid chromatography-mass spectrometry system, equipped with a Phenomenex Aerus Widepore XB-C8 analytical column (2.1 x 50 mm; 3.6 µm packing). Mobile phase A was H2O (0.1% formic acid); mobile phase B was acetonitrile (0.1% formic acid). Chromatographic conditions were as follows:
   - Flow rate: 0.4 mL/min
   - Column oven: 25 °C
   - Gradient program (linear):
     - 0 min: 5% B
     - 5 min: 5% B
     - 18 min: 65% B
     - 21 min: 65% B
     - 24 min: 5% B

Recombinant protein stocks (-5 mg/mL in phosphate-buffered saline) were diluted 10-fold into either: 6 M guanidine hydrochloride, 100 mM phosphate, pH 7 buffer (denaturing, non-reducing conditions); 6 M guanidine hydrochloride, 100 mM phosphate, pH 7 buffer containing 100 mM DTT (denaturing, 100 mM DTT conditions), or 1x phosphate-buffered saline (native, 100 mM DTT conditions). The resulting solutions were allowed to stand (ambient temp, 20 min), and then analysed by LC-MS (10 µL; -5 µg). Mass spectra were integrated across the entire peak in the resulting total ion current chromatograms.

Statistics and reproducibility
No statistical methods were used to predetermine sample size. The experiments were not randomised and investigators were not blinded to allocation during experiments and outcome assessment.

Reporting summary
Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability
Structure factors and coordinates generated in this study have been deposited in the Protein Data Bank under accession codes 7DB8, 7D6Y and 7XTP for the elf4E-VH-ICS, elf4E VH-S4 and elf4E-VH-S4s complexes, respectively. The source data generated in this study are provided in the Supplementary information or Source Data file. Antibodies used in this study are provided in the Supplementary information. Amino acid and nucleic acid sequences of the VH domains used in this study are listed in the Supplementary information. Source data are provided with this paper.

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Author contributions
C.J.B., I.A., P.N., T.C., C.S.V., and D.P.L. conceived and designed the study. Y.F., Y.C.L., and S.R.R. performed all cell biology experiments associated with the manuscript. Y.C.L. carried out all the yeast surface display experiments and analysis of the affinity maturation results. K.H., A.H.E., A.P., and Y.X.C. performed the directed evolution experiments, constructed the phage library, carried out the phage panning and preliminary binding analysis of the initial selected clones. K.Y. undertook recombinant protein purification and thermal stability experiments. J.S. collected and processed X-ray crystallography as well as carrying out the SPR experiments and associated analysis. D.L. performed functional analysis of the elucidated crystal structures. Z.G. designed and undertook the mass spectrometry experiments as well as spectra analysis. D.T. performed recombinant protein purification and fluorescence polarization experiments. C.J.B. supervised the structural, biophysical and cellular studies. I.A. supervised directed evolution and phage selection experiments. C.J.B. and I.A. wrote the manuscript with input from all co-authors.

Competing interests
P.N., I.A., K.H. and T.C. are shareholders of DotBio Pte. Ltd. I.A. K.H., A.H.E., A.P., K.Y. and Y.X.C., are employees of DotBio Pte. Ltd. The stabilizing mutations introduced to the VH domains described in this paper, as well as the phage display libraries created, are the subject of the following patent applications: WO2016072938A1 (World Intellectual Property Organization, Patent Cooperation Treaty), US20170320934A1 (US Patent and Trademark Office, granted), US105330282 (US Patent and Trademark Office, granted), JP2018500879A (Japan Patent Office, granted), EP3215537A4 (European Patent Office, granted), EP3215537B1(European Patent Office, granted), EP4008729A1 (European Patent Office, pending) and CN107001477A (China National Intellectual Property Administration, pending). T.C., I.A. and P.N. are inventors in these patent applications, while Nanyang Technological University is the assignee. These patents are licensed exclusively to DotBio Pte. Ltd. Y.F., Y.C.L., J.S., S.R.R., M.G., D.L., Z.G., C.S.V., D.P.L., D.T. and C.J.B. have no competing interests to declare.

Additional information
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Correspondence and requests for materials should be addressed to Ignacio Asial or Christopher J. Brown.

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