Evolving E3 ligase towards recognising novel substrates for targeted protein degradation

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Abstract

Targeted protein degradation offers a novel therapeutic approach, enabling the selective degradation of disease-associated proteins that are otherwise difficult to target. Currently established strategies, such as PROTACs, frequently suffer from off-target effects and poor pharmacokinetics. This study presents a potential alternative strategy that could leverage the ubiquitin-proteasome system to degrade non-canonical targets by delivering an evolved E3 ligase capable of recognizing novel, disease-associated substrates. Specifically, we set out to evolve the degron bound by SIAH to enable recognition of NLRP3, a protein implicated in various inflammatory and neurodegenerative diseases. The previously established Phage-Assisted Continuous Evolution (PACE) was chosen as the evolution method due to its success in reprogramming peptide-specific enzymes such as proteases. While the optimization of the selection system outlined here is still ongoing, we are confident that this alternative approach toward targeted protein degradation could address some of the major challenges of other drugs in this field.

Introduction

Targeted protein degradation (TPD) represents a novel therapeutic approach that could address disease-causing proteins which traditionally have been challenging to target. Most commonly, this induction of selective degradation has been achieved by small molecule drugs such as PROTACs (proteolysis-targeting chimaeras) and molecular glues that recruit target proteins to a ubiquitin ligase labelling it for downstream proteolysis, with slight mechanistic differences [1]. Although promising examples exist, these drugs frequently suffer from off-target effects, toxicity, and poor pharmacokinetic properties, making their development into safe and efficient therapeutics challenging [1]. Here, we present a novel approach to using the ubiquitin-proteasome system (UPS) for TPD.

In mammals, the regulated degradation of misfolded and damaged proteins is primarily managed by the Ubiquitination-Proteasome System. This process begins with a cascade involving three enzymes - E1, E2, and E3 ubiquitin ligases - that work together to recognize and modify target proteins with ubiquitin. In humans, the ubiquitin activation step is typically carried out by UBA1 (E1), which transfers the ubiquitin to one of approximately 40 E2 ubiquitin-conjugating enzymes. The E3 ligase then identifies the target protein by recognizing short peptide motifs called degrons, post-translational modifications, or other signals [2]. Depending on the type of E3, ubiquitin is transferred onto the target protein either by an E3-guided E2 or directly by the E3 itself. Additional ubiquitin units are subsequently added to the initial ubiquitin, branching out as a polyubiquitin chain. The fate of the ubiquitinated protein, whether through proteolytic or non-proteolytic mechanisms, is determined by the length and linkage of the polyubiquitin chain [3,4].

A change in UPS activity and therefore stability of certain proteins, sometimes caused by missing E3 substrate recognition, has been associated with autoimmune and neurological diseases, as well as cancer [5,6]. By reprogramming the substrate specificity of E3 ligases through directed evolution, we aim to target these proteins for TPD while avoiding the limitations of existing methods. Specifically, the evolution process could counter-select toxicity and off-target activity, which restrict the clinical use of PROTACs and molecular glues. Additionally, the established platform for E3 evolution could be adapted to evolve novel, target-specific E3 ligases as needed. Finally, the evolved E3s could also leverage well-established delivery systems, such as lipid nanoparticle-encapsulated mRNA [7], to enable tissue-specific delivery.

We aim to reprogram the substrate specificity of E3 ubiquitin ligases to enable ubiquitination of non-canonical targets using Phage-Assisted Continuous Evolution (PACE) [8]. In PACE, the desired trait—such as enzyme activity—is directly tied to phage propagation, allowing only phages with beneficial mutations to replicate. This approach, combined with error-prone replication of the phage genome, generates a large, diverse phage library that can be subjected to adjustable selection pressures. Compared to other directed evolution methods, PACE offers unprecedented exploration of sequence space, completing over 100 evolutionary cycles in a few weeks [9]. It has been used successfully to evolve substrate recognition for novel DNA and protein targets [8,10], showcasing its strong potential in reprogramming E3 substrate specificity.

Results

E3 ligase, substrate, and target selection

Achieving TPD with a reprogrammed E3 ligase requires a careful choice of the E3 to evolve. After screening well-characterised human E3s, we chose to evolve SIAH1 and SIAH2 (collectively referred to as SIAH1/2), both of which belong to the RING family of E3 ligases. SIAH 1/2 are functionally similar proteins with a sequence identity of 86%, an almost identical substrate-binding domain [11], and are primarily implicated in cellular stress responses such as hypoxia, with a host of known canonical substrates [12,13]. With lengths of 282 and 324 amino acids, respectively, SIAH1/2 fall within the optimal size range for PACE, enabling efficient expression in *Escherichia coli* and packaging into M13 phage. Additionally, their small size significantly increases the chance that impactful mutations occur by chance compared to larger E3 family proteins. SIAH2 has been reliably expressed in *E. coli* and used for ubiquitination-based assay, showing it can be functionally active in a bacterial system [14]. This also indicated that no additional regulatory proteins need to be present for ubiquitination, drastically simplifying the selection system and evolution. Because of structural and functional similarities, we assume that SIAH1 is expressed and functions similarly in *E. coli.* Finally, the binding of SIAH1 to a respective peptide sequence has been characterised extensively [15,16] providing a foundation for identifying potential target sequences for subsequent evolution toward the recognition of non-canonical targets. The SIAH family of E3 ubiquitin ligases identifies target proteins through a PxAxVxP degron motif [17]. In this motif, the conserved Pro, Ala, Val, and Pro residues are oriented towards the binding pocket of SIAH (Figure 1). Specificity is primarily determined by the binding pockets for Ala and Val residues at positions 3 and 5, as they are too small to fit bulkier side chains [18]. Additionally, the Pro residue at position 7 interacts with the side chain of Trp178 in SIAH in a coplanar arrangement, which confers an additional degree of specificity. Among canonical substrates, the VXP motif is the most highly conserved [16,19], while variations in the remaining residues are generally more tolerable and can thus most likely be targeted for evolution.

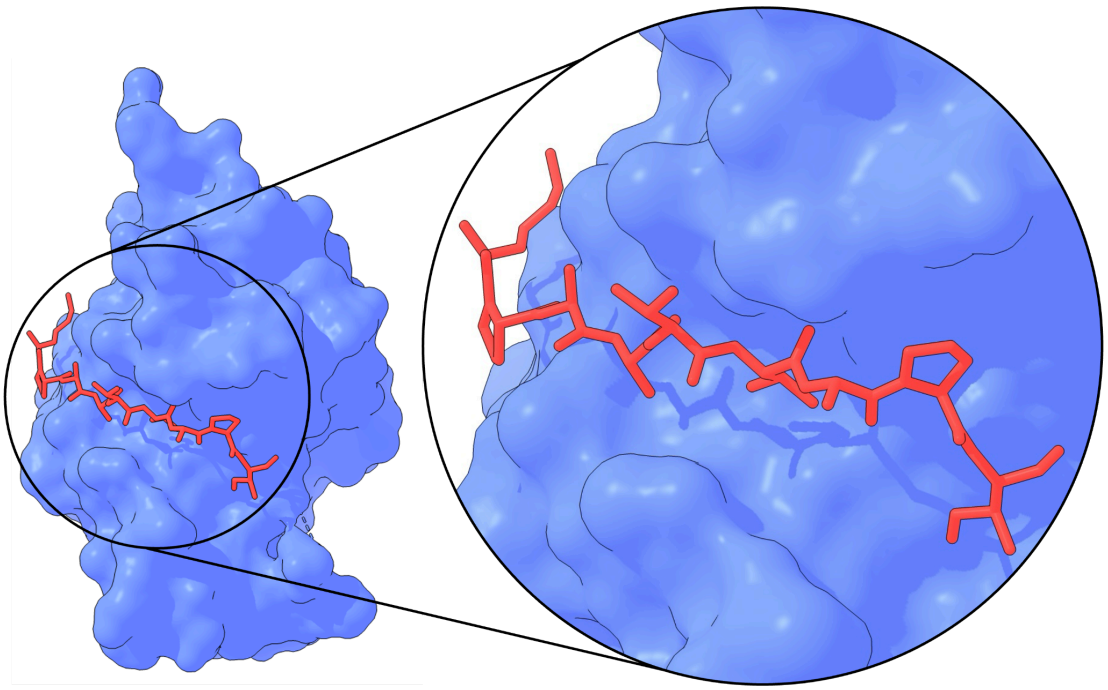

Figure 1: SIAH1 monomer bound to peptide substrate; PDB: 2A25 [15,16]. Peptide residues (red) alternate between facing towards and away from SIAH1 (blue).

We plan to evolve SIAH1/2 to recognize a non-canonical substrate containing a VXP motif by replacing one residue at a time from a canonical substrate motif with the respective amino acid of the final target. This “substrate walk” strategy has been used previously to lower the evolutionary hurdle that has to be passed to adapt to the selection pressure [10].

Canonical targets of SIAH1/2 were selected for their known ubiquitination, structural compatibility, and therapeutic potential while avoiding features that could interfere with the evolutionary logic at hand. We chose EGLN3 as our main substrate to develop the evolutionary logic, with EGLN1 and α-Synuclein serving as alternatives (Supplementary Table 1). NLRP3 was selected as a non-canonical evolutionary target since it contains two easily accessible VXP motifs (Supplementary Figure 1) and for its clinical relevance in inflammatory bowel disease, as well as neurodegenerative and hepatic diseases. This makes any evolved E3 ligase easily deliverable, as mRNA encapsulated in lipid nanoparticles (LNPs) has already been established for treating liver diseases [20]. The two VXP motifs (200-VSP-203 and 707-VLP-710) are found in unstructured, surface-exposed regions, with proximal lysines crucial for ubiquitin attachment; notably, K689 is natively polyubiquitinated, inhibiting NLRP3 activation and inducing TPD [21], suggesting that NLRP3 can indeed be ubiquitinated in this region. To minimise interference from NLRP3's size in the evolutionary system, we designed peptide fragments containing the respective VXP motif, surrounding residues, and necessary lysines near the degron (Supplementary Result 1).

Overview of the PACE Logic

PACE relies on the ability of phages to propagate in host cells expressing *gIII,* a gene essential for the phage replication cycle, which is conditional to the biological activity of interest. In this method, M13 phages are engineered to carry a protein of interest (POI) on a selection plasmid (SP) in place of *gIII*. Inside a bioreactor, these phages infect host cells carrying a genetic circuit designed to select active variants of the POI. This circuit is encoded by accessory plasmids (AP). When the POI encoded by the phage demonstrates the desired phenotype, *gIII* expression is triggered. The constant inflow of new cells carrying the AP and outflow of inactive gene variants from the reactor allows for only the SP population encoding an active POI to persist in the lagoon and propagate faster than their rate of dilution [8]. The stringency of the system can be adjusted by using stricter evolution logic, or by modulating the flow rate through the turbidostat. Cells also carry the mutagenesis plasmid (MP6), which increases mutagenesis rates by expressing additional proteins that increase DNA methylation and deamination (*dam* and *cda1*), inhibit cellular repair pathways (*ugi*) and retain mutagenic bases in the cell (*emrR*) [22]. Mutagenesis is induced by the addition of arabinose and increases the library size of SP variants that can be selected in the PACE reactor. Simultaneous mutagenesis and selection for the desired activity allows for the evolution of the POI in a continuous and high-throughput manner.

In order to evolve SIAH1/2, we linked the ubiquitinase activity of the E3 ligase to a synthetic genetic circuit that uses a split-modified bacteriophage T7 RNA polymerase [23,24]. The genetic circuit coding the evolution logic was split into three plasmids: one SP and the two accessory plasmids AP1 and AP2 (Supplementary Figure 2). The SP carried a SIAH1/2 variant and was packaged in an M13 filamentous phage (from now on referred to as SIAH1-SP and SIAH2-SP). On the AP1, ubiquitin-activating enzyme E1 and ubiquitin-conjugating enzyme E2 were cloned together with ubiquitin fused to the N-terminal subunit of the T7 RNA polymerase (N-term RNAP). Additionally, the gene coding for pIII (*gIII*) was placed under the control of a T7 promoter. On AP2, the substrate of SIAH1/2 was fused to the C-terminal RNAP (C-term RNAP). This modular design, with the substrate-RNAP fusion on its own plasmid, facilitates cloning and allows for easy substrate switching using competent cells that are already transformed with AP1 and MP6, thereby enabling efficient substrate walking to modulate the system's stringency. In addition to this positive selection strategy, a negative selection system was designed to punish promiscuous E3 ligase activity. This system could be used later in parallel to select against variants with off-target activity (Supplementary Figure 3). With these genetic components in place prior to phage infection, the ubiquitin-N-term RNAP fusion protein should be loaded onto E2 by E1. Upon infection by SP-encoding phages, the expression of SIAH1/2 would facilitate the recruitment of the loaded E2. If the substrate is recognised by SIAH1/2, this interaction would result in the conjugation of ubiquitin to the substrate fused to C-term RNAP.

This effectively brings the two T7 RNAP subunits together, thereby inducing the expression of *gIII* from the T7 promoter (Supplementary Figure 3). Expression of *gIII* would then allow the further propagation of phages. Active variants able to generate sufficient pIII levels would be able to propagate, and phages with high activity would be preferentially selected (Figure 2).

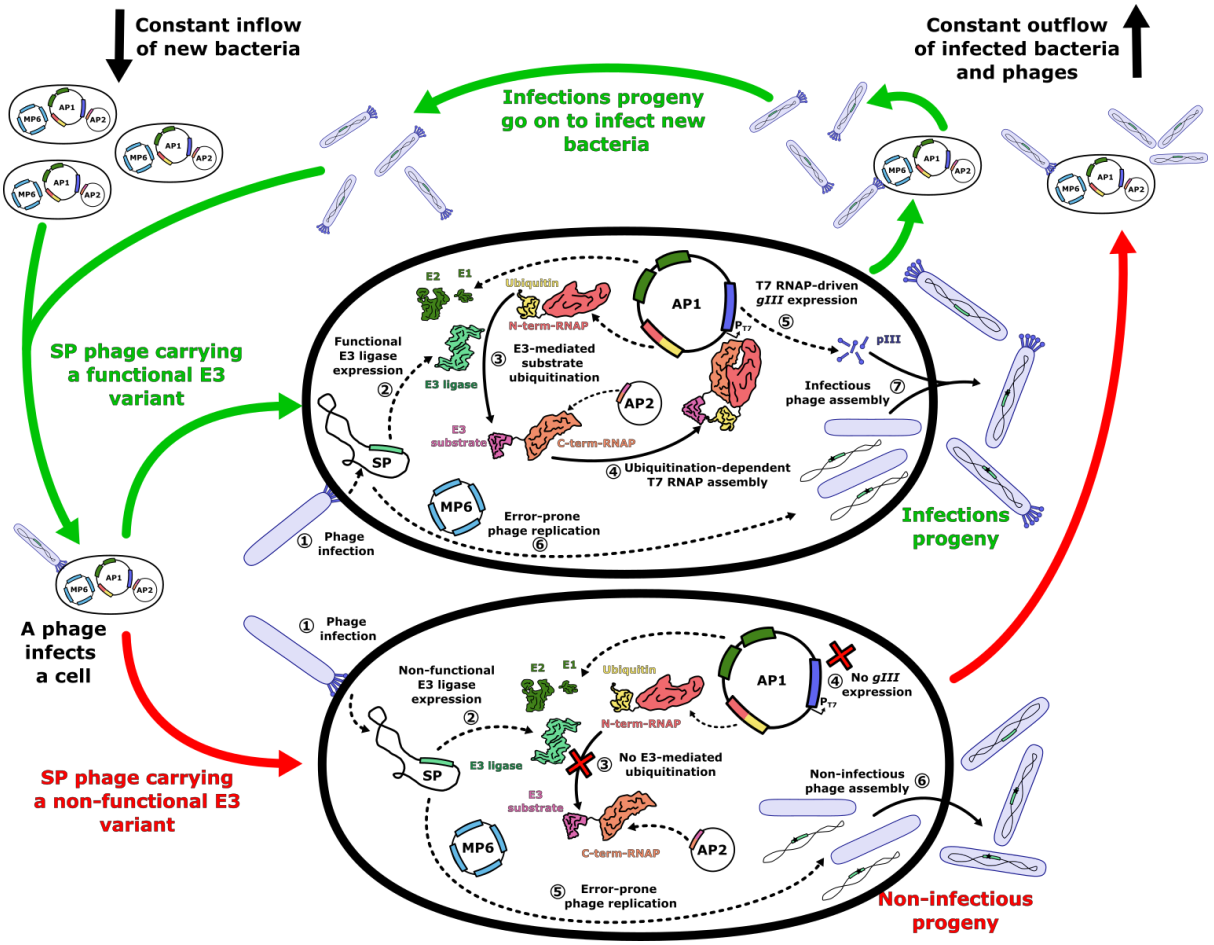

Figure 2: PACE logic. The PACE system operates within a lagoon with a constant inflow of new host cells and an outflow of phages and infected host cells. Upon infection of a host cell with a selection phage carrying a functional E3 ligase variant (green arrows), the E3 ligase ubiquitinates its target, which is fused to the C-terminal subunit of a split T7 RNA polymerase (RNAP), using ubiquitin fused to the N-terminal subunit of the split RNAP. The ubiquitin-mediated proximity of the split RNAP subunits assembles a functional T7 RNAP, which transcribes the *gIII* gene required for the assembly of infectious progeny. In the case of a non-functional E3 variant (red arrows), the lack of assembled T7 RNAP prevents *gIII* expression, resulting in non-infectious phage progeny. During phage genome replication, the mutation plasmid MP6 increases the mutation rate, generating new E3 variants in the process. Infectious progeny carrying new E3 variants then go on to infect fresh host cells, repeating the cycle.

Phage propagation is independent of the SP

To validate E3-dependent *gIII* expression if a recognisable substrate is present, we fused EGLN3 to C-term RNAP and conducted an overnight phage propagation assay. Initially, host cells were infected with a low phage titer of 10^6 PFU/mL and incubated overnight. The following day, phage titers were quantified using qPCR. We observed a greater increase in phage titer for SIAH1-SP and SIAH2-SP compared to those carrying a nonspecific protein (UN-SP) (Figure 3A), indicating phage-specific *gIII* expression. To determine if our system was dependent on the substrate fused to the C-term RNAP, we replaced the original substrate (EGLN3) with α-Synuclein, another well-characterised target of SIAH1/2. Phage propagation assay of SIAH1-SP and SIAH2-SP was negatively affected by the change of substrate (Figure 3B). However, this was likely not ubiquitination-specific as UN-SP also showed a slight decrease in propagation. The size of the SP was shown to be irrelevant to phage propagation ability (Supplementary Figure 4).

In parallel, we decided to determine the importance of the degron that is recognised by SIAH1/2 within the substrate. We introduced single amino acid substitutions into the native degron sequence of EGLN3, assuming these changes would affect recognition by SIAH and consequently phage propagation (Supplementary Results 2). Strikingly, phage propagation was unaffected by the disruption of the VXP motif and appeared independent of the presence of phages encoding SIAH1/2 (Figure 3C).

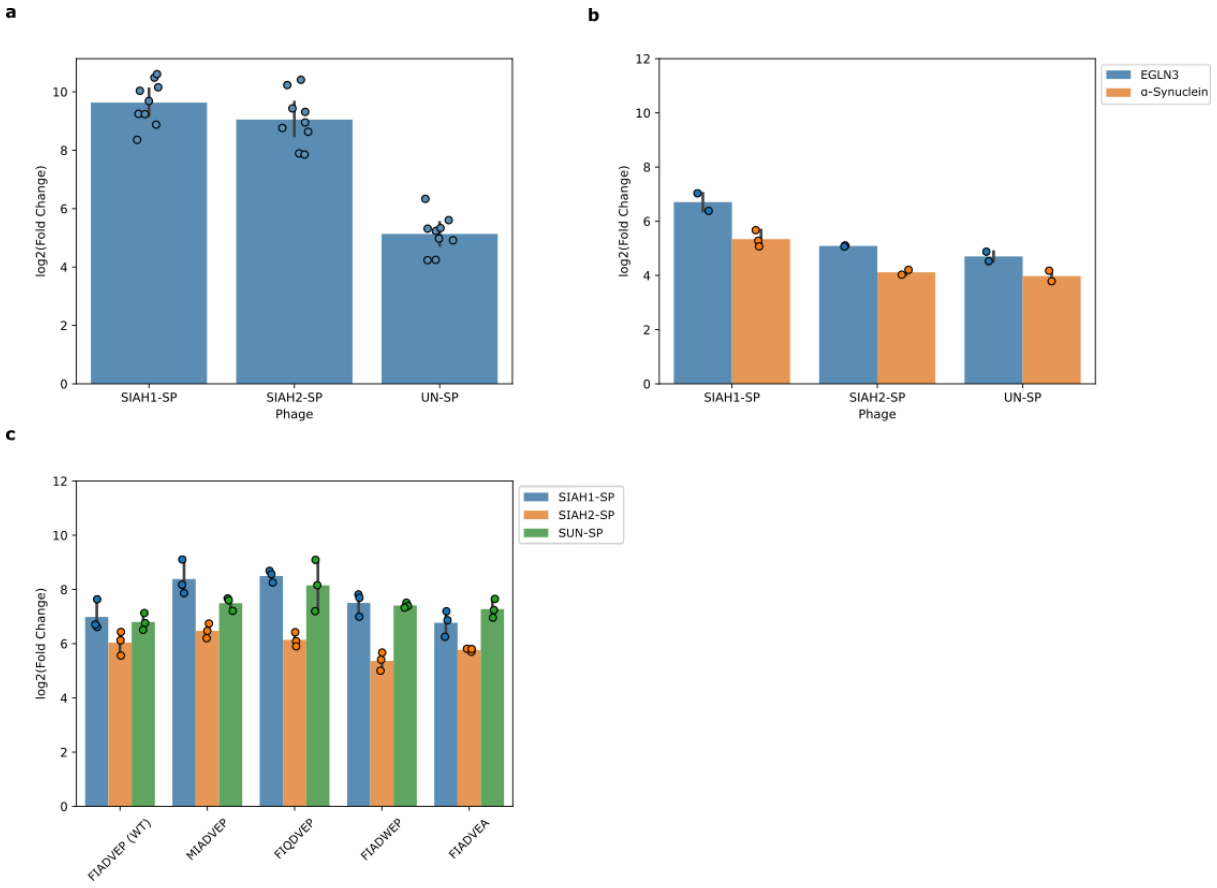

Figure 3: Phage propagation under different SP and substrate combinations. The log2-fold change in phage titers following an overnight incubation period on bacteria containing the respective components is shown. The fold changes were normalised relative to the initial phage titers. **(a)** SP-dependent phage propagation on cells expressing the native substrate EGLN3 (S2060 transformed with pES1076 and pES2008). **(b)** Substrate-dependent phage propagation using different SPs; EGLN3 (S2060 transformed with pES1033 and pES2009) compared to α-Synuclein (S2060 transformed with pES1035 and pES2009). **(c)** Phage propagation of different SPs in the presence of a set of the canonical EGLN3 degron compared to different point mutations (S2060 transformed with pES2008 and mutated versions of pES1076).

Validating hypotheses for background phage propagation

We hypothesised that ubiquitination-independent phage propagation could result from either leaky transcription from the T7 promoter or spontaneous assembly of the split RNAP subunits, both of which would enable *gIII* expression independently of E3 ligase activity. To test these hypotheses, we quantified phage propagation in cells expressing only a single RNAP subunit. In cells lacking either C-term RNAP or N-term RNAP, phage propagation was repressed, confirming that both RNAP subunits are essential for *gIII* expression (Figure 4). This finding suggests that any potential leaky transcription of *gIII* under T7 promoter control is insufficient to drive propagation; instead, the spontaneous assembly of the split-RNAP subunits likely explains the E3-independent phage propagation observed in our previous experiments.

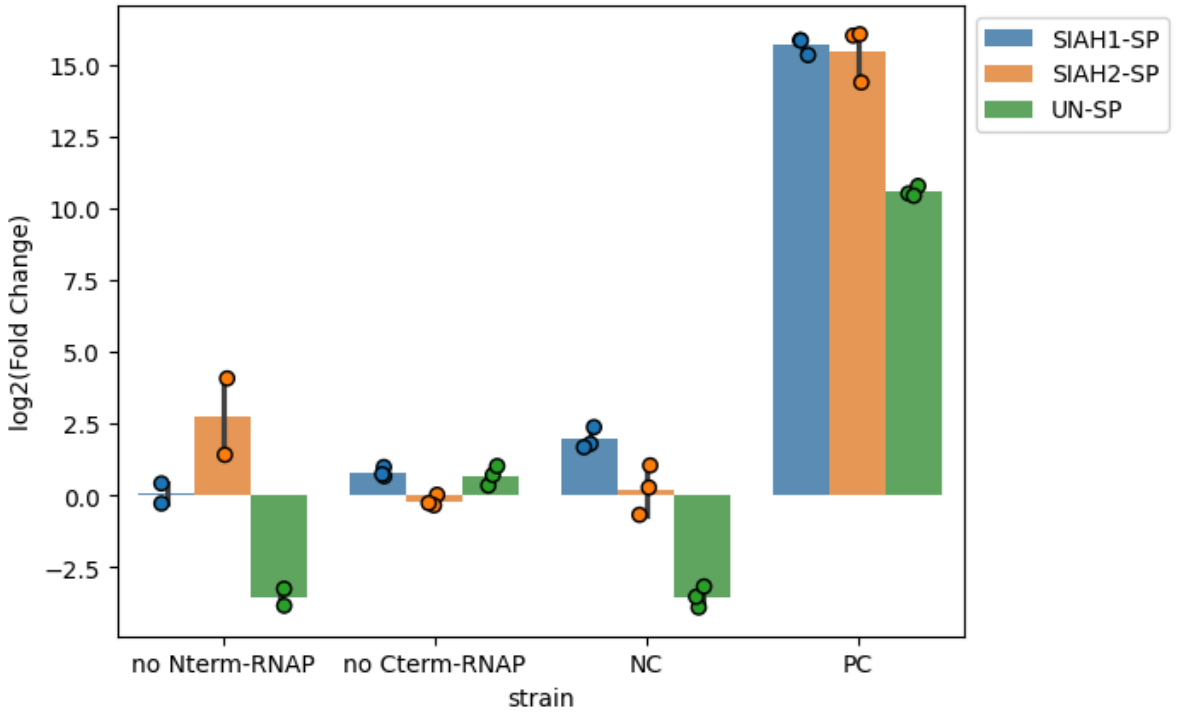

Figure 4: Phage propagation in the presence and absence of split-RNAP components. The log2-fold change in phage titers following an overnight incubation period on bacteria strains lacking either component of the split-RNAP is shown. The fold changes were normalised to the initial phage titers. no Nterm-RNAP: S2060 transformed with pES1076, pES2037, and DP6; no Cterm-RNAP: S2060 transformed with pES2008 and DP6 only; NC: negative control, S2060 only; PC: positive control, S2208 strain.

Since the non-covalent assembly of RNAP and thereby *gIII* expression in the absence of E3 can already occur way before phages enter the cell, we decided to induce the expression of the N or C-terminal RNAP fusion proteins just before the addition of phages. This was achieved by replacing the previously used constitutive promoter by either a vanillic acid promoter (pVan) [25] or a phage shock inducible promoter [26], potentially reducing the expression of *gIII* prior to phage addition. In case of pVan, the repressor coding gene (VanR) was also added to the respective plasmids to control gene expression. We hypothesised that the controlled expression of at least one RNAP subunit will circumvent the nonspecific assembly of the RNAP. This module can also be easily incorporated into the PACE system by controlling the time cells are exposed to vanillic acid before entering the phage lagoon.

SP Plasmid drift

Before initiating PACE evolution of SIAH1 using the AP1+AP2 system, selection stringency must be considered. A common challenge in PACE is the washout of phages due to excessive initial evolutionary stringency [8]. To mitigate this, selection stringency in PACE can be reduced by generating a library of phages carrying SIAH1/2 variants through random mutagenesis or “drift”. By using a drift plasmid (DP), DNA is mutagenized while providing *gIII* independently of selection, creating genetic diversity with minimal selection pressure [27]. Here, we used DP6, a plasmid previously described [28] to induce drift to SIAH1-SP via Phage-assisted non-continuous evolution (PANCE). Briefly, strain S2060-DP6 was infected with SIAH1-SP phages in media containing arabinose to induce the mutagenesis machinery and tetracycline to induce expression of *gIII*. Already after 4 passages, the fraction of mutated phages was shown to be higher than the control (Figure 5). This library of SP-bearing phages could then be used to start PACE with the proposed selection system.

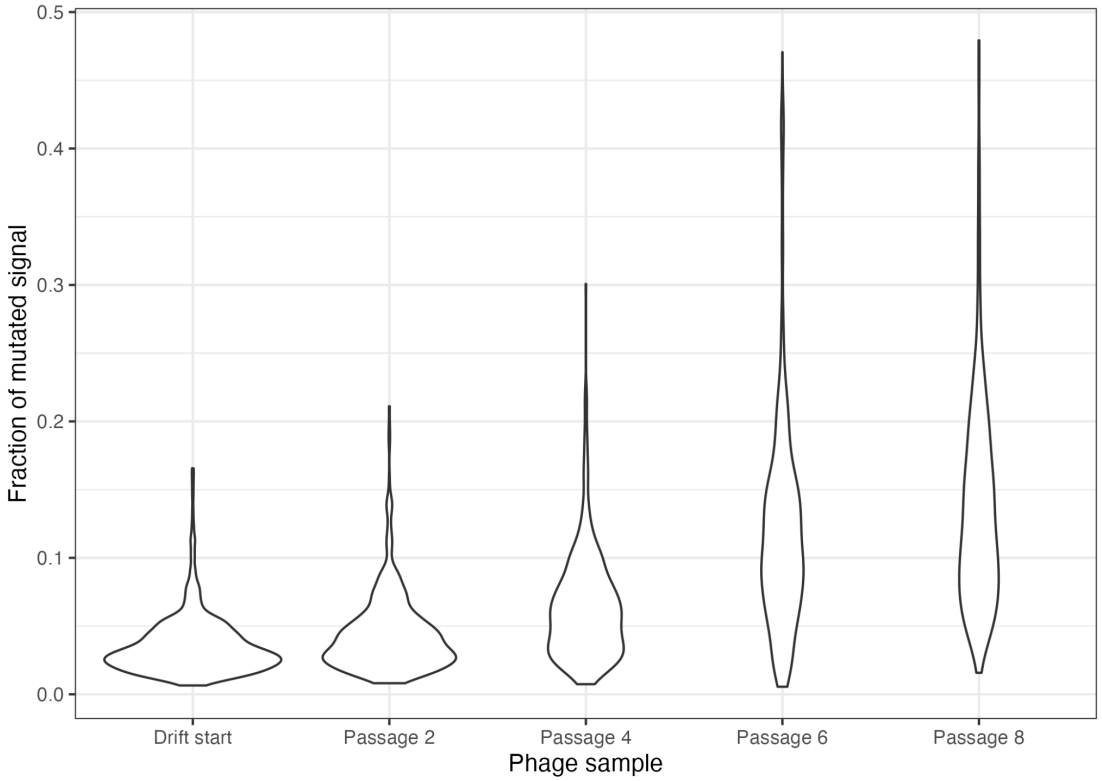

Figure 5: Signal coming from mutated bases in SIAH-SP subject to eight drift passages. For each position, the fraction of mutated signal is calculated as the fraction of chromatographic signal coming from uncalled bases (see methods and materials).

Discussion

Here we present the design and development of a PACE-based selection system aimed at evolving E3 ligases for TPD. While other therapeutic approaches utilising the cells UPS for TPD, such as PROTACs, are currently in development, we hope to offer an alternative to those strategies in the future with the use of evolved E3 ligases for TPD. Although this approach is still very much in its infancy, the establishment of a platform for E3 ligase evolution represents one major milestone on the path towards the development of TPD drugs that circumvent some of the previously outlined problems of PROTACs among others. To evolve E3s capable of recognising non-canonical substrates, we relied on the established PACE method which has previously been used to reprogram the specificity of other enzymes that recognise peptide or nucleotide motifs [8,10,29]. To link the phage propagation to the activity of E3, we designed a selection system based on two split T7-RNAP parts fused to E3 substrate and ubiquitin, respectively. Phages carrying an active E3 capable of recognizing the substrate would then link the two RNAP parts with the help of E1 and E2 to allow *gIII* transcription and thereby phage propagation. We were able to show that phage propagation in this system indeed relies on both RNAP parts being present. However, our findings indicate that the spontaneous assembly of RNAP in the absence of E3 remains a significant obstacle in the selection system, allowing phage propagation to occur partially independent of E3 activity. By reducing the expression levels of RNAP parts through the use of inducible promoters, we aimed to lower the background phage propagation rate before the addition of phages, with experiments currently being performed.

Once E3-dependent phage propagation above the background signal is established, a stepwise transition from the canonical E3 substrate to the recognition of the final target (“substrate walk”) will be implemented in our PACE-based selection system to evolve SIAH1/2 towards recognizing NLRP3 for ubiquitination. If a SIAH1/2 variant capable of targeting NLRP3 is identified, the effects of this monoubiquitination will be studied *in vivo* to confirm whether polyubiquitination and subsequent degradation via the UPS can be achieved. Crucially, potential off-target effects of the evolved SIAH1/2 will need to be monitored and actively selected against in additional PACE rounds if they become problematic. While optimization of the outlined selection system is ongoing, we are confident that this approach highlights the potential of evolved E3 ligases for therapeutic use as TPD drugs.

Materials & Methods

Materials

The enzymes used in the experiments were obtained from New England Biolabs. Protocols and enzyme-specific reaction conditions, if not mentioned otherwise, were followed as provided by the manufacturer. NucleoSpin Gel and PCR Clean-up kit (Macherey-Nagel) was used for the purification of PCR products and gel-extracted DNA fragments, following the manufacturer's instructions. QIAprep Spin Miniprep Kit (Qiagen) was used for plasmid isolation from bacterial cultures, as per the protocol provided by the manufacturer. Chemicals used for the preparation of buffers, solutions, and other reagents were purchased from Sigma-Aldrich. The EcoFlex MoClo Kit (Kit #1000000080, Addgene), and the Plasmid #74090 and Plasmid #74088, obtained from Addgene, were used for Golden Gate assembly of the modular constructs. All DNA fragments for the experiments were codon optimised for *E. coli,* synthesised and ordered from Twist Bioscience and are listed in Supplementary Table 3. Oligonucleotide primers were ordered from Microsynth AG and are listed in Supplementary Table 4. The bacterial strains used in this work are listed in Supplementary Table 5. Sanger sequencing and ONT sequencing were carried out by Microsynth AG.

Selection of canonical SIAH1/2 substrates

Canonical SIAH1/2 targets were used to validate the evolutionary logic. They were selected for their known ubiquitination, structural compatibility, and therapeutic potential while avoiding features that could interfere with the evolutionary logic at hand. The primary criterion for substrate selection was documented ubiquitination by SIAH1/2, with clear identification of ubiquitination sites and availability of sufficient structural information, either through experimental structural elucidation or high-confidence AlphaFold predictions. The databases UbiBrowser and UbiNet were used to identify SIAH1/2 canonical substrates [30,31]. Additionally, the substrates were required to exist as a cytoplasmic monomer, as dimerisation could disrupt the evolutionary system. The size of the substrate was restricted to a maximum of approximately 300 aa as an oversized substrate could impair the functionality of split-RNAP fusion proteins. Substrates with potential therapeutic applications were prioritised. This consideration was crucial if we were unable to perform a substrate walk. This would allow us to increase the substrate recognition efficiency of SIAH1/2 for the canonical therapeutic target with PACE.

Plasmid construction
Supplementary Table 2 lists all the plasmids used in this study and provides detailed information about their construction.

Golden Gate Cloning
DNA vectors were constructed by Golden Gate assembly using the EcoFlex MoClo kit (Addgene kit #1000000080) [32]. Level 0 and Level 1 assemblies were performed as described in the publication, Level 2 assemblies were performed with plasmid amounts mentioned in the publication, but the following cycling protocol was used: 1h at 42°C, (5 min at 42°C, 5 min at 18°C) x 99 cycles, 1 h at 42°C, 15 min at 80°C. We encountered significant challenges primarily with level 2 assemblies. To determine whether the issue was specific to the Golden Gate cloning method, we attempted alternative cloning strategies, including Gibson Assembly. However, this approach did not yield improved results. It is possible that the considerable size of the plasmid and the associated metabolic burden on the host cells negatively impacted the transformation efficiency of correctly assembled level 2 plasmids. In contrast, smaller vectors containing fewer inserts and reduced protein expression demands may exhibit higher transformation efficiency due to their reduced size and metabolic load on the cells.

Gibson Assembly
Gibson assembly was carried out using the *NEB HiFi DNA Assembly Mix* (#E2621S, NEB) in a scaled-down 5 µL reaction. Inserts and vector backbones were first amplified by PCR to introduce overlapping overhangs. After amplification, the products were treated with *DpnI* to remove template DNA, followed by gel extraction using the *NucleoSpin Gel and PCR Clean-up Kit* (740609.50, Macherey-Nagel). The assembly reaction contained 50-100 ng of vector in a 1:2 molar ratio of vector to insert. The vector and insert were mixed with 2.5 µL of *NEB HiFi Assembly Mix* and incubated at 50°C for 60 min.

Plasmid transformation into competent bacteria
For single plasmid transformation, DH5α cells (18265017, ThermoFisher Scientific) were made chemically competent using the Mix & Go *E. coli* Transformation Kit & Buffer Set (T3001 and T3002, Zymo Research), following the manufacturer's protocol. The transformation was performed by adding 1-5 µL of assembly reaction to 20-50 µL of competent cells, incubated on ice for 30 min, followed by heat shock (42°C, 30 s), recovery in LB at 37°C, 750 rpm, 60 min and then spread on LB agar plates containing appropriate antibiotic and incubated overnight at 37°C. For co-transformation of multiple plasmids, S2060 (Addgene #105064) or S2060+DP6 were made chemically competent using the Mix & Go *E. coli* Transformation Kit & Buffer Set, following the manufacturer's protocol. Transformation was performed by pre-mixing 200 ng of the two accessory plasmids in an equimolar ratio before adding 50 µL of competent cells. The cells were then incubated on ice for 30 min, heat shocked for 30 s at 42°C, and recovered in LB for 60 min at 37°C and 750 rpm.

Recovered cells were spread on LB agar plates containing appropriate antibiotics at 0.6x working concentration. If the cells contained the DP6 plasmid, the LB plates also contained 100 mM glucose. It was observed that the cells demonstrated a markedly reduced growth rate when transformed with all three necessary plasmids, likely due to the presence of numerous plasmids and the high expression of a multitude of proteins can impose a significant burden on the cells.

Phage production

The splitC (Addgene #138523) and splitD (Addgene #138521) plasmids were combined with a donor plasmid containing the desired phage insert together with an RBS flanked by AGT/GGC overhangs using SapI Golden Gate assembly: 1h 37°C, (5 min 37°C, 5 min 18°C) x 99 cycles, 1 h 37°C, 15 min 80°C. Following assembly, 2 µL of the ligation reaction was transformed into competent S2208 cells. Cells were incubated on ice for 30 min, heat-shocked at 42°C for 30 s, and then returned to ice for 2 min. The entire transformation was diluted in LB (without antibiotics) and grown to saturation at 37°C. A phage plaque assay was performed to determine phage titer, and a single plaque was picked for propagation. The resulting phage was filter-sterilised with a 0.22 µm filter and the sequence was confirmed by Sanger sequencing (Microsynth AG) using the primer oLS670.

Determination of phage titer by phage plaque assay

S2208 *E. coli* cells were cultured to an OD_{600} of 0.5. LB-agar plates without antibiotics were prepared as the bottom layer. A 2 mL mix of LB and hot LB-agar was combined with 1 mL of S2208 cells at OD_{600} of 0.5, vortexed, and poured over the bottom agar to form the top layer. After the top layer solidified, a 10-fold serial dilution of the phage was prepared, and 4 µL of each dilution was spotted onto the top agar. Plates were incubated overnight at 37°C, and plaques were counted the next day to determine the phage titer using the following formula: phage titer (pfu/mL) = (# of plaques)*(dilution factor)*100

Determination of phage titer by qPCR

To determine phage titer, the sample containing phages was centrifuged to pellet any debris (8,000 rpm, 2 min), and the supernatant was collected. The qPCR reaction mix was prepared as follows: 3 µL of FirePol polymerase (08-36-0000S, Solis BioDyne), 0.33 µM each of primers oLS-1662 and oLS-1663, and 2 µL of the supernatant sample. The qPCR was performed with LightCycler 480 II (Roche) with the following settings: pre-incubation at 95ºC for 12 min, followed by 45 cycles of denaturation at 95ºC for 15 s, annealing at 53ºC for 30 s, and elongation at 72ºC for 10 s. Excitation wavelength was set to 465 nm, and the detection wavelength was set to 510 nm. qPCR results with CT > 30 were considered to resemble the absence of phages.

Site-directed mutagenesis for substrate degron modification

To introduce the single substitution at degron position 5 (V > W), the native EGLN3 sequence was PCR-amplified from pES1076 using the primers o069: 5'-TGGGAACCGATCTTCGATCGCCT-3', and o066: 5'-ATCCGCAATAAAGCTTTTGCCTTCCGGA-3'. For P > A at position 7, primers o067: 5'-GCGATCTTCGATCGCCTGCTG-3', and o068: 5'-TTCCACATCCGCAATAAAGCTTTTGC-3' were used. For F > M at position 1, primers o060: 5'-ATGATTGCGGATGTGGAACCGATCT-3', and o061: 5'-GCTTTTGCCTTCCGGAAAAATGCG-3' were used. For A > Q at position 3, primers o062: 5'-CAGGATGTGGAACCGATCTTCGA-3', and o063: 5'-AATAAAGCTTTTGCCTTCCGGAAAAATGC-3' were used. The amplified products were 5'-phosphorylated, recircularized, and DpnI digested using the KLD Enzyme Mix (NEB#M0554S). The KLD-treated products were transformed into competent cells as previously described.

Phage propagation Assay

A saturated overnight culture was diluted and grown to an OD_{600} of 0.5. Phages were added to each condition to achieve a final titer of 10^6 PFU/mL. The plate was sealed with a breathable membrane and incubated overnight at 37°C. Phage titer was then determined via qPCR or phage plaque assay.

Phage-assisted non-continuous evolution (PANCE) for SP drifting

The PANCE method was conducted as described by [33]. For each passage of PANCE was performed by growing the bacterial strain containing the AP plasmids and either DP6 or MP6 was grown in Chloramphenicol and Tetracycline antibiotics at 1x and 2x working concentration, respectively, and 100mM glucose to an OD_{600} of 0.5. Arabinose was added to reach a final concentration of 40 mM to induce the expression of the mutagenesis machinery and *gIII* expression on DP6 was induced by adding 20 µg/mL. This culture was infected with 10^6 pfu/mL of phage and then incubated at 37°C, shaking at 250 rpm, overnight. The following day, the phage titer was determined using qPCR as described above. Phages were inoculated again to 10^6 pfu/mL to initiate the next round of evolution.

Analysis of SIAH1 drift using Sanger sequencing

Data were analysed and visualised using R Statistical Software v4.4.1 [34]. Sanger sequencing result files were processed using the sangerseqR package v1.40.0 [35]. For each nucleotide position, the fraction of mutated signal was calculated as the ratio of the maximum peak amplitude for each base to the total peak amplitudes within the respective base-calling window. To account for signal degradation commonly observed at the extremities of Sanger sequencing reads, only positions between 50 and 650 were considered.

Data analysis and availability

Raw data and code used to analyse it are available on GitHub: https://github.com/Student-Biolab-Zurich/idec2024-data-analysis.

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Contributions

N. A., M. S. and K. B. V. wrote the initial draft of this manuscript. N. A., G. C. A., M. B., P. J. A., S. M., P. C. L. N. and J. W. carried out experiments. N. A., G. C. A., S. M. and M. S. were involved in the project conceptualisation. M. S. designed the selection logic. N. A. and G. C. A. designed the cloning strategy. S. M. and K. B. V. wrote programs for data analysis. S. M., L. S., M. S. and K. B. V. were consulted for analysing lab results. J. B. and P. C. L. N. acquired the project funding. G. S. provided the lab space.

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