Development of a fluorescent three-hybrid system for the identification of protein-protein associators

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Abstract

The chemically-induced dimerization of proteins is fundamental to many key regulatory pathways. A number of natural products exert their downstream effect through the stabilization of a protein complex, either by direct binding to two distinct protein partners or via an allosteric mechanism. Here, we report a bacterial three-hybrid system, with dual life/death and fluorescent reporters, which detects protein association and is compatible with high-throughput screening. We use rapamycin mediated mTOR-FKBP12 (mammalian target of rapamycin—FK506 Binding Protein 12 kDa) dimerization to validate this platform; the addition of rapamycin results in the association of these target proteins, leading to the expression of two essential life/death reporter genes and a fluorescent signal. We further used this system to quantify the activity of rapamycin by utilizing the fluorescent readout, exemplifying its potential in screening and for ranking large *in vivo* libraries for compounds that upregulate the association of any two given proteins.

KEYWORDS

protein-protein interaction, rapamycin, two-hybrid system

1 | INTRODUCTION

Chemically-induced protein dimerization is involved in many key regulatory pathways throughout the biological world.[1] A number of natural products exert their downstream effect by the stabilization of naturally occurring interactions. This can occur via an allosteric effect, whereby an initial interaction between the dimerizer and the target protein results in the formation or uncovering of a new binding interface on the protein, enabling a protein-protein interaction (PPI) that is not otherwise observed. Alternatively, the dimerizer can act by the direct binding of two or more target proteins. This can either involve the binding of one target protein preferentially to create a new, high affinity protein-ligand binding surface for the second protein, or binding to a pre-existing interface between the two proteins can increase the lifetime or affinity of a given PPI.[2] The former is best

characterized by the rapamycin-mTOR interaction.[3] The latter is typically induced by large macrolide natural products; these include the 14-3-3 adapter proteins, whereby stabilization of these chaperone proteins with a range of targets has been demonstrated with natural products such as fusicoccin A and cotylenin A.[2,4]

Within the same family as rapamycin, other notable examples include the immunosuppressants FK506 and cyclosporin, both of which are now licensed for therapeutic use.[5–7] Rapamycin is one of the most widely studied examples of this family. By binding to a small hydrophobic pocket on FKBP12, a new FKBP12-rapamycin binding surface is generated that displays a strong affinity for mTOR (0.2 nM Kd).[8] Subsequent binding to mTOR results in the inhibition of wide reaching mTOR signaling pathways,[9] which acts to regulate critical pathways in cell growth and metabolism, as well as proliferation and survival.[10]

Whilst a variety of techniques have been developed and deployed for the identification of PPI inhibitors,[11] there are very few examples for the *de novo* discovery of compounds that upregulate the direct association of two proteins.[12] Increasingly, chemically-induced protein dimerization is being used for the investigation of complex signaling networks, with sequestering of target proteins providing spatiotemporal control of intracellular proteins.[13] This typically involves the fusion of protein domains that have prior characterized ligands to the proteins of interest.[14–16] Taking advantage of chemically-induced protein dimerization for therapeutic or research use first requires a known natural protein binder or dimerizer, However, not all desirable protein targets have an identified natural product ligand that can be used as a starting point for these studies. The stabilization of existing interactions, or the artificial association of two naturally unrelated proteins, represents both a significant research and therapeutic potential in the PPI field.

We have previously reported a platform for the identification of PPI inhibitors that combines a bacterial reverse two-hybrid system (RTHS), a well-established means of investigating PPIs,[17–19] with an *in vivo* library generation technique called split-intein circular ligation of peptides and proteins (SICLOPPS). This combined approach allows for the intracellular generation and screening of cyclic peptide libraries of up to a hundred million members for inhibitors of a given PPI.[20–24] Further developments of this approach include the construction of a bidirectional fluorescent two-hybrid system (FTHS).[25] Here, we report the construction and validation of a three-hybrid system (3HS) incorporating both a life/death selection and a fluorescent marker for combination with SICLOPPS libraries, allowing for the high-throughput identification of cyclic peptides that induce or upregulate the dimerization of two target proteins. Such a system could enable the identification of new starting points for small-molecule stabilizers or inducers of dimerization.

2 | MATERIALS AND METHODS

All molecular biology reagents were purchased from Fisher Scientific, New England Biolabs or Promega unless otherwise stated, and used in accordance with manufactures protocols. All *de novo* synthesized genes were purchased from Integrated DNA Technologies (IDT). LB agar vegitone was purchased from Sigma Aldrich. Minimal media plates were composed of 10x minimal media salts (K_2HPO_4 (603 mM), KH_2PO_4 (330 mM), $(NH_4)_2SO_4$ (76 mM), sodium citrate.$2H_2O$ (17 mM)), 50% (v/v) glycerol, $MgSO_4$ (1 M).

Fluorescence measurements were recorded on a CLARIOstar microplate reader (BMG LABTECH).

pZE21-GFPaav and repressilator (pZS1-ITIrLLtCL) plasmids were a gift from Michael Elowitz, California Institute of Technology (USA), (Addgene plasmids #26643 and #26489, respectively). pRSET-*tdTomato* was donated by Prof. Tsien from the University of California (USA).

Strain BW27786, CRIM plasmids pAH68, pAH63, pINT-ts, pAH69 and recombineering plasmids pCP20, pKD46, pKD3 were all sourced from the Coli Genetic Stock Centre (CGSC), Yale University (USA).

Please see Supporting Information for primer sequences and GenBank accession numbers for plasmids.

2.1 | Generation of the BW27786Δ*hisB* strain

Deletion of *hisB* was achieved following the λ Red recombineering protocol previous detailed.[26] pKD4 was used as a template for two rounds of PCR for the generation of a Kan^R-FRT fragment, using first the primers "pKD4 fw1" and "pKD4 rv1." The 1525 bp product was subsequently used as a template for a second round of PCR, with primers "pKD4 fw 2" and "pKD4 rv 2." The 1553 bp product was subsequently transformed into electrocompetent BW27786 cells containing the pKD46 helper plasmid. The cells were grown overnight at 37 °C on LB agar supplemented with kanamycin (25 μg/mL) and L-arabinose (1 mM). Colonies were analyzed by PCR with the primers "kt" and "HisBfw," with a product at 1117 bp confirming the deletion of *hisB* and insertion of the Kan^R-FRT fragment. This strain (BW27786Δ*hisB*::kan) was made chemically competent following standard protocols before transformation with the pCP20 plasmid containing the FLP recombinase. This was recovered in SOC at 30 °C before growth on LB agar supplemented with carbenicillin (100 μg/mL) overnight at 30 °C. A single colony was picked and grown overnight in LB broth at 30 °C before streaking on LB agar with no antibiotics. This was incubated overnight at 42 °C, before analysis of colonies by PCR with the primers "HisB fw" and "HisH rv," with a product at 192 bp confirming the excision of the kanamycin gene. The final chromosomal sequence can be found in the Supporting Information.

2.2 | Drop-spotting assays

All drop-spotting assays were performed from an overnight growth of the strain in LB broth at 37 °C, 200 rpm, before serial dilution in 10% glycerol/H_2O and applied to the plate as 2.5 μL drops. LB agar or minimal media agar was prepared following manufacturers instruction before supplementation with the relevant antibiotics for strain maintenance and the stated final concentrations of spectinomycin/IPTG/3-AT. The rapamycin solutions were prepared in 10% DMSO and added to the 10% glycerol solution prior to culture dilution, not included in the agar mixture. All plates were incubated at 37 °C for 1 night (LB agar) or 3 nights (minimal media).

2.3 | Generation of the FKBP12/FBD SNS126 strain

FKBP12 and mTOR *FBD* genes were synthesized by IDT, codon optimized for *E. coli*. *FKBP12* and the *FBD* were cloned into *XhoI* and *KpnI*, and *SalI* and *SacI*, restriction sites respectively in the plasmid pTHCP14 as previously reported.[23] This was transferred to the integration vector pAH68 and integrated on to the HK022 site on the SNS126 chromosome,[27] utilizing the CRIM plasmid system following the published protocol.[28] The final strain was maintained with carbenicillin (50 μg/mL) and spectinomycin (25 μg/mL). The final plasmid sequence can be found in the Supporting Information.

2.4 | Generation of the Three-Hybrid-System (3HS)

The PLtet0-1 promoter/operator sequence (TRE) was obtained from the plasmid pZE21-GFPaav,[29] amplified by PCR and ligated into the pTHCP14 plasmid backbone by standard molecular biology techniques using restriction sites SacI and NdeI. tdTomato was obtained from prSET-tdTomato and ligated downstream of the TRE with KpnI and SacI restriction sites. HIS3 and aadA genes were amplified from the SNS126 chromosome by PCR and sequentially inserted downstream of tdTomato, utilizing SalI and MfeI, and MfeI and EcoRI restriction sites respectively. The tetR gene was amplified by PCR from the plasmid pZS1-ITIrLLtCL[29] and ligated downstream of the P22/434 repressor sites, before addition of a downstream transcriptional terminator sequence. The two operons from this pTHCP14-3HS plasmid were transferred to the integration plasmid pAH63 via BamHI and SpeI restriction sites, and the pAH63-AF_3HS plasmid was integrated onto the λ site of the BW27786ΔhisB strain following the published protocol.[28] The pAH68-FKBP12/FBD plasmid was subsequently integrated onto the HK022 site to generate the full three-hybrid system. The 3HS strain was maintained with carbenicillin (50 μg/mL) and kanamycin (10 μg/mL). The final plasmid sequence can be found in the Supporting Information.

2.5 | Liquid-based fluorescence assays

Cell strains were grown overnight in LB broth at 37 °C, 200 rpm with the relevant antibiotics for maintenance of the cell (3HS = carbenicillin (50 μg/mL), kanamycin (10 μg/mL)). This was used to inoculate (1%) M9 media, supplemented with the same maintenance antibiotics and the noted supplements (final concentrations = IPTG (25 μM), rapamycin (10 μM), ATc (100 ng/μL, spectinomycin (60 μg/mL), 3-AT (3 mM)). These were grown at 30 °C, 200 rpm for 24 hours. Two-hundred microliters of the culture was then transferred to a black half-area greiner plate in triplicate, and the emission spectra between 550 and 650 nm was recorded at a bandwidth of 2 nm, following excitation at 514 nm.

3 | RESULTS AND DISCUSSION

3.1 | Design of the three-hybrid system (3HS)

We based the design of our 3HS on a previously reported reverse two-hybrid system RTHS,[27] which utilizes bacteriophage P22 and 434 repressor proteins lacking their N-terminal dimerization domains. The native dimerization domain is replaced by the proteins of interest, whose interaction causes the formation of an active repressor complex that inhibits downstream expression of two essential reporter genes, leading to cell death on selective media (Figure S1). Whilst this system has previously been used for the validation of individual protein-protein interactions,[30–33] it cannot be used for the high-throughput selection of molecules that bring two proteins together (e.g., from a SICLOPPS library), as the positive phenotype will be cell death and so active molecules cannot be readily identified. We therefore set out to design a bacterial 3HS for use in high-throughput screening for protein-protein associators. The previous RTHS used two essential reporter genes, with both conferring a life/death response, and so the potential for false positives arising from selection pressure on the bacterial host (leading to mutations) is relatively high. We therefore added a fluorescent gene as a third reporter to the redesigned construct to allow for the selection of life/death and/or fluorescence. The direction of the RTHS was "reversed" by inverting the link between the P22/434 repressors and the reporter gene construct; the gene encoding the tetracycline repressor protein (TetR) was inserted downstream of the P22/434 sensitive promoter, along with the insertion of a tetracycline promoter/operator sequence upstream of the reporter construct. In this system, the TetR sensitive promoter (P_L-tet-01)[34] is used to control expression of the three reporter genes. Thus, in the absence of target protein dimerization, the P22/434 operators are unoccupied, leading to TetR expression. This in turn binds to the tetracycline operator to repress expression of the three reporter genes (Figure 1A). Induced dimerization of the targeted proteins forms the P22/434 repressor, which inhibits expression of the TetR. In the absence of TetR, the tetracycline operator is unoccupied, allowing expression of the three reporter genes and subsequent growth/fluorescence of the host cell (Figure 1B). The reporter genes comprise of two life/death selectable markers; HIS3 for histidine selection in an auxotrophic strain, and the spectinomycin resistance gene (aadA). tdTomato[35] was included as the third reporter gene due to its bright fluorescence, enabling high-throughput selection based on fluorescence (e.g., by FACS). These three genes were incorporated downstream of the PL-tet-01, with a ribosomal binding site between each to enable the transcription of all three genes as a single operon, all controlled by the activity of the TetR. To ensure rapid switching between the on and off states, we added an ssRA degradation tag (AANDENYALAA) to the C-terminus of the TetR protein, which directs TetR to the ClpX protein degradation machinery and would reduce the half-life of TetR to approximately 5 minutes.[29]

3.2 | Verifying the function of FKBP12/FBD RTHS

The FKBP-rapamycin-mTOR interaction was chosen as a proof-of-concept for our 3HS. Previous studies have identified the FBD (FKBP12-binding domain) of mTOR, comprising of amino acids (aa's) 2025-2148, as a minimum domain necessary for rapamycin mediated association.[36] This 11 kDa domain was used alongside the full length FKBP12 (12 kDa), with both genes codon optimized for E. coli expression. These were fused to the C-terminals of the P22 and 434 repressor DNA-binding domains (to generate P22-FKBP12 and 434-FBD respectively), and were placed downstream of an IPTG sensitive promoter, separated with a ribosomal binding site to enable co-expression. To confirm the suitability of this interaction for our new 3HS system, this construct was initially validated with the previously

reported RTHS strain containing a reporter complex that will bring about cell death upon successful target protein association (Figure 2A).[27] Following integration of the fusion proteins at the HK022 site via the CRIM plasmid system (Figure S2),[28] the tolerance of *E. coli* to both the expression of the fusion protein constructs and varied levels of rapamycin was confirmed by a drop-spotting assay, with 10 μM rapamycin found to be non-toxic to the cells (Figure S3). Following this, the rapamycin mediated association of the two proteins was investigated by drop spotting of 10-fold serial dilutions of the FKBP12/FBD RTHS on selective media with and without IPTG (10 μM) and rapamycin (10 μM). A previously reported RTHS monitoring the homodimerization of C-terminal binding proteins (CtBP) was used as a negative control.[37] Neither IPTG nor rapamycin alone had any effect upon the growth of the FKBP12/FBD RTHS. However, in the presence of both IPTG (10 μM) and rapamycin (10 μM), growth of the FKBP12/FBD RTHS was repressed by ∼100-fold, decreasing to a similar level as to the CtBP control system (Figure 2B). These results confirmed that the rapamycin-induced dimerization of P22-FKBP12

and 434-FBD functions correctly in *E. coli* and can be used in our proposed 3HS.

3.3 | Construction and verification of the 3HS reporter complex

The three-reporter gene construct (Figure 1) was assembled sequentially, with *HIS3*, *aadA* (Spec[R]), and *tdTomato* placed downstream of the TetR sensitive promoter. This construct, in the absence of the TetR, results in the constitutive expression of the reporter genes. This served as a useful control for investigating the maximal activity of the reporter construct, representing the event of a strong protein-protein association in the full system.

Next, the *tetR* gene was placed downstream of a P22/434 sensitive promoter. This was then transferred to the reporter construct plasmid, with the two sides of the 3HS separated by a transcriptional terminator (Figure S4). This plasmid, and the intermediate lacking the
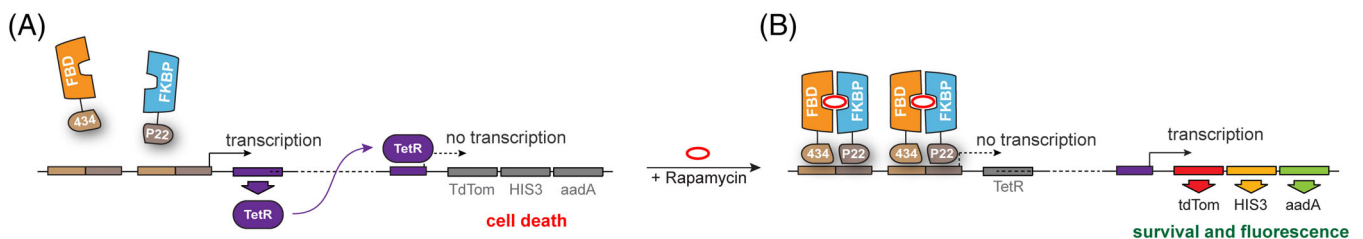

FIGURE 1 Design of the 3HS. A, The two target proteins (FKBP and FBD) are expressed as fusions with the 434 and P22 DNA-binding domains. In the absence of a dimerization-inducer, the 434 and P22 operators are unoccupied, allowing expression of the TetR protein. This in turn binds to the TetR sensitive promoter to prevent expression of the three reporter genes, leading to cell death. B, In the presence of a dimerization inducer (rapamycin) the 434/P22 repressor complex is reconstituted and binds to its operators, preventing the expression of TetR. The absence of TetR binding allows expression of the three reporter genes, leading to cell survival and fluorescence
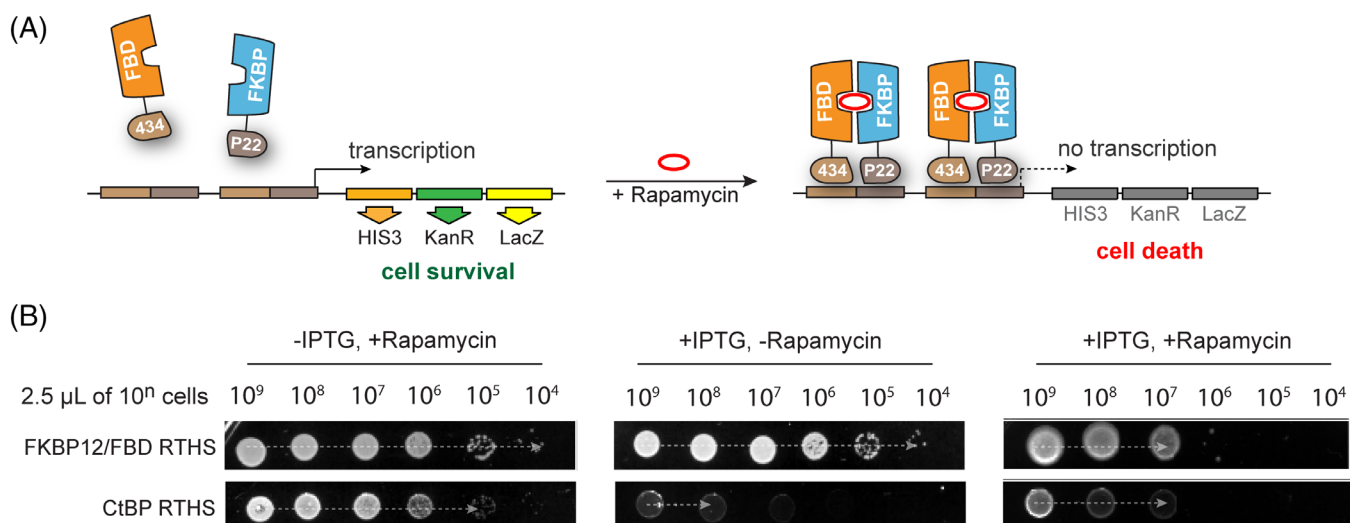

FIGURE 2 Characterizing the rapamycin-induced FKBP12/FBD PPI in a bacterial RTHS. A, The rapamycin-induced association of FKBP12 and FBD in the RTHS inhibits expression of the three reporter genes, leading to cell death in selective media. B, Drop-spotting shows rapamycin-induced cell death in the presence of IPTG for the FKBP12/FBD RTHS, whilst the CtBP (control) RTHS shows cell death in the presence of IPTG only, with minimal effect from rapamycin

tetR, were independently integrated into the λ site on *E. coli* (Figure S5).[28]

To enable selection with the yeast HIS3 protein, an auxotroph strain lacking the native *E. coli hisB* enzyme was required. This *hisB* deletion mutant of the *E. coli* BW27786 strain was generated utilizing the λ Red recombinase system.[26] Briefly, a kanamycin resistance gene (Kan[R]) was inserted in place of the second half of the *E. coli hisB* gene (aa's 132-236), corresponding to the activity of imidazoleglycerol-phosphate dehydratase. After positive selection, this was subsequently removed via 5′ and 3′ FRT sites using flippase recombinase, yielding a strain deficient in the ability to biosynthesize histidine. The newly generated $\Delta hisB$ strain of BW27786 confirmed a dependency on the analogous yeast derived HIS3

reporter gene for efficient growth in histidine depleted media. Drop-spotting of serial dilutions was used, with growth on minimal media agar supplemented with 3-Amino-1,2,4-triazole (3-AT), a competitive inhibitor of the dehydratase (Figure 3A). The $\Delta hisB$ strain displayed full repression of growth upon addition of 3-AT (top row, 2.5 mM), with the original *hisB* containing variant showing minimal repression (bottom row). As a further control, the growth of the 3HS was assessed in the presence of anhydrotetracycline (ATc), a selective and non-toxic TetR inhibitor. As expected, inhibition of TetR by ATc restored the growth of the 3HS in the $\Delta hisB$ strain. These results confirmed not only the successful deletion of the *hisB* gene, but also the function of the *HIS3* reporter gene and the TetR mediated inhibition.
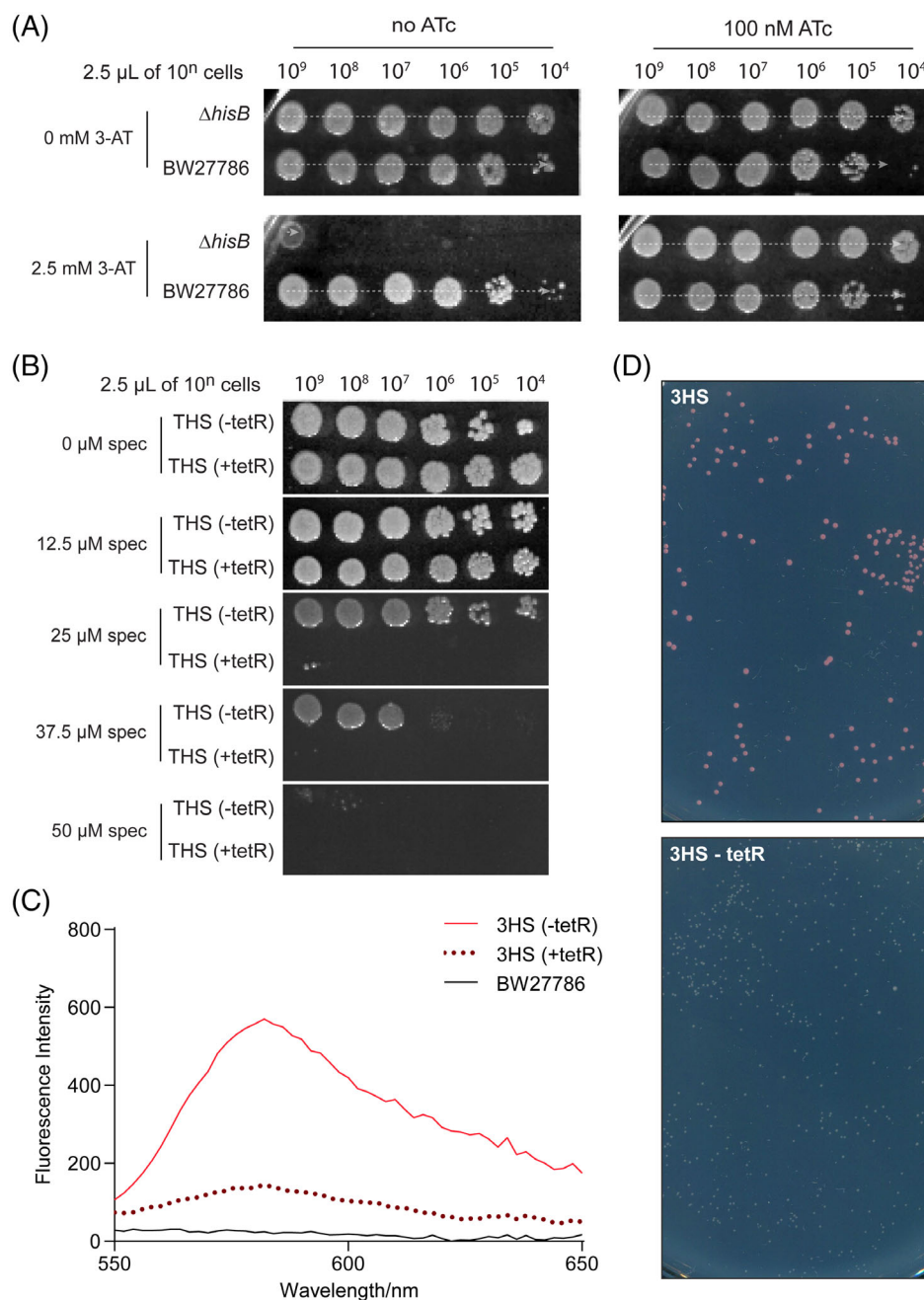

FIGURE 3 Verifying function of the 3HS. A, Drop-spotting shows limited cell growth of the $\Delta hisB$ strain when in the presence of the inhibitor 3-AT in comparison to the BW27786 parent strain, alleviated by the repression of TetR caused by the addition of ATc (100 nM). B, Drop-spotting over a range of spectinomycin conditions highlights the TetR dependency on antibiotic resistance, with inhibition of growth observed in the presence of the TetR at 25 μM. C, Fluorescence spectra of the 3HS +/− TetR following excitation at 512 nm, showing the TetR dependent inhibition of *tdTomato*, with minimal background fluorescence in the presence of the repressor. D, Images of minimal media plates supplemented with 2.5 mM 3-AT, with growth of the 3HS both with (top) and without (bottom) the TetR. Increased growth and fluorescence was observed in the absence of TetR only

The activity of the spectinomycin resistance gene was also analyzed via a drop-spotting assay (Figure 3B). The full (+*tetR*) and -*tetR* control strains were grown in the presence of increasing concentrations of spectinomycin on LB agar, with a clear contrast in growth between the two strains observed at 25 μg/mL, confirming both the activity of the *aadA* (SpecR) gene and again the ability of the TetR to control this expression. Finally, the function of the third reporter gene, *tdTomato*, was investigated. The FKBP12/FBD 3HS (+/−*tetR*) was grown for 72 hours in a liquid minimal media, before the fluorescence was measured following excitation at 554 nm (Figure 3C). A control of the un-integrated BW27786 parent cell line was measured to ensure no autofluorescence. The full 3HS was also plated onto selection media containing 2.5 mM 3-AT, alongside a control 3HS strain lacking the TetR. The 3HS (−*tetR*) colonies were red and grew as expected, whereas the colonies of the full strain were not colored and were visibly smaller due to the lack of tdTomato and HIS3 gene product respectively (Figure 3D). The visible difference between the 3HS and control suggests that the 3HS can be used to monitor the compound-induced dimerization of two proteins.

3.4 | The effect of rapamycin on the 3HS

Following the construction of the system and the validation of the TetR controlled reporter construct, the P22-FKBP12 and 434-FBD genes, under the control of an IPTG dependent expression system, were subsequently integrated onto the HK022 site on the full 3HS (-Figure S6). In this strain, the addition of IPTG leads to the expression of the P22-FKBP12 and 434-FBD fusion proteins. The dimerization of these is dependent upon the presence of rapamycin, with association resulting in the inhibition of TetR expression and ultimately upregulating reporter gene expression.

We probed the ability of the constructed 3HS to detect a molecule-induced protein association event. The concentrations of 3-AT and spectinomycin were initially optimized. The FKBP12/FBD 3HS was drop-spotted with 3-AT (1, 2, 3, or 4 mM) (Figure S7) and spectinomycin (0, 10, 20, 30, 40, 50, 60, 70, or 80 μg/mL) (Figure S8), with all conditions +/− rapamycin (10 μM). The optimal conditions were identified as 3 mM 3-AT and 60 μg/L of spectinomycin, whereby the contrast between the on and off states (+/− rapamycin) was at its
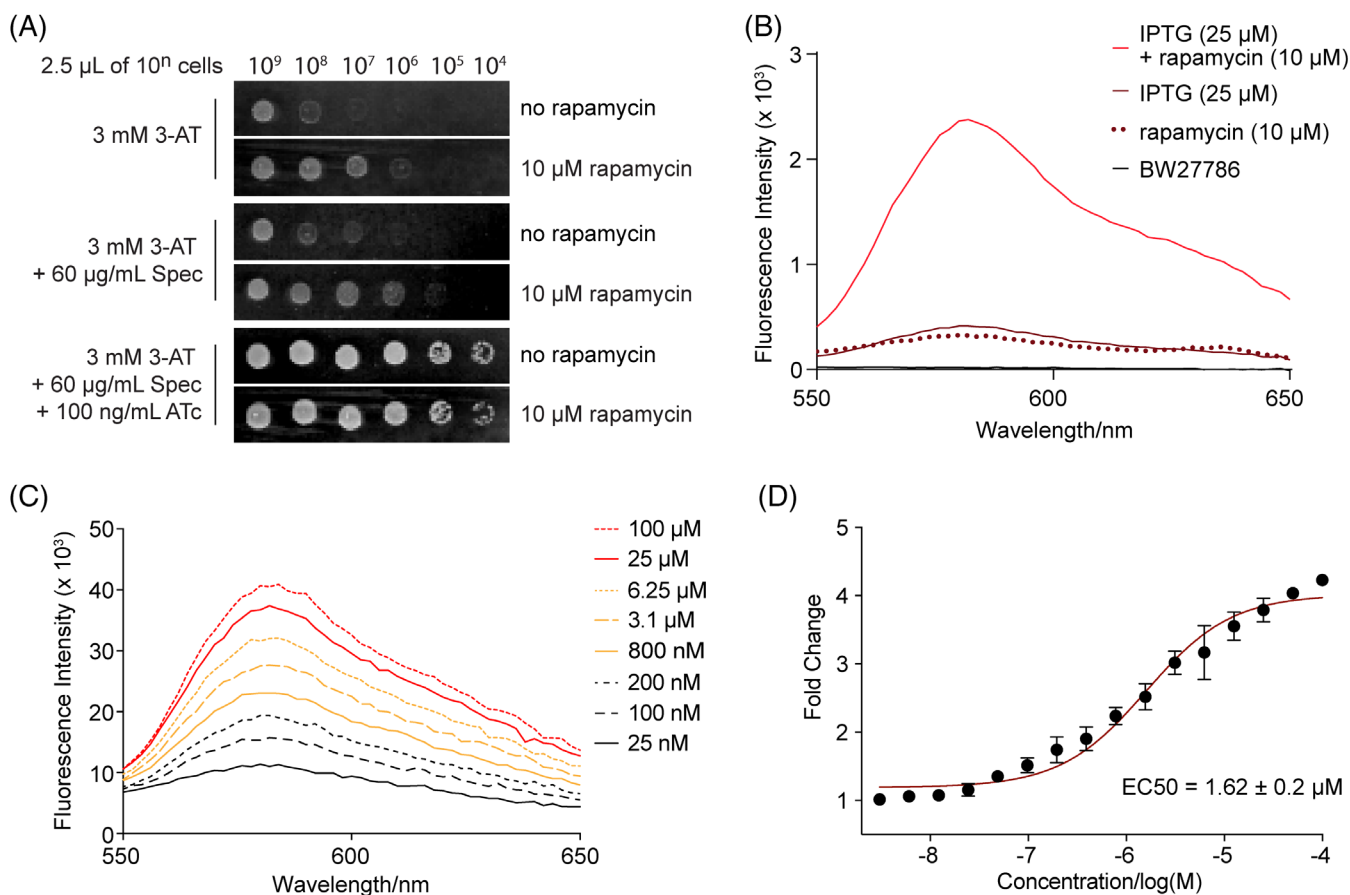

FIGURE 4 The effect of rapamycin on the 3HS. A, Drop-spotting shows the increased growth with rapamycin (10 μM) in the presence of 3 mM 3-AT alone (top) and 3 mM 3-AT +60 μg/mL spectinomycin (middle), with all growth inhibition alleviated by addition of ATc (100 ng/μL). B, Fluorescence spectra following excitation at 512 nm of the 3HS with rapamycin alone (10 μM), IPTG alone (25 μM), and both combined, showing the rapamycin dependent expression of tdTomato. C, Fluorescent spectra of the 3HS after excitation at 514 nm, grown with a range of rapamycin concentrations. All cultures contain IPTG (25 μM). D, Plotting of emission maxima at 582 nm, panel C, as a function of rapamycin concentration. Error bars represent SD, n = 3

maximum; the inclusion of rapamycin in the culture medium resulted in an approximate >100-fold increase in cell growth (Figure 4A). The growth difference afforded by 3-AT alone was improved over 10-fold with the addition of spectinomycin, demonstrating the benefits of the dual-marker selection system (Figure 4A). ATc (100 ng/μL) was again used as a control to ensure that the growth inhibition and subsequent alleviation with rapamycin was a direct result of TetR mediated inhibition and not an off-target effect of rapamycin. As expected, there was no effect from rapamycin on the 3HS in the presence of ATc (Figure 4A).

To probe the function of tdTomato, liquid minimal media supplemented with the previous selection conditions (3 mM 3-AT, 60 μg/mL spectinomycin) was used to grow the 3HS. When both IPTG (25 μM) and rapamycin (10 μM) were added to the culture medium, a > 5-fold increase in fluorescence (c.f. IPTG-only control) was observed at 582 nm upon excitation of the cells at 514 nm (Figure 4B). These results confirmed the expected function of the reporter genes in our 3HS, to identify a specific chemical inducer of protein-protein association, with a > 100-fold growth increase and > 5-fold fluorescence increase observed in the presence of rapamycin. This fluorescent reporter represents an alternative (or additional) means to characterize chemical inducers of protein-protein association.

We used the fluorescence reporter in our 3HS to monitor the binding of rapamycin to FKBP12 and FBD. Growth of the full 3HS in liquid minimal media with a range of rapamycin concentrations yielded a dose-dependent change in the fluorescent emission maxima at 582 nm (Figure 4C). Analysis of the fold change in fluorescence of the 3HS as a function of rapamycin concentration resulted in an EC50 of 1.6 ± 0.2 μM for the rapamycin induced association of FKBP12 and FBD in this system (Figure 4D).

4 | CONCLUSIONS

Following the validation of the system with successful growth and fluorescence assays, it would be possible to combine this 3HS with a SICLOPPS library[38] to identify cyclic peptide analogues of rapamycin. However, rapamycin binds within a small, well-defined binding site, with interactions with both partner proteins inducing the association. Initial binding to a hydrophobic pocket in FKBP12 generates a new rapamycin-FKBP12 interface, which is then able to bind a shallow hydrophobic pocket on mTOR.[39] The likelihood of mimicking this interaction with a cyclic hexapeptide is low, given that the two rapamycin interactions have K_d's of 0.2 nM and 0.4 nM, respectively. Thus, the approach would be better suited for bringing together proteins with pre-existing but weak interactions in the absence of a third component, with either the binding strength or lifetime enhanced by an external compound. Such examples include the 14-3-3 family of proteins, whereby stabilization of a range of partner proteins with the 14-3-3 chaperones has been demonstrated.[40] Targeting a ready-made binding site should ease the pressure on identifying both a high-affinity cyclic peptide ligand for two targets simultaneously, instead

enabling a SICLOPPS screen that looks for an increase in the binding between the two partner proteins. It should also be noted that the triple reporter system used in our 3HS allows for a combination of life/death and fluorescence screening (e.g., using a fluorescence-activated cell sorter).[25] The use of SICLOPPS with a number of alternative targets to identify initial scaffolds for inducers is currently being investigated.

CONFLICT OF INTEREST

The authors declare no competing interests.

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Additional supporting information may be found online in the Supporting Information section at the end of this article.

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