



High-throughput characterization of protein–protein interactions by reprogramming yeast mating

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Edited by Brenda Andrews, University of Toronto, Toronto, ON, Canada, and accepted by Editorial Board Member James J. Collins September 29, 2017 (received for review April 11, 2017)

High-throughput methods for screening protein–protein interactions enable the rapid characterization of engineered binding proteins and interaction networks. While existing approaches are powerful, none allow quantitative library-on-library characterization of protein interactions in a modifiable extracellular environment. Here, we show that sexual agglutination of *Saccharomyces cerevisiae* can be reprogrammed to link interaction strength with mating efficiency using synthetic agglutination (SynAg). Validation of SynAg with 89 previously characterized interactions shows a log-linear relationship between mating efficiency and protein binding strength for interactions with K_d s ranging from below 500 pM to above 300 μ M. Using induced chromosomal translocation to pair barcodes representing binding proteins, thousands of distinct interactions can be screened in a single pot. We demonstrate the ability to characterize protein interaction networks in a modifiable environment by introducing a soluble peptide that selectively disrupts a subset of interactions in a representative network by up to 800-fold. SynAg enables the high-throughput, quantitative characterization of protein–protein interaction networks in a fully defined extracellular environment at a library-on-library scale.

high-throughput screening | protein–protein interaction networks | protein engineering | synthetic biology | yeast mating

Many powerful methods have been developed for the high-throughput screening of protein–protein interactions. Yeast two-hybrid (1) assays can be used to intracellularly screen pairwise interactions and have been extended to the screening of large protein interaction networks using next-generation sequencing (2–4). However, intracellular assays are limited by a lack of control over the binding environment and suffer from frequent false-positive and false-negative results (5, 6). Phage (7) and yeast (8) display has enabled the high-throughput binding characterization of large protein libraries, but can only screen binding against a limited number of targets simultaneously due to the spectral resolution of existing fluorescent reporters (9). Both approaches also require the expression and purification of recombinant target proteins, which is limiting if the target is unstable or expresses poorly. Single molecular interaction sequencing (10) can characterize protein interaction networks in a cell-free environment, but requires purified proteins and a dedicated flow cell for the analysis of each network and condition. While each approach expands protein interaction screening capabilities, none allows for cell-based, quantitative, library-on-library interaction characterization in an extracellular environment that can be modified as desired.

Yeast mating in an aerated liquid culture depends critically on an intercellular protein–protein interaction that drives agglutination between MATa and MAT α haploid cells (11, 12). The MATa sexual agglutinin subunit, Aga2, and the MAT α cognate, Sag1, interact with a K_d of 2–5 nM and initiate the irreversible binding of two haploid cells and subsequent cellular fusion to create a single diploid cell (13). Cellular agglutination and mating are highly efficient, occurring in a matter of hours, and each mating event forms a stable and propagating diploid strain (14).

Here, we reprogram yeast mating by knocking out Sag1 and replacing wild-type agglutination with engineered or natural binding pairs that act as synthetic agglutination (SynAg) proteins. SynAg proteins are expressed on the cell surface as fusions to Aga2 using yeast surface display, which has been used to functionally display proteins with diverse structural properties, such as single-chain antibody fragments (15), green fluorescent protein (16), and the human epidermal growth factor receptor ectodomain (17). Thus, we expect that SynAg will be broadly compatible with soluble proteins of interest. We first show that interaction strength between one MATa SynAg protein and one MAT α SynAg protein can be quantitatively assessed by coculturing the two haploid strains and measuring their mating efficiency with a flow cytometry assay (Fig. 1A). We then extend the approach to one-pot, library-on-library characterization by barcoding SynAg gene cassettes, coculturing many MATa and MAT α strains, and using next-generation sequencing to count interaction frequencies for all possible MATa–MAT α SynAg protein interactions (Fig. 1B). Finally, we demonstrate that the binding environment can be systematically manipulated to probe the influence of external variables on protein interaction networks.

Significance

De novo design of protein binders often requires experimental screening to select functional variants from a design library. We have achieved high-throughput, quantitative characterization of protein–protein binding interactions without requiring purified recombinant proteins, by linking interaction strength with yeast mating. Using a next-generation sequencing output, we have characterized protein networks consisting of thousands of pairwise interactions in a single tube and have demonstrated the effect of changing the binding environment. This approach addresses an existing bottleneck in protein binder design by enabling the high-throughput and quantitative characterization of binding strength between designed protein libraries and multiple target proteins in a fully defined environment.

Author contributions: D.Y., D.B., and E.K. designed research; D.Y. and S.B. performed research; D.Y. contributed new reagents/analytic tools; D.Y. analyzed data; and D.Y., S.B., D.B., and E.K. wrote the paper.

Conflict of interest statement: The University of Washington has filed a patent application based on the findings in this article (US Appl 15/407,215). D.Y., D.B., and E.K. are listed as coinventors on the patent application.

This article is a PNAS Direct Submission. B.A. is a guest editor invited by the Editorial Board.

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Data deposition: The data reported in this paper have been deposited in the National Center for Biotechnology Information (NCBI) BioProject (accession no. PRJNA380247) and NCBI BioSample database (accession nos. SAMN06642476, SAMN06642477, SAMN06642478, SAMN06642479, SAMN06642480, SAMN06642481, SAMN06642482, SAMN06642483, SAMN06642484, and SAMN06642485). All code for sequencing analysis is fully available on GitHub (https://github.com/dyounger/yeast_synthetic_agglutination).

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This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1705867114/-DCSupplemental.

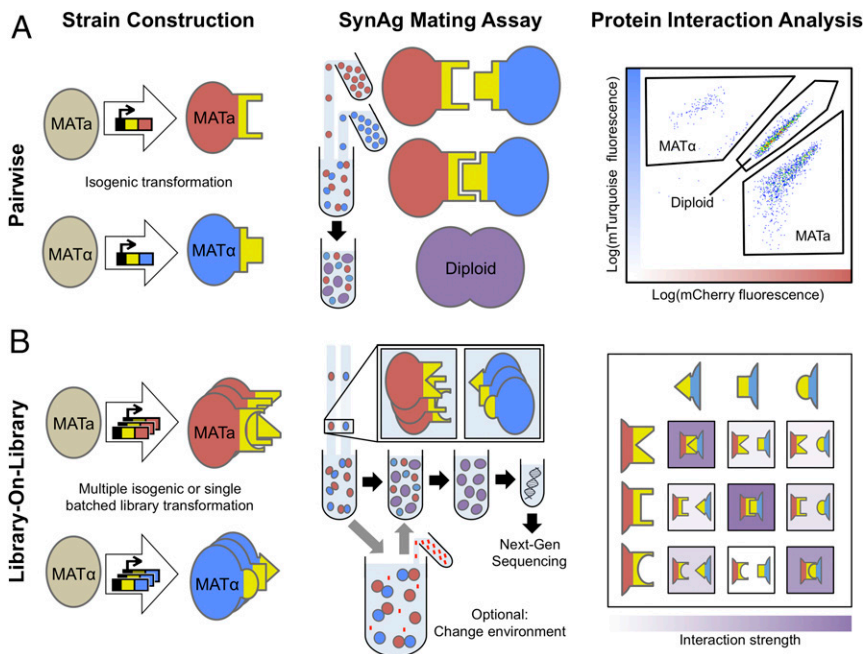


Fig. 1. Yeast SynAg workflow. (A) Pairwise characterization of a protein interaction involves isogenic yeast transformations followed by a SynAg mating assay and flow cytometry. (B) Library-on-library protein interaction network characterization involves library construction followed by a SynAg mating assay, diploid DNA isolation, and next-generation (Next-Gen) sequencing. Changing the binding environment during the SynAg mating assay is optional.

Results

Reprogramming Sexual Agglutination. To characterize protein-protein interactions using yeast mating, we first genetically replaced native sexual agglutination with SynAg interactions. To validate our approach, we used members of the well-characterized BCL2 family (18), which regulate apoptosis in human cells via binding interactions between pro-survival and pro-apoptotic family members, as SynAg proteins (Fig. S1). Six pro-survival BCL2 homologs were expressed on MATa cells. Binding peptides from seven pro-apoptotic BCL2 proteins were expressed on MATα cells. Previously, the pairwise interaction strength between the pro-survival homologs and pro-apoptotic peptides was semiquantitatively characterized using a competition binding assay that showed over 10,000-fold differences in interaction strength (19). Nine de novo binding proteins, also exhibiting a wide range in interaction strength for the pro-survival homologs, were expressed on MATα cells. Previously, the pairwise interaction strength between pro-survival homologs and engineered binders were quantitatively characterized using biolayer interferometry (20, 21).

Isogenic yeast strains were generated for the expression of each SynAg protein by transforming Sag1-deficient strains with a fragment containing a SynAg surface expression cassette and a mating type-specific fluorescent reporter. Pairs of SynAg-expressing haploid cells were cocultured in nonselective liquid media for 17 h to allow agglutination-dependent mating. Flow cytometry was performed to differentiate between mCherry-expressing MATa haploids, mTurquoise-expressing MATα haploids, and diploids that expressed both fluorescent markers (Fig. 1A). Diploid percent was used as a metric for mating efficiency to quantitatively characterize the interaction strength between a MATa SynAg protein and a MATα SynAg protein.

We found that complementary SynAg proteins expressed on the surface of yeast are necessary and sufficient to replace the function of Aga2 and Sag1. Wild-type *Saccharomyces cerevisiae* haploid cells mated with an efficiency of $63.6 \pm 3.1\%$ in standard laboratory conditions and a knockout of Sag1 in the MATα haploid eliminated mating with wild-type MATa (Fig. 2A). In the Sag1 knockout, expression of an interacting SynAg protein pair recovered mating efficiency to $51.6 \pm 7.9\%$, while expression of a noninteracting SynAg protein pair showed no observable recovery (Fig. 2B). SynAg-dependent recovery of mating occurred

with a variety of natural and engineered proteins and peptides ranging from 26 to 206 amino acids, indicating a large engineerable space for SynAg.

Pairwise Characterization of Protein Interactions. Mating efficiency and affinity, measured with biolayer interferometry, are related log-linearly ($R^2 = 0.878$) for protein interactions across over five orders of magnitude of K_d (Fig. 2C). We tested proteins with binding affinities ranging from below 500 pM to above 300 μM, which gave mating efficiencies up to 35.4% and down to below 0.2%. All three interactions with affinities worse than 100 μM gave mating efficiencies with no statistically significant difference from the Sag1 knockout negative control. The strong log-linear relationship between mating efficiency and affinity over multiple orders of magnitude contradicted our expectation of avidity as the main driving force for yeast agglutination (13). We expected that upon the formation of a single interaction between cells, newly localized protein pairs would rapidly bind, making off-rate largely irrelevant. However, both on-rate and off-rate showed a correlation with mating efficiency, and neither provided as good a fit as K_d (Fig. S2A and B).

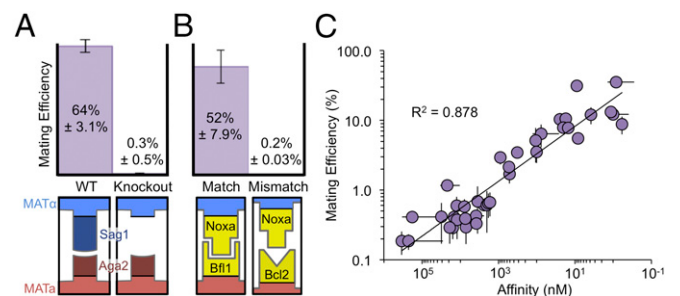


Fig. 2. SynAg for characterizing pairwise protein interactions. (A) Mating efficiency of wild-type *S. cerevisiae* haploid cells is eliminated with the knockout of the MATα sexual agglutinin protein Sag1. (B) Complementary binding proteins expressed on the surface of yeast haploid cells recover mating efficiency. (C) Mating efficiencies for SynAg protein pairs with a K_d from below 500 pM to above 300 μM show a log-linear relationship with affinity, measured with biolayer interferometry (SD, $n = 3$) (21).

SynAg proteins must be adequately expressed on the cell surface to accurately correlate mating efficiency and affinity. To test the effect of surface expression strength on mating efficiency, we constructed an inducible SynAg expression cassette with a synthetic transcription factor (22) that could be tuned to different levels by changing the inducer concentration (Fig. S2C). Surface expression strength was measured by labeling with FITC-conjugated anti-myc (8) and measuring fluorescence with flow cytometry. While mating efficiency was highly dependent on surface expression strength at low levels of expression, the effect saturated above 4,000 arbitrary units (au) (Fig. S2D). Of all SynAg proteins tested, one BCL2 homolog showed surface expression strength below 4,000 au and, subsequently, minimal recovery of mating efficiency regardless of its mating partner (Fig. S2E). An alternative semifunctional truncation improved surface expression and enabled affinity-dependent mating (23) (Fig. S2F).

Barcoding and Recombination of Interaction Libraries. A chromosomal barcoding and recombination scheme was developed for one-pot, library-on-library protein interaction network characterization, which uses a similar approach to previous work on plasmid-based recombination (3). We began by constructing MATa and MAT α parent strains, ySYNAGa and ySYNAG α , into which SynAg expression cassette libraries were transformed (Fig. S3). These strains contain complementary lysine and leucine auxotrophic markers for diploid selection and express CRE recombinase (24) after mating when induced with β -estradiol (25). For small libraries, SynAg cassettes were assembled with isothermal assembly (26) in one of two standardized vectors, pSYNAGa or pSYNAG α , for integration into yeast (Fig. S4 A and B). In addition to the surface expression cassette, each vector backbone contains a randomized SynAg protein-specific barcode and a mating type-specific lox recombination site and primer binding site. Sanger sequencing (27) was used to match barcodes with their corresponding SynAg proteins. The construction of SynAg libraries is comparable in time and cost to the construction of yeast two-hybrid or yeast surface display libraries, and identical methods can be used for DNA preparation and transformation.

Unidirectional CRE-induced chromosomal translocation (28) in diploid cells resulted in the combining of barcodes representing two interacting SynAg proteins onto the same chromosome (Fig. 3A). After recombination, interacting SynAg proteins were identified from a mixed culture using Illumina next-generation sequencing (29). To test the approach, haploid cells containing SynAg cassettes were mated, induced with β -estradiol, and lysed. The yeast lysate was used as a template for a PCR assay using mating type-specific primers. Amplification indicated that the primer binding sites had been combined onto one contiguous DNA strand, and hence that recombination had occurred. Sanger sequencing of the amplicon confirmed that recombination resulted in the expected chromosomal translocation.

Library-on-Library Characterization of Protein Interactions. The frequency with which pairs of barcodes corresponding to interacting SynAg proteins appear in diploid lysate following a library-on-library mating was observed to be log-linear with biolayer interferometry affinity measurements ($R^2 = 0.872$) (Fig. 3B). As before, we validated our approach with previously characterized protein interactions involving six pro-survival BCL2 homologs and nine de novo binding proteins. Here, all 15 SynAg strains were combined in a single mating. The batched mating percent for each interaction in the combinatorial matrix was calculated from next-generation sequencing counts, providing a relative strength for each protein interaction. We found that protein interactions spanning five orders of magnitude of K_d led to a more than 500-fold difference in batched mating percent. In addition to the de novo binding proteins, seven proapoptotic peptides with diverse binding profiles for the pro-survival homologs were added to a batched mating (30), and the observed interactions were consistent with previous work (19) (Fig. 3 C–F).

A comparison of the pairwise and library-on-library SynAg methods showed a near-perfect 1:1 agreement (Fig. S5). To compare the two approaches, pairwise mating efficiency was normalized so that the mating efficiency of all tested pairs summed to 100. A paired two-sided t test of normalized pairwise mating percent and batched mating percent gave a P value of 0.80, indicating no statistically significant difference between the two methods.

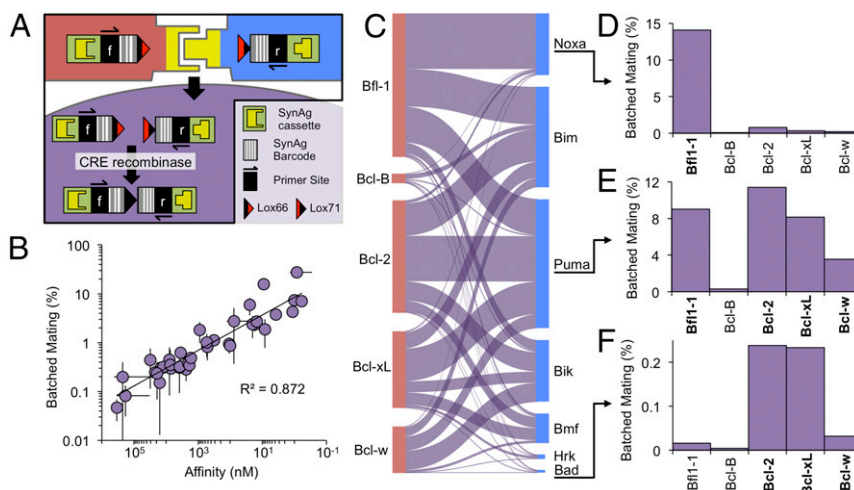


Fig. 3. SynAg for one-pot, library-on-library protein network characterization. (A) Each SynAg protein expression cassette is flanked by a barcode unique to a particular SynAg protein and a mating type-specific primer binding site and lox recombination site. CRE recombinase expression in diploid cells combines MATa and MAT α barcodes onto the same chromosome for next-generation sequencing. (B) One-pot, library-on-library mating assay gives a strong log-linear relationship between batched mating percent and affinity for protein interactions with a K_d from below 500 pM to above 300 μ M (SD, $n = 2$). (C) All interactions between five pro-survival BCL2 homologs and seven proapoptotic peptides (BH3 domains of Noxa, Bim, Puma, etc.) were characterized with a library-on-library SynAg assay. The height of each purple bar represents the frequency of diploid formation from a given pair of SynAg-expressing haploid strains. Interaction profiles for the BH3 domain of Noxa (Noxa.BH3; D), Puma.BH3 (E), and Bad.BH3 (F) are shown in greater detail, with bolded BCL2 homologs indicating an affinity below 1 μ M, according to a competition assay (19).

Large Protein–Protein Interaction Library Characterization. For the construction of large chromosomally integrated libraries, a “landing pad” approach (31) was used to achieve high-efficiency transformations from an integration requiring four-fragment homologous recombination (Fig. S4C). At the SynAg cassette integration locus, both ySYNAGa and ySYNAG α were transformed with an expression cassette consisting of *SceI* endonuclease driven by a galactose-inducible promoter and flanked by *SceI* cut sites. Galactose induction before transformation with a SynAg protein library resulted in DNA nicking at the site of integration, which dramatically improved transformation efficiency (32). Next-generation sequencing of genomic DNA extracted from transformed yeast libraries was used to pair each SynAg protein variant to its distinct barcode and to count relative barcode frequencies in the naive library before mating.

A single-pot batched mating was used to characterize 7,000 distinct protein–protein interactions. A partial site-saturation mutagenesis library of a de novo Bcl-xL binding protein, XCDP07 (21), consisting of 1,400 distinct variants was characterized for interactions with five prosurvival BCL2 homologs. For each variant, interaction strength (the number of times a particular variant was observed to have mated with Bcl-xL divided by the number of times that variant was observed in the naive library) and specificity (the percent of observed matings with Bcl-xL minus the percent of observed matings with the next highest homolog) were determined. As a proof of principal, interactions involving SynAg variants with premature stop codons were analyzed (Fig. 4A). Only eight of 55 premature stop codons included in the library resulted in even a single mating, and only six resulted in more than two matings. These six variants contained stop codons at residue 93 or later, which leaves the central binding helix intact. Two variants, with stop codons at residues 113 and 114, showed improved interaction strength and specificity for Bcl-xL. These early terminations resulted in the removal of the C-terminal myc tag from the 116-residue full-length protein, which may have negatively impacted binding. The same analysis was repeated with similar results for a site-saturation mutagenesis library of a Bcl-2 binder, 2CDP06 (Fig. S6A).

Favorable mutations from a yeast surface display library were correctly identified using library-on-library SynAg, but with additional information about relative binding affinities and specificities (Fig. 4B and C). In particular, two mutations at the interface periphery, L47R and A48T, were found to improve interaction strength with Bcl-xL. Both mutations were enriched by fluorescence-activated cell sorting of an XCDP07 site-saturation mutagenesis surface display library incubated with fluorescently labeled Bcl-xL and unlabeled competitor homologs (21). Unlike a traditional one-sided yeast surface display assay, SynAg provided detailed

information about binding affinities and specificities to each target (Fig. 4C). We observed moderately improved on-target specificity for L47R, mostly through relative weakening of the interactions with Bcl-w and Bcl-B. We observed that A48T more dramatically weakened all off-target interactions with a 16.5% increase of on-target binding. SynAg was also able to confirm two favorable mutations in a Bcl-2 binder, 2CDP06, that were previously identified with yeast surface display enrichment (21) (Fig. S6B).

Characterizing the Effect of Environmental Changes. To demonstrate the characterization of a protein interaction network in a new extracellular environment, we added a soluble competitive binder at the start of a batched mating, which selectively inhibited protein–protein interactions up to 800-fold (Fig. 5A and B). A proapoptotic BCL2 peptide, the BH3 domain of Bad (Bad.BH3), was found to interact selectively with five prosurvival BCL2 homologs (Fig. 3F). No interaction was observed with Bcl-B. Library-on-library matings with and without 100 nM Bad.BH3 were normalized to one another with the assumption that interactions involving Bcl-B were unaffected. Normalization accounted for differences in total sequencing reads between conditions.

The addition of 100 nM Bad.BH3 resulted in specific inhibition of interactions involving its expected binding partners: Bcl-2 and Bcl-xL. Interactions involving these two homologs were inhibited by at least 16-fold and up to 800-fold (Fig. 5B–D). No change was observed for all strong protein–protein interactions involving prosurvival homologs that weakly interact with Bad.BH3: Bfl-1, Bcl-B, and Bcl-w. Weak protein–protein interactions involving these three homologs showed reduced mating efficiency in the presence of Bad.BH3, which can be attributed to an increased concentration of bulk protein in the media that serves to block nonspecific interactions. Considered together, all protein–protein interactions involving Bcl-xL and Bcl-2 were strongly inhibited, with normalized mating percent fold changes of 209 and 162, respectively. The weaker Bad.BH3 binders, Bcl-w and Bfl-1, displayed a normalized mating percent fold change of 2.6 and 1.5, respectively. All aggregate fold changes were consistent with previous characterization of Bad.BH3 interactions with the five prosurvival homologs (Fig. 3F) and with previous work (19).

Discussion

We showed that the mating of *S. cerevisiae* can be reprogrammed by the surface expression of arbitrary SynAg proteins that replace the function of the native sexual agglutinin proteins, Aga2 and Sag1. Using SynAg, we demonstrated quantitative library-on-library characterization of up to 7,000 distinct protein–

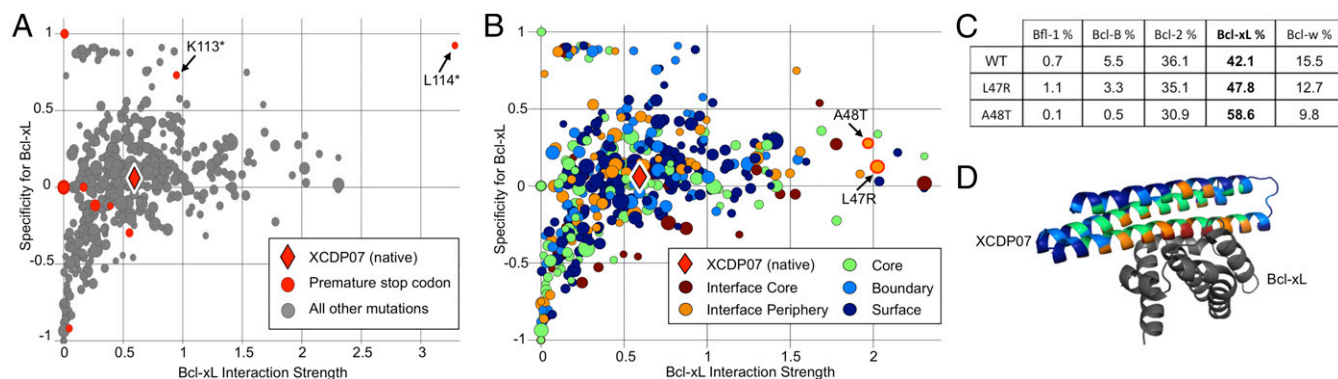


Fig. 4. SynAg for large library protein interaction characterization. Interaction strength versus specificity plots are shown for a site-saturation mutagenesis library of the de novo Bcl-xL binder, XCDP07, with premature stop codon variants in red (A) and two confirmed affinity and specificity improving mutations highlighted (B). For each protein variant, the diameter is a function of its representation in the naive library, which is used as a measure of confidence. (C) Prosurvival BCL2 homolog mating distribution for wild-type (WT) XCDP07 and two point mutants that showed enrichment in a one-sided yeast surface display screen (21). (D) Feature-colored cartoon model of XCDP07 bound to Bcl-xL.

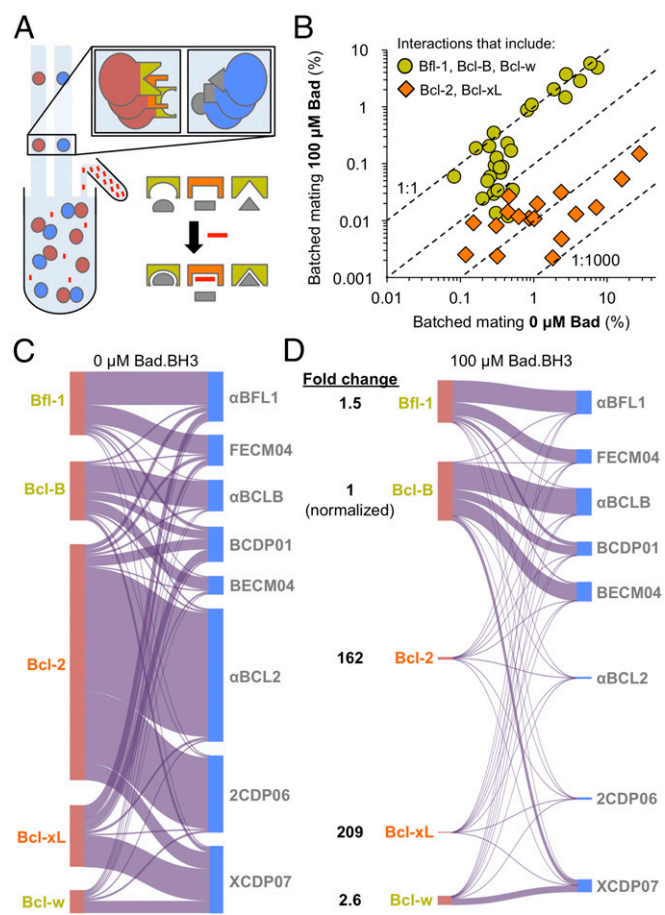


Fig. 5. SynAg for characterizing the effect of environmental changes on protein interactions. (A) The agglutination environment can be manipulated by adding arbitrary molecules (red) that disrupt interactions involving particular proteins (orange) but not others (yellow). (B) Addition of a competing peptide, Bad.BH3, to a mating between five pro-survival BCL2 homologs and eight *de novo* binding proteins results in the isolated disruption of interactions involving the peptide's binding targets, Bcl-2 and Bcl-xL (Fig. 3F). Dashed lines representing 1:1, 1:10, 1:100, and 1:1,000 differences between conditions are given for reference. The protein-protein interaction network is visualized without (C) and with (D) the addition of 100 μM Bad.BH3. Fold changes are given for the aggregate interactions of each BCL2 homolog.

protein interactions in a single pot. Additionally, we showed how SynAg could be used for characterizing protein interaction networks in different environments by adding an exogenous competitor to the mating environment. To date, tools for screening libraries of protein interactions are limited by throughput, a fixed intracellular environment, or accuracy. Previous strategies for developing library-on-library screening platforms have used cell-free systems, which are far less scalable. In contrast, SynAg combines the scalability of a cellular assay with the feature of environmental manipulation on a library-on-library scale.

The BCL2 protein network was chosen to demonstrate and validate SynAg due to previous characterization that showed a wide range of interaction strengths. We expect any of the diverse classes of proteins that can be functionally expressed on the surface of yeast (33) to be compatible with SynAg. Some proteins do not functionally display on the yeast surface, and would therefore not be compatible with SynAg. For example, the detection of interactions requiring specific posttranslational modifications may not be possible (34). Additionally, SynAg is likely ill-suited for the screening of homodimer libraries. Oligomeric proteins are known to display on the yeast surface as functional assemblies (35), which

means that homodimers would already be bound and not accessible for agglutination with a neighboring yeast cell expressing the same protein. Further studies are required to investigate these and other possible restrictions to SynAg.

SynAg provides a high-throughput platform for screening environment-responsive protein interactions and interaction-inhibiting drug candidates. Engineered protein-protein interactions that respond to environmental changes, such as pH, are valuable for biosensors (36) and drug delivery (37). SynAg may enable the rapid identification of functional variants using one-pot screening of design libraries rather than individual testing of protein pairs. SynAg also has potential applications for pharmaceutical development. Drug-induced protein interaction inhibition is a powerful therapeutic strategy for treating cancers, inflammation, and infectious diseases (38). SynAg may streamline preclinical drug profiling workflows by testing candidate compounds on protein interaction networks, enabling simultaneous screening for efficacy and specificity.

In addition to its utility for characterizing protein interactions, SynAg provides a unique ecological model for studying prezygotic genetic isolation. Previous work described the large diversity in sexual agglutination proteins across yeast species and suggested that coevolution of these proteins may drive speciation by genetically isolating haploid pairs (39). Here, we have created a fully engineerable synthetic prezygotic barrier that can be used as a model to study complex ecological phenomena such as speciation and sexual selection, similar to the use of engineered *Escherichia coli* for modeling predator-prey dynamics (40).

Methods

DNA Construction. Isogenic fragments for yeast transformation or plasmid assembly were PCR-amplified from existing plasmids or yeast genomic DNA with Kapa polymerase (Kapa Biosystems), gel-extracted from a plasmid digest (Qiagen), or synthesized by a commercial supplier (Integrated DNA Technologies). Plasmids were constructed with isothermal assembly (26) and verified with Sanger sequencing (27). Site-saturation mutagenesis library DNA was prepared with overlap PCR using custom NNK primers for each codon (21). A complete list of plasmids used in this study is provided in Table S1. Sequences for all cloning primers, fragments, and plasmids are available upon request.

Yeast Methods. Unless otherwise noted, yeast transformations were performed with a standard lithium acetate transformation (41) using ~300 ng of plasmid digested with PmeI. Yeast peptone dextrose (YPD), yeast peptone galactose (YPG), and synthetic drop out (SDO) media supplemented with 80 mg/mL adenine were made according to standard protocols. Saturated yeast cultures were prepared by inoculating 3 mL of YPD from a freshly struck plate and growing for 24 h at 30 °C.

Yeast Strain Construction. EBY100α was generated from a mating between EBY100a and a leucine prototroph W303α variant. Following sporulation and tetrad dissection, replica plating was used to identify MATα haploids auxotrophic for lys and trp (42). Plating on 5-fluoroorotic acid was used to select strains with URA3-inactivating mutations (43). Final ySYNAG strains were constructed with many rounds of chromosomal integration, with each consisting of a single transformation, auxotrophic or antibiotic selection, and PCR to verify integration into the expected locus. A complete list of yeast strains used in this study is provided in Table S2.

Yeast Site-Saturation Mutagenesis Library Construction. Site-saturation mutagenesis libraries were transformed into yeast using nuclease-assisted chromosomal integration (Fig. S4C). Before transformation, yeast strains were grown in YPG for 5 h. Library transformations were conducted with 2 μg of each fragment and 10⁸ cells and reagent volumes. Cells were washed in 1 mL of YPD and resuspended in YPD to a total volume of 5 mL. A dilution series was plated on SDO-trp to quantify transformation efficiency. The remaining culture was grown for 5 h, washed twice with 5 mL of SDO-trp, and grown in 20 mL of SDO-trp for 17 h. Two-milliliter 25% glycerol aliquots were stored at -80 °C.

Protein Purification. DNA encoding the BH3 domain of Bad (Bcl-2 agonist of cell death protein, residues 103–131) was inserted into a modified pMAL-c5x vector resulting in an N-terminal fusion to maltose binding protein and a

C-terminal 6-histidine tag. The vector was transformed into BL21(DE3) * *E. coli* (New England Biolabs) for protein expression. Protein was purified from soluble lysate first with nickel affinity chromatography (NiNTA resin; Qiagen) and then by size exclusion chromatography (Superdex 75 10/300 GL; General Electric). Purified protein was concentrated via centrifugal filter (Millipore), snap-frozen in liquid nitrogen, and stored at -80°C .

Surface Expression Screening. Ten microliters of saturated cells was washed with 1 mL of phosphate-buffered saline with 0.1% bovine serum albumin (PBSF), incubated in 50 μL of PBSF media with 1 μg of FITC-anti-myc antibody (Immunology Consultants Laboratory, Inc.) for 1 h at 22°C , washed with 1 mL of PBSF, and read with the FL1.A channel on an Accuri C6 cytometer. SynAg expression cassettes contain an HA tag between Aga2 and the gene of interest that can be used as an alternative epitope tag for labeling SynAg variants containing premature stop codons.

Pairwise Mating Assays. In total, 2.5 μL from a saturated MATa culture and 5 μL from a saturated MAT α culture were combined in 3 mL of YPD media and incubated at 30°C in a shaking incubator for 17 h. Five microliters was added to 1 mL of water, and cellular expression of mCherry and mTurquoise was characterized with a Miltenyi MACSQuant VYB cytometer using channels Y2 and V1, respectively. A standard yeast gate was applied to all cytometry data, and FlowJo was used for analysis and visualization.

Yeast Library Preparation. Precharacterized yeast libraries were prepared by manually combining individually transformed isogenic yeast strains with known barcodes (Fig. S1). Saturated isogenic yeast strains of the same mating type were pooled with equal cell counts, measured with an Accuri c6 flow cytometer.

Next-generation sequencing was used to characterize the barcodes and relative population distribution of yeast site-saturation mutagenesis libraries. A 2-mL glycerol stock was thawed, washed once with 1 mL of YPD,

and grown in 50 mL of YPD for 24 h. Genomic DNA was then prepared for next-generation sequencing.

Library-on-Library Mating Assays. In total, 2.5 μL of saturated MATa library and 5 μL of saturated MAT α library were combined in 3 mL of YPD and incubated at 30°C in a shaking incubator for 17 h. When characterizing interactions in the presence of Bad.BH3, the peptide was added at a concentration of 100 nM. One milliliter was washed twice in 1 mL of SDO-lys-leu and transferred to 50 mL of SDO-lys-leu with 100 nM β -estradiol for 24 h. Genomic DNA was prepared for next-generation sequencing.

Preparation for Next-Generation Sequencing. Fifty-milliliter yeast cultures were harvested by centrifugation and lysed by heating to 70°C for 10 min in 2 mL of 200 mM LiOAc and 1% SDS (44). Cellular debris was removed with centrifugation, and the supernatant was incubated at 37°C for 4 h with 0.05 mg/mL RNase A. Following an ethanol precipitation, a 2% agarose gel was run to verify genomic DNA extraction. Two rounds of qPCR were performed to amplify a fragment pool from the genomic DNA and to add standard Illumina sequencing adaptors and assay specific index barcodes. Both PCR assays were terminated before saturation to minimize PCR bias. The first PCR assay was run for 25–30 cycles, and the second PCR assay was run for five to seven cycles. The final amplified fragment was gel-extracted, quantified with a Qubit, and sequenced with an MiSeq sequencer (Illumina).

Code Availability. Sankey diagrams were generated with SankeyMATIC (sankeymatic.com).

ACKNOWLEDGMENTS. We thank M. Dunham for technical discussions, A. Rosenberg for data analysis support, and M. Parks and the UW BIOFAB for assistance with the construction of many plasmids and yeast strains used in this study. This work was supported by US National Science Foundation (NSF) Award 1317653. D.Y. and S.B. are supported by the NSF Graduate Research Fellowships Program.

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