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Detection of a Peptide Biomarker by Engineered Yeast Receptors

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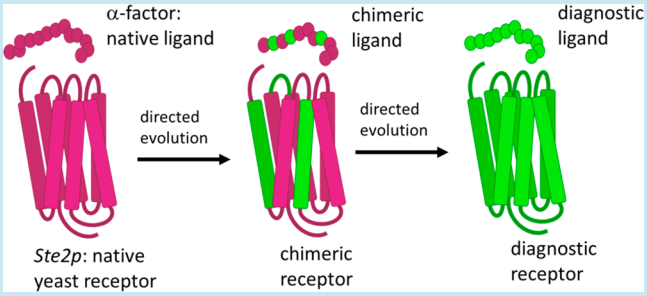
Supporting Information

ABSTRACT: Directed evolution of membrane receptors is challenging as the evolved receptor must not only accommodate a non-native ligand, but also maintain the ability to transduce the detection of the new ligand to any associated intracellular components. The G-protein coupled receptor (GPCR) superfamily is the largest group of membrane receptors. As members of the GPCR family detect a wide range of ligands, GPCRs are an incredibly useful starting point for directed evolution of user-defined analytical tools and diagnostics. The aim of this study was to determine if directed evolution of the yeast Ste2p GPCR, which natively detects the α-factor peptide, could yield a GPCR that detects Cystatin C, a human peptide biomarker. We demonstrate a generalizable approach for evolving Ste2p to detect peptide sequences. Because the target peptide differs significantly from α-factor, a single evolutionary step was infeasible. We turned to a substrate walking approach and evolved receptors for a series of chimeric intermediates with increasing similarity to the biomarker. We validate our previous model as a tool for designing optimal chimeric peptide steps. Finally, we demonstrate the clinical utility of yeast-based biosensors by showing specific activation by a C-terminally amidated Cystatin C peptide in commercially sourced human urine. To our knowledge, this is the first directed evolution of a peptide GPCR.



KEYWORDS: *directed evolution, G-protein coupled receptors, diagnostics, substrate walking, receptor engineering*

Directed evolution is commonly used to evolve proteins toward user-defined goals. For enzymes, directed evolution only needs to uncover mutations that alter the ligand binding site to accept a non-native ligand. For proteins such as membrane receptors, directed evolution is much more challenging, as the evolved receptor must not only accommodate a non-native ligand, but also maintain the ability to transduce the detection of the new ligand to any associated intracellular components.

The GPCR (G-protein coupled receptor) superfamily is the largest group of membrane receptors. Given the incredible range of molecules GPCRs detect, including proteins, small molecules, ions, and photons,[1] this superfamily is under-utilized as a starting point for directed evolution of user-defined analytical tools and diagnostics. To date, directed evolution of GPCRs has only been successful for ligands that are closely related to the native ligand,[2] and has been limited to small molecules.[3,4] Specifically, directed evolution of peptide GPCRs could yield user-defined sensors capable of interrogating or analyzing any peptide-dependent biological state. Here we investigate the feasibility of using directed evolution to yield a point-of-care (POC) biosensor to detect a human peptide biomarker.

Access to low-cost, POC diagnostics enables screening, diagnosis and treatment monitoring for several critical diseases. POC diagnostics reduce hospital stays, increase adherence to treatment, and have wider economic benefits compared to laboratory testing.[5] POC diagnostics, such as lateral flow sandwich immunoassays, nucleic acid amplification tests, and colorimetric chemical reactions, are available for proteins, DNA/RNA, and small molecule disease biomarkers. However, there is a technology gap for assaying peptides and small proteins biomarkers smaller than 15 kDa, including known biomarkers for several cancers,[6] Alzheimer's,[7] diabetes,[8] renal failure,[8] and tuberculosis.[9] These biomarkers are frequently degradation products of larger proteins[10,11] and often appear in easy-to-access specimens such as urine[12] and blood,[11] suitable for POC diagnostics. These biomarkers are often too small to be simultaneously bound by two antibodies as required for sandwich immunoassays and instead require more sophisticated assays like immunoturbidimetric measurements[13] and mass spectrometry,[9] which can prove impractical and prohibitively expensive at the POC level. Thus, a cost-effective POC peptide diagnostic can improve early detection and treatment management for a range of diseases.

Baker's yeast (*Saccharomyces cerevisiae*) is a particularly promising vehicle for a POC peptide diagnostic because it is cheap, robust, and easily engineered with standard techniques. Active dry yeast is simple to manufacture and can be freeze-dried and reconstituted to provide easy distribution without requiring a cold-chain. A yeast-based biosensor (YBB)

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diagnostic tool would not require power for operation, and could be operated with minimal training because all detection and processing happens automatically at the cellular scale. Also, signal amplification can easily be built into the system using standard Synthetic Biology techniques.[14] Yeast contains a native G-protein coupled receptor (GPCR)[15] which detects the 13 amino acid α-factor peptide. We hypothesized that Ste2p may be a useful starting point for directed evolution to produce receptors for peptide biomarkers that cannot currently be assayed at the POC.

Directed evolution has been used to evolve GPCRs to detect small molecules,[2,4,16,17] but not peptides. Directed evolution experiments ideally start with proteins that have a low basal activity that can be improved upon. However, most peptide biomarkers have sequences that are very dissimilar from the α-factor, so Ste2p is not likely to have basal activity for the peptide biomarker. The sequence dissimilarity, representing a large evolutionary distance, is challenging as many simultaneous mutations would likely be required for the receptor to detect the biomarker. To overcome this difficulty, we chose substrate walking as an alternative technique for covering large evolutionary distances.[17] In substrate walking, an enzyme is evolved along a pathway of chimeric substrates which contains features of both the native and desired substrates before being evolved for the desired substrate. Substrate walking with peptide-receptor pairs has not been explored, so we investigated the feasibility and constraints of this strategy.

Here, we chose a peptide biomarker of chronic kidney disease (CKD). CKD, the 12th leading cause of death worldwide,[18] affects 10% of the adult American population[19] and is a significant, emerging burden in low and middle income countries.[20] As CKD worsens, the kidneys lose the ability to clear cystatin C from the body, and cystatin C accumulates in the serum[21] and urine.[22] Clinically, cystatin C satisfies the criteria for a universal biomarker as it is independent of age, weight, and muscle mass[13,23] and is more sensitive to early onset renal failure compared to creatinine, a commonly used biomarker for CKD.[24] Cystatin C is typically measured with a nephelometer, which is costly and requires substantial infrastructure to use and maintain.[25] Because a biomarker protein may not be consistently degraded in the body, it is necessary to standardize the peptide length and sequence. We used a tryptic digest to ensure heterogeneous degradation products are cleaved into standard peptides. Though cystatin C is a 13 kDa protein, trypsin digestion of cystatin C yields an 11 amino acid peptide[26] that can serve as a CKD biomarker.

In this study, we present a generalizable method for producing peptide biomarker receptors using substrate walking and directed evolution, and validate the approach by developing a YBB that can detect a modified peptide fragment of cystatin C, a biomarker for renal failure. Finally, we demonstrate the clinical feasibility of the proof-of-concept YBB by showing functionality in commercially sourced pooled human urine, a relevant sample matrix. To our knowledge, this is the first demonstration of engineering a GPCR to detect fully orthogonal peptide sequences.

Results and Discussion. Evolution of a Receptor for Cystatin C, a Peptide Biomarker of Kidney Failure. Toward the goal of determining if GPCRs can be evolved for new peptide ligands, we developed a fluorescence-activated cell sorter (FACS)-based high throughput screen. We coupled receptor activation to green fluorescent protein (GFP) expression by cloning GFP downstream of the Ste2p-activated FUS1 promoter. We used constitutive RFP expression to control for cell-to-cell variations in size and protein synthesis rate, and identified activated receptors by a ratio of GFP expression to RFP expression. Multiple rounds of enrichment were required to isolate rare receptors from the library due to stochastic fluorescence at the single cell level (Supporting Information, Supplement Figure 7). For Ste2p, there are approximately 8×10^3 possible single amino acid mutants and 6×10^7 possible double amino acid mutants. We typically created libraries of approximately 10^6 receptor mutants using error-prone PCR (epPCR).

An initial directed evolution experiment targeting Ste2p for the 11 amino acid long cystatin C tryptic peptide Cys7 (Table 1) failed to produce any mutant receptors with detectable sensitivity, so we proceeded with a substrate walking strategy. We calculated the step size for each peptide as the evolutionary change per amino acid based on the BLOSUM62 matrix.[27] On the basis of this rudimentary analysis (Supplementary Figure 6 and Supplementary Table 3) we estimated a maximum permissible step size. We designed an evolutionary path containing chimeric peptide ligands named Cys1−Cys7, where Cys1 is similar to the native ligand, α-factor, and Cys7 is the trypsinized cystatin C fragment (Table 1). The intermediate ligands were designed qualitatively by previous knowledge of how specific amino acid substitutions in α-factor affect the native α-factor- Ste2p interaction[28−30] (Supporting Information).

Table 1. Chimeric Ligands[a]

Name	Sequence
α-factor	WHWLQLKPGQPMY
Cys1	- HALALKPGEPMY
Cys2	- - ALALKPGEPMY
Cys3	- - ALDFKPGEPMY
Cys4	- - ALDLAVGEPMY
Cys5	- - ALDFAVGEPMK
Cys6	- - ALDFAVGEYMK
Cys 7 – Cystatin peptide	- - ALDFAVGEYNK

[a]Chimeric ligands used to traverse the evolutionary landscape in a step-wise fashion. The sequences of the native ligand, α factor, and chimeric ligands, as well as the step-size scores are provided (see Methods).

A visualization of the directed evolution strategy is provided in Figure 1.

To evolve for activity for the chimeric peptide ligand Cys1 (Table 1), we mutagenized the wildtype Ste2p receptor using epPCR and exposed the mutant library to 100 nM Cys1 during enrichment sorting (Methods). Though the wild type receptor had no detectable response to Cys1 at a saturating ligand concentration of 10 μM (Figure 2), receptor Cys1H4 and receptor Cys1H5, both isolated from this experiment, detected ligand Cys1 with nanomolar sensitivity (Figure 3A, Supple-

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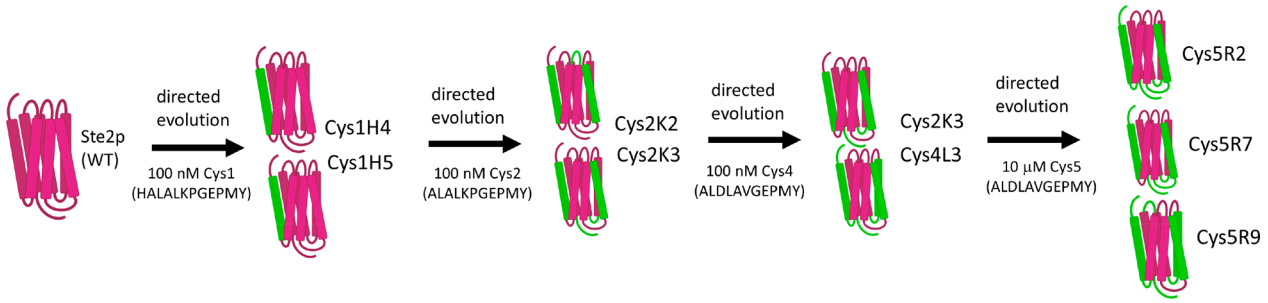

Figure 1. Substrate walking directed evolution strategy to evolve a receptor to detect a peptide biomarker. Mutant receptors and experimental conditions used in the directed evolution of Ste2 to detect a cystatin C peptide are detailed above. Though evolved to detect peptide Cys5, receptors Cys5R2, Cys5R7, and Cys5R9 can detect the cystatin C peptide Cys7 (ALDFAVGEYNK).

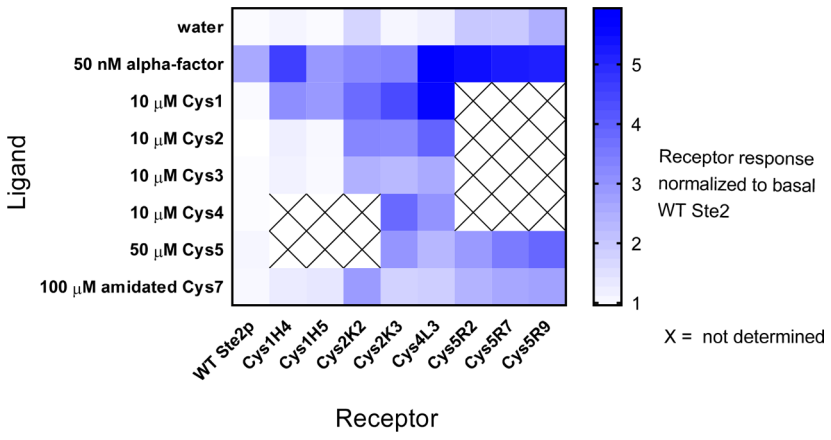

Figure 2. Response of native and mutant receptors to chimeric ligands. Response of mutant receptors to chimeric intermediates throughout the evolutionary pathway is shown here. Responsiveness for Cys7, the diagnostic peptide, is gradually gained through the course of evolution. For peptides Cys1−Cys3, each data point is the average for $n = 2$ technical replicates. For all other peptides, each data point is the average of $n = 3$ technical replicates. The experiment was replicated one additional time in our laboratory with comparable results.

mentary Table 1). As expected, Cys1H4 and Cys1H5 showed no response to chimeric ligand Cys2 even at 10 μM, the top of the linear range of detection for peptide Cys1 (Figure 2). Thus, we further mutagenized receptors Cys1H4 and Cys1H5 using epPCR and exposed the library to 100 nM Cys2 during enrichment sorting. From this sort, we isolated receptors Cys2K2 and Cys2K3, which responded to Cys2 with nanomolar sensitivity (Figure 3B, Supplementary Table 1).

Fortuitously, both receptors Cys2K2 and Cys2K3 responded to chimeric peptide intermediate Cys3 with micromolar sensitivity (Supplementary Figure 4B, Supplementary Table 1), and receptor Cys2K3 responded to peptide Cys4 with micromolar sensitivity (Supplementary Table 1). Directed evolution to further evolve sensitivity to chimeric ligand Cys4 by mutagenizing receptors Cys2K2 and Cys2K3 via epPCR and exposing the mutagenized library to 100 nM Cys4 yielded receptor Cys4L3. Subsequent analysis of receptor Cys4L3 with a new stock of Cys4 peptide showed a sensitivity of 1 μM. However, the receptor showed a 2.5-fold improvement in sensitivity in detecting ligand Cys4 over parent receptor Cys2K3 (Figure 3C, Supplementary Table 1) and was used for further directed evolution.

Next, we used epPCR to mutagenize receptors Cys4L3 and Cys2K3 to evolve activity for the intermediate Cys5. Due to the increasing EC50 values of our receptors, we increased the concentration of peptide used during directed evolution. Though an initial attempt using 5 μM Cys5 peptide failed to produce receptors, a sort using 10 μM Cys5 produced receptors Cys5R2, Cys5R7, and Cys5R9.

Using these three receptors as a starting point for directed evolution to detect intermediate Cys6 at 100 μM, a 10-fold increase in ligand concentration from the previous directed evolution experiment, failed. This was surprising as Cys5 and Cys6 differ by only one amino acid. We believed that the failed evolution was due to poor binding of the ligand as peptides Cys5 and Cys6 contain mutations on the C-terminal end, which is the region responsible for binding to the GPCR in the native Ste2p-α-factor interaction.[31] As we wanted to maintain sensitivity of the receptor toward clinically relevant concentrations of cystatin, we ceased directed evolution for higher concentrations of ligand. Instead, we looked toward the interaction of native peptide ligands and their GPCRs from other species to aid our understanding of the system. For nonyeast eukaryotes, many bioactive peptide ligands of GPCRs are amidated on the C-terminus.[32] As C-terminal amidation increases peptide hydrophobicity,[32] we hypothesized that amidated versions of our peptide intermediates may improve binding to the GPCRs. Receptors Cys5R2, Cys5R7, and Cys5R9 were found to respond to C-terminally amidated Cys7 at 100 μM, 50 μM, and 500 μM, respectively (Figure 3D, Supplementary Table1). Interestingly, receptor Cys4L3, isolated from the previous round of sorting, also responded to 100 μM amidated Cys7 (Supplementary Table 1). Ste2p responds to 1 mM amidated Cys7 (Supplementary Table 1). However, the most sensitive mutant, receptor Cys5R7, shows a

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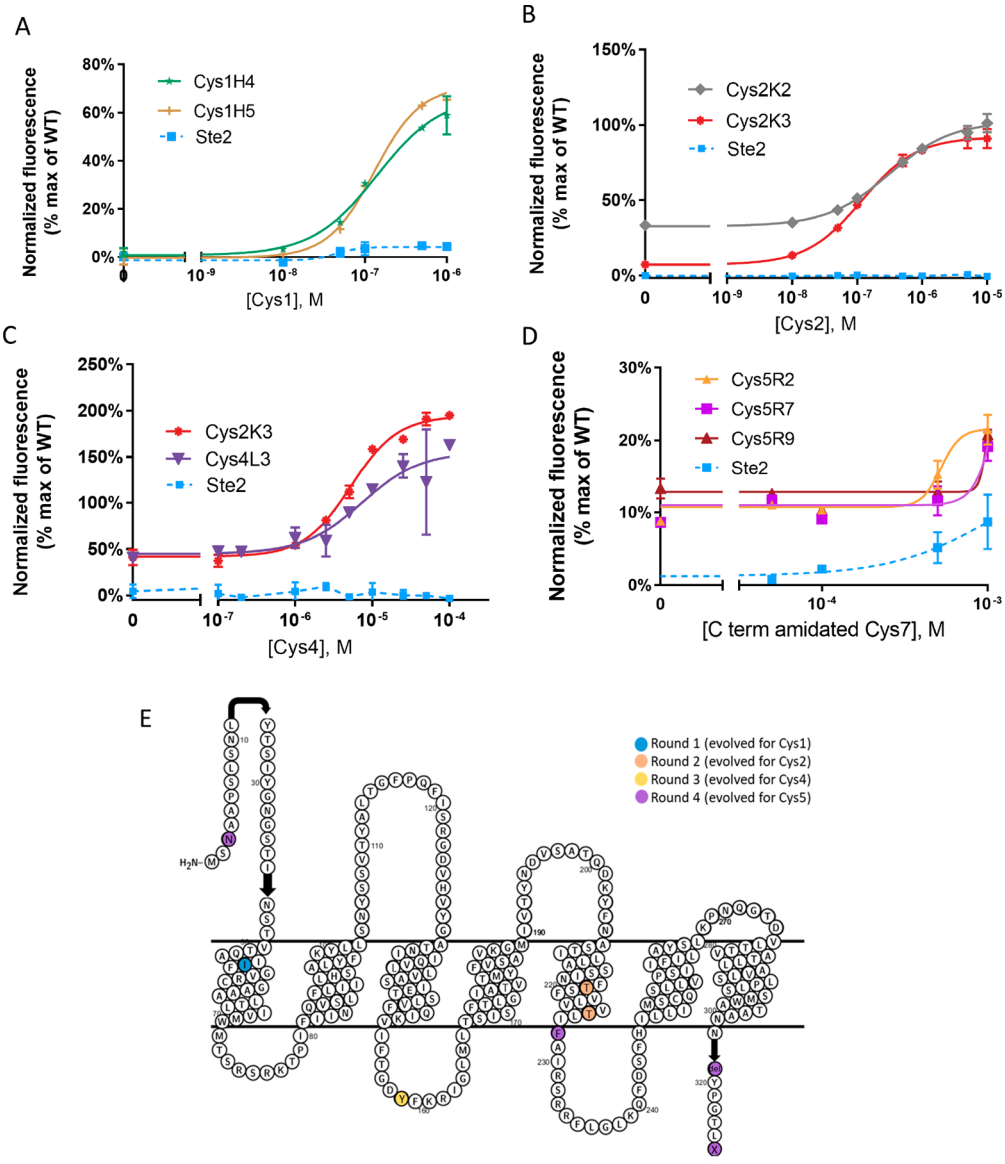

Figure 3. Evolved receptors respond in a dose-dependent manner to target ligands. (A) The native Ste2p receptor does not respond to chimeric ligand Cys1. Evolved receptors Cys1H4 and Cys1H5 respond in a dose dependent manner to ligand Cys1. Each data point is the average shown for $n = 2$ technical replicates. (B) The native Ste2p receptor does not respond to chimeric ligand Cys2, but evolved receptors Cys2K2 and Cys2K3 respond in a dose dependent manner to ligand Cys2. (C) The native Ste2p receptor does not respond to chimeric ligand Cys4. Evolved receptors Cys2K3 and Cys4L3 respond in a dose dependent manner to ligand Cys4. (D) Evolved receptors Cys5R2, Cys5R7, and Cys5R9, and native receptor Ste2p respond to amidated biomarker Cys7. The evolved receptors display significantly higher responsiveness and sensitivity when detecting amidated biomarker Cys7 as compared to the native receptor. (A–D) Unless otherwise noted, each data point is the average shown for $n = 3$ technical replicates and error bars represent standard error of measurement. For all receptor–ligand pairs, EC50 and Hill slope values are reported in Supplementary Table 1. (E) Snake plot showing mutations acquired during directed evolution for receptor Cys5R7, which displayed the highest sensitivity toward the cystatin C peptide Cys7.

20-fold improvement in sensitivity for amidated Cys7. A diagram showing the locations of acquired mutations for receptor Cys5R7 is provided in Figure 3E. Along the course of evolution for carboxy terminated peptides, the substrate walking method worked to evolve receptors that detect peptides for which Ste2p cannot detect at all.

We sequenced the STE2 mutants in the strains isolated in the different rounds of evolution and analyzed the sequences with respect to known information on Ste2p. We hypothesize that the new receptor function is a result of mutations that do not solely affect ligand binding. To summarize the course of evolution, we first observed a mutation that likely affected the binding pocket (M54I), then mutations that are likely to be responsible for global stability (M218K, M218T, K225T), followed by mutations that may affect interaction with other signaling network proteins (truncations, T167R, N158Y, N158F). A detailed reasoning of the proposed function of these mutations is provided in the Supplement (Analysis of Mutations in Cystatin Receptors).

Clinical Relevance of Evolved Receptor. To test whether the proof-of-concept YBB diagnostic would be effective in a clinically relevant sample matrix, we examined the receptors' ability to respond to their respective target ligands in urine. Given that our biosensor detects a trypsinization fragment of

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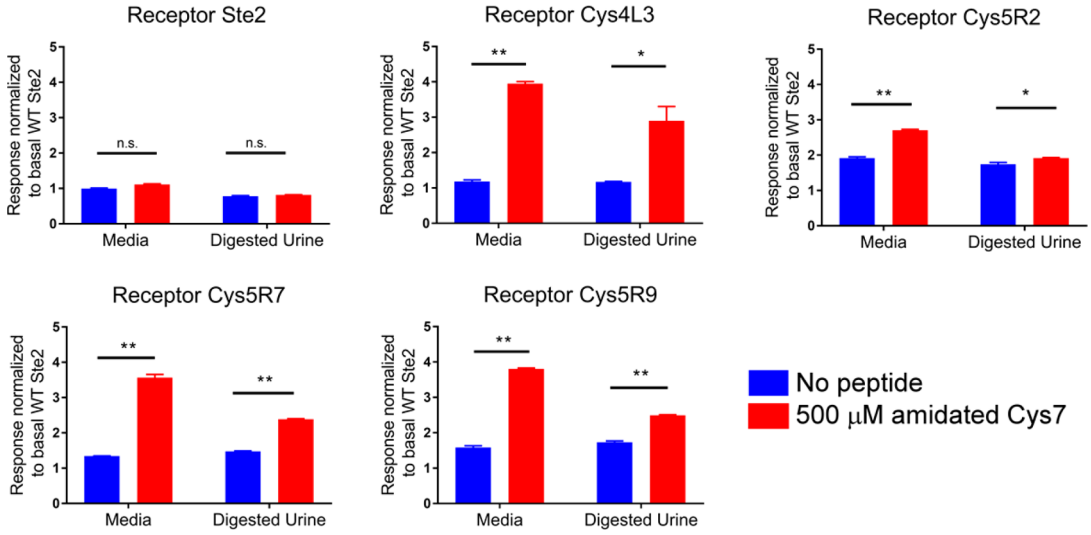

Figure 4. Engineered receptors are sensitive and specific in human urine. Ste2p and the Cys7 responsive receptors were exposed to pooled human urine digested with trypsin and spiked with Cys7. The ability of receptors Cys4L3, Cys5R2, Cys5R7, and Cys5R9 to detect the Cys7 fragment in human urine is comparable to the receptor performance in media. Trypsinized urine does not spontaneously activate the receptor. Each data point is the average shown for $n = 3$ samples and error bars represent standard error. $^{**}p < 0.001$. $^{*}p < 0.05$ as determined by two-way ANOVA. This experiment was not reproduced beyond the technical replicates.

cystatin C, practical application of the biosensor would require us to trypsinize the human sample matrix, which may liberate peptides that would stimulate the receptor. When tested in trypsinized urine, receptors Cys4L3, Cys5R2, Cys5R7, or Cys5R9 did not show any spontaneous activity (Figure 4) and all four receptors were able to detect 500 μM amidated Cys7 in pooled human urine (Figure 4). The most sensitive receptor, receptor Cys5R7, detects the trypsinized cystatin C fragment at 50 μM sensitivity. Patients with CKD have been reported to have urinary cystatin C concentrations ranging from 29 to 743 nM[22] and commercially available pooled urine from CKD patients has a minimum cystatin C concentration of 750 nM.[33] As this is 67-fold lower than our receptor's sensitivity threshold, a concentration step coupled with the trypsinization and C-terminal amidation[34] may bring the sensitivity within the range of the current assay. Further optimization of receptor sensitivity would likely be required before field applications of the biosensor can be established.

Step-Size Analysis. Our initial step-size analysis using the BLOSUM62 matrix failed to predict the difficulty of the evolutionary leap from Cys4 to Cys7, so we sought to improve this analysis using a more biophysical approach. We recently conducted a detailed study of the promiscuity of Ste2p to determine the physiochemical properties of peptides that are predictive of receptor response.[35] We created a quantitative sequence–activity relationship (QSAR)-based model that could qualitatively predict receptor response for peptides that differed from α-factor. We used this model to analyze the chimeric peptides and model responses for both the wild-type Ste2 receptor and Cys1H4, which we had previously found to have increased promiscuity. Cys1H4 was predicted to respond to intermediates Cys1-Cys4 while Ste2 was predicted to not respond to any chimeric peptide (Figure 5). This implies that the first evolutionary step increased promiscuity, enabling further directed evolution.

The physiochemical model identified the specific features and positions, namely the hydrophobicity of residue 13, that had the greatest predictive power for receptor response. In the

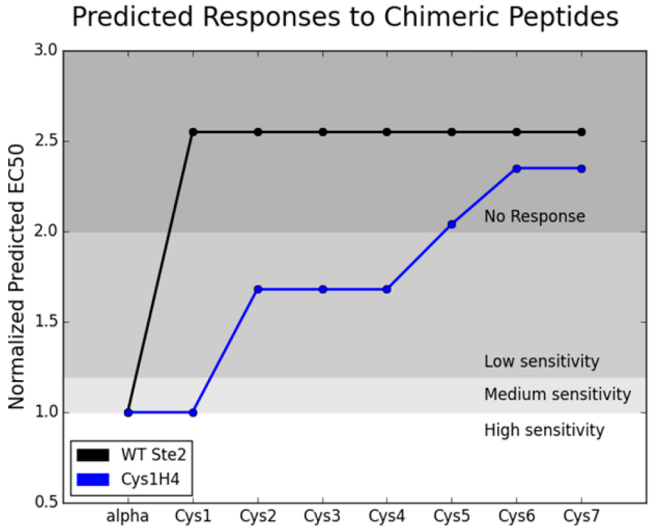

Figure 5. Prediction of receptor responses to chimeric ligands. The results of receptor response modeling for all peptides in the pathway are shown. Increasing predicted normalized response indicates decreasing sensitivity.

model for receptor Cys1H4, only residues 2, 4, 9, 11, and 13 contributed significantly to determining response. In our work, residues 4 and 9 were left unchanged. Our model reveals that the step from Cys4 to Cys7 was particularly challenging because residues 11 and 13 were changed simultaneously. Thus, intermediate steps Cys5 and Cys6 were required, in which only one highly predictive residue was changed at a time. The predicted importance of the hydrophobicity of residue 13 was also borne out experimentally as the change from hydrophobic tyrosine to polar lysine resulted in a large decrease in sensitivity. Amidation of this residue partially restored the hydrophobic character and enabled receptor response. Thus, physiochemical modeling can identify highly predictive features and provide valuable insight into which residues and properties will be most difficult to evolve experimentally.

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Conclusions. We present in this work, to our knowledge, the first demonstration of engineering a GPCR to sense a completely orthogonal peptide sequence. Our work demonstrates the feasibility of applying mutagenesis and screening to enrich rare receptors and traversing a large evolutionary space using the substrate walking strategy for peptides. Evolution starting from Ste2p has resulted in three mutant receptors that recognize a C-terminally modified biomarker for CKD. Furthermore, YBBs are promising POC diagnostics. In this work, we demonstrate that YBBs can detect a modified biomarker peptide in human urine. Although sensitivity of the proof-of-concept YBB has not yet been optimized, previously reported YBBs for human and plant peptide pathogens using GPCRs as the detection element demonstrate nanomolar and micromolar sensitivity.[36] Additionally, several naturally occurring GPCRs can detect peptide ligands at picomolar[37] and subpicomolar[37] concentrations, which are ripe to be exploited with the methodology we present here. Specifically, further directed evolution using a lower concentration of biomarker may optimize YBB sensitivity.

In general, we anticipate Ste2p directed evolution to work particularly well for peptide ligands that share three features with α-factor: length, a central type II β-turn, and C-terminal hydrophobicity. In the current study, we chose a target ligand that largely obeyed these rules. First, our target cystatin C peptide is 11 amino acids long, which differs in length from the 13-amino acid long α-factor. The native Ste2p receptor can detect α-factor variants between 9[38] and 17[39] amino acids in length, though activity is inversely proportional to the length of the peptide, falling by as much as 100 fold with the 17th amino acid. Second, the type II β-turn spanning residues 7−10 of α-factor plays a key role in orienting the N-and C-termini of α-factor to provide an ideal interaction with Ste2p.[31] Disrupting the type II β-turn can reduce receptor activity by as much 200 fold,[40] and eliminating the turn can abolish ligand binding.[41] Even so, peptide Cys7 from Table 1 has a disrupted β-turn;[42] however, we were able to find mutant receptors that detected Cys7. This is consistent with previous work showing that α-factor variants with alanine substitutions at the predicted β-turn (amino acids 7−10) are still detected by the native Ste2p.[31] While it is generally thought type II β-turns are helpful for Ste2p binding, it does not appear to be an essential condition. Additionally, as an estimated 25% of all residues in all proteins are in β-turns, and β-turns tend to be located at solvent-exposed surfaces,[43] we do not anticipate that finding easily accessible peptide biomarkers that contain β-turns will be problematic. Finally, Cys7 contains at least two hydrophobic residues in the C-terminal region. The C-terminus is reported to be important for binding of the ligand to the receptor,[31] mediated by the presence of hydrophobic and aromatic residues.[44]

Making small steps through the evolutionary pathway with chimeric peptides was critical to obtain receptors for the CKD biomarker. Random mutagenesis proved too inefficient to simultaneously provide the combination of mutations needed to detect the cystatin biomarker, and it was necessary to gradually traverse the evolutionary pathway using substrate walking. We initially estimated permissible steps between peptide intermediates with a BLOSUM-based distance approach. This approach treated each residue of the peptide as equally important to determining receptor response, and did not predict the importance of C-terminal effects. When we instead employed a physiochemical model that included a residue position previously derived for this system, we were able to identify which residue changes were behind our experimental difficulties during directed evolution. Insights from this model may be informative for designing future chimeric peptide series. As we continue to understand sequence/specificity relationships between the receptor and its peptide ligand, targeted libraries may allow for larger evolutionary steps along the pathway.

Through substrate walking, we have enabled the directed evolution of GPCRs with sensitivity to orthogonal peptide targets. The successful use of this method to create a GPCR for a modified CKD biomarker highlights the potential application of our method to create YBBs as useful detection reagents. Despite broadening of specificity, the receptors capable of detecting the cystatin peptide fragment did not respond to any proteins or peptides in human urine. YBBs have great potential for many POC diagnostics, for example by eliminating the need for expensive affinity enhancements[45] and, when coupled to a colorimetric output,[36] replacing the LC/MS[46] detection step downstream of microdialysis frequently used in neuropeptide detection schemes. However, this technology is not limited to diagnostic applications, and has potential for a range of applications including environmental monitoring and synthetic communication. YBBs for the quorum sensing peptides of Gram-positive bacteria could be used to monitor the presence, concentration, and coordinated activities of organisms in our environment that pose a threat to human health, such as pathogenic species of *Streptococcus* and *Staphylococcus*.[47] This technology could also be used in exogenous control of eukaryotic cell behavior, as GPCRs comprise one of the largest groups of eukaryotic membrane receptors. For synthetic biology, our substrate walking directed evolution approach could be used to create orthogonal peptide/receptor pairs that do not exist in nature, contributing to the synthetic communications toolkit. These new communication systems could be used to precisely control cellular behavior, and enable cell-to-cell communication within and between species.

In summary, YBBs have great potential as POC diagnostics, but until now have been hampered by an inability to create receptors for specific, non-native target stimuli. In this work, we enable development of YBBs by demonstrating a platform approach for creation of peptide-responsive GPCRs of novel specificity. By combining directed evolution with stepwise substrate walking, we used a multistep pathway to evolve a receptor for a modified version of a clinically validated renal failure biomarker. The success and generalizable nature of this receptor creation method demonstrate the potential of evolved YBBs as low-cost and easily distributable diagnostic and monitoring devices for diverse applications.

METHODS

Strains, Plasmids, Reagents, and Media. Yeast strain MPY578t5[3] was a gift from Bryan Roth. STE2 was obtained from the genomic DNA of strain ESM356-1 and yeGFP and natNT2 were obtained from plasmid pCT191, which were both kindly given from Michael Knop's lab. p416GPD from the Mumberg plasmids was obtained from Addgene.[48] All oligonucleotides were synthesized by Integrated DNA Technologies (Coralville, USA). Pooled human urine (lot W09051) was purchased from Lee Biosolutions (St. Louis, USA). Custom peptides with carboxy C-termini were synthesized by Abbiotec (San Diego, USA) or New England Peptides (Gardner, USA). α-Factor mating pheromone was purchased from Sigma-Aldrich

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(St. Louis, USA). All C-terminally amidated peptides were kindly synthesized by the Mrksich lab at Northwestern University (Evanston, USA). All restriction enzymes were purchased from New England Biolabs (Ipswich, USA).

Yeast requiring nonselective media were cultivated in YPAD media prepared with Yeast Extract-Peptone-Dextrose premix (Beckton, Dickinson & Company, Sparks, USA) according the manufacturer's directions and supplemented with 80 mg/L adenine hemisulfate (Sigma-Aldrich, St. Louis, USA). When appropriate, 100 mg/L nourseothricin (Gold Biotechnology, St. Louis, USA) was added to YPAD media and used for selection.

Yeast with the uracil auxotrophy were cultivated in complete synthetic media without uracil prepared with -His-Ura dropout supplement (Clontech, Mountain View, CA) according to manufacturer's instructions, and supplemented with 20 μg/mL histidine and 80 mg/L adenine hemisulfate (Sigma-Aldrich, St. Louis, USA) To aid recovery from cell sorting, yeasts were grown in synthetic dextrose with casamino acids (SDCAA media),[49] supplemented with 100 μg/mL streptomycin and 100 U/mL pencillin (ThermoFisher Scientific, Pittsburgh, USA). Yeasts were passaged in CSM-Ura before each sorting round.

Strain and Plasmid Construction. For flow cytometry, fluorescent reporter genes were integrated into the genome of yeast strain MPY578t5 [MATa, far1::LYS2 fus1::FUS1-HIS3 sst2::PSST2-G418R ste2::LEU2 fus2::PFUS2-CAN1 *ura3 lys2 ade2 trp1*, the last five amino acids of GPA1 (KIGII) being replaced with the homologous mammalian Gαi (DCGLF)]. yeGFP was integrated chromosomally under control of the Fus1 promoter with a NatNT2 selectable marker to create strain JB005. A clone was sequence verified using primers JB079 and JB080 (see Supplemental File 2 for details of sequence at this locus.) A constitutively expressed mKate construct under the GPD promoter was chromosomally integrated at the TRP1 locus to create strain JB013 (see Supplemental File 3 for details of sequence at this locus). Transformations were done via a standard chemical method.[50]

STE2 was expressed from a single-copy centromeric plasmid. STE2 was amplified from ESM356-1 genomic DNA and cloned into p416GPD immediately downstream of the GPD promoter using standard molecular biology techniques[51] to create plasmid pJB036 (see Supplemental File 4 for plasmid sequence). A control strain expressing wild-type STE2 was made by transforming JB013 with pJB036 to create strain JB015. Transformed colonies were plated on CSM-Ura plates. The phenotype was verified by looking for GFP production in response to alpha factor peptide using flow cytometry.

Strain notations and primer sequences can be found in Supplementary Tables 2 and 3.

STE2Mutagenesis. To create a library of mutant receptors, STE2 on plasmid pJB036 was subjected to error prone PCR using the GeneMorphII random mutagenesis kit (Agilent Technologies) and primers AVA015 and AVA016, according to the manufacturer's instructions, which resulted in 0−6 base mutations/kb as determined by sequencing 10 random clones. The primers used for epPCR added 40 base pairs of homologous sequence on each side of the mutagenized gene to match the digested backbone (Supplementary Table 3), allowing the mutagenized receptor library to be introduced into yeast using homologous recombination (gap repair) with plasmid p416GPD digested at the *Bam*HI and XhoI restriction sites. The receptor libraries were transformed into yeast strain yJB013 using electroporation,[52] which typically resulted in libraries of 10^6 mutants. Libraries were passaged in selective media, either CSM-Ura or SDCAA-Ura, before screening and selection.

Receptor Induction and Flow Cytometry. Yeast were grown overnight at 30 °C and 225 rpm in 250 mL Erlenmeyer flasks and then diluted to an OD_{600} = 0.1 in the appropriate medium, typically CSM-Ura or SDCAA-Ura, or commercially sourced pooled human urine. Immediately after dilution, the peptide ligand was added. The culture was incubated for 2.5 h at 30 °C with 225 rpm shaking. Cells were centrifuged at 2000*g* for 5 min and supernatant was removed. Cells were resuspended in 1x PBS in preparation for flow cytometry.

Analytical flow cytometry was performed with the BD LSR II (BD Biosciences, San Jose, USA); yEGFP was read on the Alexa-Flour 488 channel, and mKate was read on the PE-Texas Red channel. Receptor output was measured by dividing the mean GFP fluorescence by the mean RFP fluorescence for a population. EC_{50} values were calculated by fitting the receptor output to a four-parameter logistic curve, and determining which ligand concentration produced an output equivalent to half of the receptor's maximum output.

When trypsinized urine was used as the sample matrix, urine was mixed with Tryp-LE Express (Life Technologies, Grand Island, USA) to a final concentration of 50%. Mixtures were incubated at 37 °C overnight. Trypsin was inactivated by boiling the samples at 99 °C for 10 min. Samples were stored at −80 °C until assayed.

High Throughput Sorting by Receptor Activity. For each round of high throughput sorting, cells were prepared identical to the analytical flow cytometry method above. The library of mutagenized Ste2p receptors was sorted by FACS using the BD FacsAria sorter (BD Biosciences, San Jose, USA); yEGFP was read on the FITC channel, and mKate was read on the PE-Texas Red channel. The top 0.2% of the population, as determined by the yeGFP to mKate fluorescence ratio, was sorted. This population was grown in either CSM-Ura or SDCAA-Ura media, and sorted again using the same yeGFP to mKate fluorescence ratio determined by the first sort. To reduce false positives, the population was typically sorted 5 or 6 times.

After all rounds of sorting were completed, colony PCR was used to amplify and isolate mutant receptor sequences. The receptor sequences were retransformed into the parental strain yJB013 with parental p416GPD backbone using either a chemical transformation method[50] or electroporation[52] to remove effects from any background mutations that may have occurred in the yeast genome or plasmid backbone during sorting. Retransformants were plated on selective CSM-Ura plates to isolate individual receptors, which were then sequenced and assayed.

Receptor Response Modeling. BLOSUM-based evolutionary step size was numerically estimated using a replacement cost calculation for amino acid residues. All predicted values were normalized to the predicted value for alpha-factor for that receptor. Evolutionary step size was numerically estimated using a replacement cost calculation for amino acid residues. For each residue that was added, removed, or mutated, a score was determined by BLOSUM62 matrix. The evolutionary difficulty or cost (C) of replacement can thus be calculated by comparing the self-replacement cost of the original amino acid (the value on the diagonal of the BLOSUM matrix, S_O) to the replacement cost of the new amino acid (R_N):

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$$C = S_O - R_N$$

Lower cost indicates that such a chemically similar replacement is evolutionarily easier to accommodate. In the case of deletions, an R_N of −11 was used. For additions, $C = 11$. The results of the analysis were insensitive to ±50% of the addition/deletion values. The step size (S) can be calculated by summing the cost for each amino acid change in the peptide and normalizing by the number of amino acids in the original peptide.

Physiochemical response modeling was performed as previously described.[35] Receptor Cys1H4 was independently found and characterized in previous work and is reported as Mut1 in those results.

Dose Response Curve Calculations. The dose response curves were modeled to fit the following four-parameter logistic curve:

$$y = a + \frac{b - a}{1 + 10^{d(\log c - x)}}$$

where a is the minimum value, b is the maximum value, c is the EC50, and d is the hill coefficient. The fluorescent receptor output, y, to a given concentration x of ligand, was determined by the following equation:

$$y = \frac{\text{GFP}}{\text{mKate}}$$

where GFP is downstream of the receptor pathway and mKate is constitutively expressed (see Strain and Plasmid Construction, Methods Section).

For graphing, all EC50 values are scaled according to interaction between Ste2p and the alpha factor, such that 0% is the basal output of Ste2p in the absence of ligand, and 100% is the output of Ste2p in the presence of 1 μM alpha factor, a saturating amount of ligand.

Linear and Dynamic Range Calculations. Data were fit to the Michaelis−Menten equation:

$$y = \frac{V_{max}x}{K_M + x}$$

where the fluorescent receptor output, y, to a given concentration x of ligand, was determined by the following equation:

$$y = \frac{\text{GFP}}{\text{mKate}}$$

where GFP is downstream of the receptor pathway and mKate is constitutively expressed (see Strain and Plasmid Construction, Methods Section).

A line, l, was extrapolated with slope equivalent to the first derivative of the Michaelis−Menten equation where x is equivalent to the lower limit of the dynamic range:

$$l = x\frac{\partial}{\partial x}\left(\frac{V_{max}x}{K_M + x}\right)\Big|_{x=\text{lower limit dynamic range}}$$

The upper bound of the linear range is the x value at which line l deviates from the Michaelis−Menten fit by 0.1%:

$$\left|\frac{V_{max}x}{K_M + x} - l\right| = .001\left(\frac{V_{max}x}{K_M + x}\right)$$

The bound of the dynamic range is defined as the lowest ligand concentration, x_{max}, needed to produce the maximal fluorescent output y_{max} where fluorescent receptor output is defined above. The response in the receptor output, y_{max}, at x_{max} is not statistically different from the response in receptor output at any ligand concentration higher than x_{max} ($^*p > .05$).

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acssynbio.7b00410.

Detailed rationale for the design of chimeric peptides, discussion of the potential functionality of each mutation, yeast strain genotypes, sequences of primers used, Supplementary Figures 1−7, Supplementary Tables 1−3 (PDF)

DNA sequence information for genetic integrations and plasmids used in this study (GenBank DNA), ye-GFP (ZIP)

DNA sequence information for genetic integrations and plasmids used in this study (GenBank DNA), mKate (ZIP)

DNA sequence information for genetic integrations and plasmids used in this study (GenBank DNA), pJB036 (ZIP)

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Author Contributions

A.V.A. designed and executed the cystatin and clinical relevance experiments, S.C.S. contributed the step-size analysis work and J.W.B. cloned the starting yeast strains. J.W.B. and K.E.T designed the FACS strategy. All authors assisted in manuscript preparation.

Notes

The authors declare the following competing financial interest(s): The authors have filed a patent application covering aspects of this technology.

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ACS Synth. Biol. 2018, 7, 696−705

REFERENCES

(1) Pierce, K. L., Premont, R. T., and Lefkowitz, R. J. (2002) Seven-transmembrane receptors. *Nat. Rev. Mol. Cell Biol. 3*, 639−650.

(2) Ault, A. D., and Broach, J. R. (2006) Creation of GPCR-based chemical sensors by directed evolution in yeast. *Protein Eng., Des. Sel. 19*, 1−8.

(3) Dong, S., Rogan, S. C., and Roth, B. L. (2010) Directed molecular evolution of DREADDs: a generic approach to creating next-generation RASSLs. *Nat. Protoc. 5*, 561−73.

(4) Armbruster, B. N., Li, X., Pausch, M. H., Herlitze, S., and Roth, B. L. (2007) Evolving the lock to fit the key to create a family of G protein-coupled receptors potently activated by an inert ligand. *Proc. Natl. Acad. Sci. U. S. A. 104*, 5163−8.

(5) Price, C. P. (2001) Regular review: Point of care testing. *Bmj 322*, 1285−1288.

(6) Petricoin, E. F., Belluco, C., Araujo, R. P., and Liotta, L. a. (2006) The blood peptidome: a higher dimension of information content for cancer biomarker discovery. *Nat. Rev. Cancer 6*, 961−967.

(7) Aluise, C. D., Sowell, R. a, and Butterfield, D. A. (2008) Peptides and proteins in plasma and cerebrospinal fluid as biomarkers for the prediction, diagnosis, and monitoring of therapeutic efficacy of Alzheimer's disease. *Biochim. Biophys. Acta, Mol. Basis Dis. 1782*, 549−558.

(8) Roscioni, S. S., De Zeeuw, D., Hellemons, M. E., Mischak, H., Zürbig, P., Bakker, S. J. L., Gansevoort, R. T., Reinhard, H., Persson, F., Lajer, M., Rossing, P., and Heerspink, H. J. L. (2013) A urinary peptide biomarker set predicts worsening of albuminuria in type 2 diabetes mellitus. *Diabetologia 56*, 259−267.

(9) Young, B. L., Mlamla, Z., Gqamana, P. P., Smit, S., Roberts, T., Peter, J., Theron, G., Govender, U., Dheda, K., and Blackburn, J. (2014) The identification of tuberculosis biomarkers in human urine samples. *Eur. Respir. J. 44*, 1719.

(10) Snell-Bergeon, J. K., Maahs, D. M., Ogden, L. G., Kinney, G. L., Hokanson, J. E., Schiffer, E., Rewers, M., and Mischak, H. (2009) Evaluation of urinary biomarkers for coronary artery disease, diabetes, and diabetic kidney disease. *Diabetes Technol. Ther. 11*, 1−9.

(11) Anderson, N. L., and Anderson, N. G. (2002) The Human Plasma Proteome: History, Character, and Diagnostic Prospects. *Mol. Cell. Proteomics 1*, 845−867.

(12) Pisitkun, T., Johnstone, R., and Knepper, M. a. (2006) Discovery of urinary biomarkers. *Mol. Cell. Proteomics 5*, 1760−1771.

(13) Coll, E., Botey, A., Alvarez, L., Poch, E., Quintó, L., Saurina, A., Vera, M., Piera, C., and Darnell, A. (2000) Serum cystatin C as a new marker for noninvasive estimation of glomerular filtration rate and as a marker for early renal impairment. *Am. J. Kidney Dis. 36*, 29−34.

(14) Lee, M. E., DeLoache, W. C., Cervantes, B., and Dueber, J. E. (2015) A Highly Characterized Yeast Toolkit for Modular, Multipart Assembly. *ACS Synth. Biol. 4*, 975−986.

(15) Bardwell, L. (2005) A walk-through of the yeast mating pheromone response pathway. *Peptides 26*, 339−350.

(16) Coward, P., Wada, H. G., Falk, M. S., Chan, S. D., Meng, F., Akil, H., and Conklin, B. R. (1998) Controlling signaling with a specifically designed Gi-coupled receptor. *Proc. Natl. Acad. Sci. U. S. A. 95*, 352−357.

(17) Chen, Z., and Zhao, H. (2005) Rapid creation of a novel protein function by in vitro coevolution. *J. Mol. Biol. 348*, 1273−1282.

(18) Codreanu, I., Perico, N., Sharma, S. K., Schieppati, A., and Remuzzi, G. (2006) Prevention programmes of progressive renal disease in developing nations. *Nephrology 11*, 321−8.

(19) Coresh, J., Selvin, E., Stevens, L. a, Manzi, J., Kusek, J. W., Eggers, P., Van Lente, F., and Levey, A. S. (2007) Prevalence of chronic kidney disease in the United States. *JAMA 298*, 2038−2047.

(20) Jha, V., Garcia-Garcia, G., Iseki, K., Li, Z., Naicker, S., Plattner, B., Saran, R., Wang, A. Y.-M., and Yang, C.-W. (2013) Chronic kidney disease: global dimension and perspectives. *Lancet 382*, 260−72.

(21) Shlipak, M. G., Katz, R., Sarnak, M. J., Fried, L. F., Newman, A. B., Stehman-Breen, C., Seliger, S. L., Kestenbaum, B., Psaty, B., Tracy, R. P., and Siscovick, D. S. (2006) Cystatin C and Prognosis for Cardiovascular and Kidney Outcomes in Elderly Persons without Chronic Kidney Disease. *Ann. Intern. Med. 145*, 237−246.

(22) Uchida, K., and Gotoh, A. (2002) Measurement of cystatin-C and creatinine in urine. *Clin. Chim. Acta 323*, 121−128.

(23) Bokenkamp, A., Domanetzki, M., Zinck, R., Schumann, G., Byrd, D., and Brodehl, J. (1998) Cystatin C — A New Marker of Glomerular Filtration Rate in Children Independent of Age and Height. *Pediatrics 101*, 875−881.

(24) van Deventer, H. E., Paiker, J. E., Katz, I. J., and George, J. a. (2011) A comparison of cystatin C- and creatinine-based prediction equations for the estimation of glomerular filtration rate in black South Africans. *Nephrol., Dial., Transplant. 26*, 1553−1558.

(25) Siemens Healthcare Diagnostics. https://usa.healthcare.siemens.com/laboratory-diagnostics (accessed 2017).

(26) Storme, M. L., Sinnaeve, B. a., and Van Bocxlaer, J. F. (2005) The use of tryptic marker-peptides for the quantitative analysis of cystatin C. *J. Sep. Sci. 28*, 1759−1763.

(27) Henikoff, S., and Henikoff, J. G. (1992) Amino acid substitution matrices from protein blocks. *Proc. Natl. Acad. Sci. U. S. A. 89*, 10915−10919.

(28) Masui, Y., Tanaka, T., Chino, N., Kita, H., and Sakakibara, S. (1979) Amino Acid Substitution of Mating Factor Saccharomyces cerevisiae: Structure-Activity Relationship. *Biochem. Biophys. Res. Commun. 86*, 982−987.

(29) Abel, M. G., Lee, B. K., Naider, F., and Becker, J. M. (1998) Mutations affecting ligand specificity of the G-protein-coupled receptor for the Saccharomyces cerevisiae tridecapeptide pheromone. *Biochim. Biophys. Acta, Mol. Cell Res. 1448*, 12−26.

(30) Shenbagamurthi, P., Baffi, R., Khan, S. A., Lipke, P., Pousman, C., Becker, J. M., and Naider, F. (1983) Structure-Activity Relationships in the Dodecapeptide alpha Factor of Saccharomyces cerevisiae. *Biochemistry 22*, 1298−1304.

(31) Abel, M. G., Zhang, Y. L., Lu, H. F., Naider, F., and Becker, J. M. (1998) Structure-function analysis of the Saccharomyces cerevisiae tridecapeptide pheromone using alanine-scanned analogs. *J. Pept. Res. 52*, 95−106.

(32) Walsh, G., and Jefferis, R. (2006) Post-translational modifications in the context of therapeutic proteins. *Nat. Biotechnol. 24*, 1241−1252.

(33) Lee Biosolutions. Urine−Cystatin C Positive.

(34) Wu, B., Wijma, H. J., Song, L., Tian, Y., Arif, M. I., Nuijens, T., Quaedflieg, P. J. L. M., Szymanski, W., Feringa, B. L., Janssen, D. B., Rozeboom, H. J., and Poloni, C. (2016) Versatile Peptide C -Terminal Functionalization via a Computationally Engineered Peptide Amidase. *ACS Catal. 6*, 5405−5414.

(35) Stainbrook, S. C., Yu, J. S., Reddick, M. P., Bagheri, N., and Tyo, K. E. J. (2017) Modulating and evaluating receptor promiscuity through directed evolution and modeling. *Protein Eng., Des. Sel. 30*, 455−465.

(36) Ostrov, N., Jimenez, M., Billerbeck, S., Brisbois, J., Matragrano, J., Ager, A., and Cornish, V. W. (2017) A modular yeast biosensor for low-cost point-of-care pathogen detection. *Sci. Adv. 3*, e1603221.

(37) Halls, M. L., and Cooper, D. M. F. (2010) Sub-picomolar relaxin signalling by a pre-assembled RXFP1, AKAP79, AC2, beta-arrestin 2, PDE4D3 complex. *EMBO J. 29*, 2772−2787.

(38) Masui, Y., Chino, N., Sakakibara, S., Tanaka, T., Murakami, T., and Kita, H. (1977) Synthesis of the mating factor of Saccharomyces cerevisiae and its truncated peptides: The structure-activity relationship. *Biochem. Biophys. Res. Commun. 78*, 534−538.

(39) Tallon, M. a, Shenbagamurthi, P., Marcus, S., Becker, J. M., and Naider, F. (1987) Synthesis and biological activity of amino terminus extended analogues of the alpha mating factor of Saccharomyces cerevisiae. *Biochemistry 26*, 7767−7774.

(40) Shenbagamurthi, P., Kundu, B., Raths, S., Becker, J. M., and Naider, F. (1985) Biological activity and conformational isomerism in position 9 analogues of the des-1-tryptophan,3-beta-cyclohexylalanine-alpha-factor from Saccharomyces cerevisiae. *Biochemistry 24*, 7070−7076.

DOI: 10.1021/acssynbio.7b00410
ACS Synth. Biol. 2018, 7, 696−705

(41) Raths, S. K., Naider, F., and Beckers, J. M. (1988) Peptide Analogues Compete with the Binding of α-Factor to Its Receptor in Saccharomyces cerevisiae. *J. Biol. Chem 263*, 17333.
(42) Petersen, B., Lundegaard, C., and Petersen, T. N. (2010) NetTurnP - neural network prediction of beta-turns by use of evolutionary information and predicted protein sequence features. *PLoS One 5*, e15079.
(43) Shepherd, a J., Gorse, D., and Thornton, J. M. (1999) Prediction of the location and type of beta-turns in proteins using neural networks. *Protein Sci. 8*, 1045−1055.
(44) Manfredi, J. P., Klein, C., Herrero, J. J., Byrd, D. R., Trueheart, J., Wiesler, W. T., Fowlkes, D. M., and Broach, J. R. (1996) Yeast alpha mating factor structure-activity relationship derived from genetically selected peptide agonists and antagonists of Ste2p. *Mol. Cell. Biol. 16*, 4700−4709.
(45) Schmerberg, C. M., and Li, L. (2013) Mass Spectrometric Detection of Neuropeptides Using Affinity- Enhanced Microdialysis with Antibody-Coated Magnetic Nanoparticles. *Anal. Chem. 85*, 915.
(46) Li, Q., Zubieta, J.-K., and Kennedy, R. T. (2009) Practical aspects of in vivo detection of neuropeptides by microdialysis coupled off-line to capillary LC with multistage MS. *Anal. Chem. 81*, 2242−2250.
(47) Kleerebezem, M., Quadri, L. E., Kuipers, O. P., and de Vos, W. M. (1997) Quorum sensing by peptide pheromones and two-component signal-transduction systems in Gram-positive bacteria. *Mol. Microbiol. 24*, 895−904.
(48) Mumberg, D., Mueller, R., and Funk, M. (1995) Yeast vectors for the controlled expression of heterologous proteins in different genetic backgrounds. *Gene 156*, 119−122.
(49) Chao, G., Lau, W. L., Hackel, B. J., Sazinsky, S. L., Lippow, S. M., and Wittrup, K. D. (2006) Isolating and engineering human antibodies using yeast surface display. *Nat. Protoc. 1*, 755−768.
(50) Gietz, R. D., and Schiestl, R. H. (2007) Large-scale high-efficiency yeast transformation using the LiAc/SS carrier DNA/PEG method. *Nat. Protoc. 2*, 38−41.
(51) Green, M. R., and Sambrook, J. (2012) *Molecular Cloning: A Laboratory Manual*, 4th ed., Cold Spring Harbor Laboratory Press.
(52) Benatuil, L., Perez, J. M., Belk, J., and Hsieh, C. M. (2010) An improved yeast transformation method for the generation of very large human antibody libraries. *Protein Eng., Des. Sel. 23*, 155−159.

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ACS Synth. Biol. 2018, 7, 696−705