EVOLUTIONARY ALTERNATIVES ON THE ROAD TO A NEW SPECIFICITY

IN A G PROTEIN-COUPLED RECEPTOR

by

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A thesis submitted in conformity with the requirements
for the degree of Doctor of Philosophy
Graduate Department of Cell and Systems Biology
University of Toronto

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Abstract

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Cells sense change in their external environment and react appropriately through the action of signaling pathways. This process is initiated by receptor proteins with high degrees of specificity for a particular stimulus. G protein-coupled receptors form the largest family of membrane protein receptors and their ability to sense a broad variety of ligands is unparalleled despite their common ancestral origin. How GPCRs evolved their unique specificities is unknown, although ligand binding affinity is often given a central role. The goal of this thesis was to assess the possible contributions of secondary mechanisms of specificity, namely ligand efficacy and downstream signaling regulation, to changes in ligand recognition. Through directed evolution, we generated a yeast pheromone receptor with an altered specificity in two steps. First, promiscuous receptors were obtained through either improved binding affinity or weaker molecular interaction with a negative regulator of signaling. Second, a ligand-discriminating receptor was obtained from a promiscuous variant solely by reducing the efficacy of the native pheromone. These findings demonstrate the importance of assessing GPCRs’ pharmacological profiles in their native context, where signaling trumps binding affinity in significance.
Acknowledgements

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List of Abbreviations

BIFC: Bimolecular fluorescence complementation

*Cgla: Candida glabrata*

EGFR: Epidermal growth factor receptor

FACS: Fluorescence-activated cell sorting

FRET: Förster/fluorescence resonance energy transfer

GFP: Green fluorescent protein

GPCR: G protein-coupled receptor

*Klac: Kluyveromyces lactis*

MAPK: Mitogen-activated protein kinase

NBD: Nitrobenzoxadiazole

ORF: Open reading frame

RGS: Regulator of G protein signaling

*Scer: Saccharomyces cerevisiae*

SDP: Specificity-determining position

SEM: Standard error of the mean

TMD: Transmembrane domain

WT: Wild-type
Chapter 1:

GENERAL INTRODUCTION

"What counts in making a happy marriage is not so much how compatible you are but how you deal with incompatibility."

— Leo Tolstoy

All organisms can sense changes in their environment and react accordingly. The perception of external stimuli is a fundamental property of life and a necessity for survival. Whether it’s a mouse noticing a cat ready to pounce, or a bacterium finding itself in a sugar-rich solution, the steps of perception are the same: the organism senses, interprets and finally reacts to the hazard or the opportunity. At the cellular level, a continuous flow of information links all three steps and enables an appropriate decision. Molecular biology has transformed our understanding of information trafficking in cells by uncovering the large networks of proteins and small molecules that are involved. These signaling networks are composed of a variety of components fulfilling specific tasks in the sensing, interpreting and responding stages, such as membrane or cytoplasmic receptors, protein-modifying enzymes, scaffolds, second messengers, transcription factors, and many others. Each of these can interact with one or more of the other components, resulting in a well-connected pathway where information can flow in multiple directions through branching points and feedback mechanisms.
The impressive size and connectivity of signaling networks has given rise to an important question: how did they become so complex? The answer may lie within a more global trend: the increasing complexity of organisms [1]. For instance, the evolution of multicellular life from unicellular organisms required more sophisticated cell-cell communications, while the rise of heterotrophy required dedicated nutrient receptors and the tunable expression of catabolic enzymes. The growing importance of cell signaling in evolution is supported by the expanding signaling protein repertoire when comparing prokaryotes to unicellular eukaryotes to metazoans [2]. Interestingly, the rapidity of this expansion has been attributed to the high level of modularity conferred by the domain-based organisation of signaling proteins. Protein domains, by acting as independent functional units, can be shuffled among each other to generate novel proteins with new functions [3, 4]. Alternatively, disordered regions of proteins are also thought to facilitate evolution by acting as reservoirs of weak but diverse domain-interacting motifs [5, 6]. In such regions, point mutations lead to the continuous creation and destructions of interaction motifs, while deleterious effects are limited in scope due to the absence of a global structure. While such theories are useful for understanding how signaling networks evolved, their applicability is limited to proteins with modular domains and/or significant disordered regions. In other cases, different angles must be considered.

The large family of G protein-coupled receptors (GPCRs) is an especially interesting case for studying the evolution of signaling proteins. Members of this family are widespread among eukaryotes and take part in numerous physiological functions such as neurotransmission, inflammation, vision, taste and olfaction. As a family, they are
characterized by shared features in their structure, function and regulation. Most notably, GPCRs are composed of seven transmembrane helices organized in a bundle. These are linked by alternating intracellular and extracellular loops of variable lengths in a “serpentine” structure, while the N- and C-terminal ends are extracellular and cytoplasmic respectively. GPCRs also share a primary mechanism of signal transduction involving the exchange of GDP for GTP in a heterotrimeric G protein, although some exceptions exist [7]. Lastly, GPCRs have common regulatory interactions. For instance, there is growing evidence that many GPCRs exist as homo- and hetero-oligomers and that these complexes enable cross-talk between receptor units affecting ligand binding and signaling [8]. GPCR monomers and oligomers are also internalized, both constitutively and following ligand-induced activation, in order to desensitize cells from further stimulation [9]. Finally, GPCRs can interact with a variety of regulators, such as arrestins and regulators of G protein signaling (RGS) [10]. In the latter case, the G protein is the true target of the RGS, but the GPCR can bring both in proximity to each other, thus acting as a scaffold [11, 12]. Clearly, GPCRs are more than just transmembrane signal transducers; they are fully-integrated parts of signaling networks, playing multiple roles in both signal transmission and suppression.

The conserved features of GPCRs suggest that they diverged from a limited set of common ancestors [13]. With over 800 members in humans alone, GPCRs form the largest family of membrane proteins. This impressive diversity is matched by the wide range of stimuli that GPCRs can sense, which includes peptides, glycoproteins, small molecules, photons, lipids, nucleotides and ions. However, it remains unclear how these
various specificities evolved from common origins. Since GPCRs are not composed of modular domains, and their ligand-binding regions are not thoroughly disordered, the evolution of GPCR specificity cannot be explained through these features. Instead, a theory tailored to GPCRs must be developed taking into account the molecular basis of GPCR-ligand recognition as well as its ability to change. Much work has focused on understanding the former. Pioneering mutational and structural analyses helped map the amino acid residues that form ligand binding pockets [14, 15]. More recently, advances in X-ray crystallography, computer modeling and the optimized expression of stable transmembrane proteins has led to an ever expanding set of solved GPCR structures [15]. These have provided a detailed view of the conformational changes that accompany the ligand binding event. But while our understanding of the molecular basis of GPCR specificity is improving, much less is known about how specificity is lost or gained. This question has important implications far beyond evolutionary biology. For instance, mutations affecting ligand specificity have been linked to diseases like hyperthyroidism and ovarian hyperstimulation syndrome [16]. Likewise, GPCR engineering often focuses on specificity. Sometimes, this involves preserving it when the goal is to optimize expression in a heterologous system for subsequent functional assays or crystallization [17-19]. Alternatively, it may be desirable to change a receptor into one that is solely activated by a synthetic ligand (RASSLs) [20-22]. One may also seek to enhance sensitivity to biological compounds, such as sugar nucleotides [23], or industrial compounds like landmine explosives [24]. For such applications, a greater understanding of the evolution of specificity would be very beneficial.
Studies focusing on changes in ligand specificity have typically used two approaches. Where relevant sequence and structural data are abundant, computational analyses have been used to compare related GPCRs with different cognate ligands and simulate changes in binding pocket architecture [25, 26]. Alternatively, where this information is lacking, an experimental approach involving mutagenesis and functional assays can be preferable. Unfortunately, due to the difficulty of studying GPCRs in their native context, what we know about GPCR specificity is largely restricted to receptors in isolation, particularly their ligand-binding region, while the role of regulatory interactions is mostly unknown. Likewise, the ease of measuring binding affinity\(^1\) rather than signaling activity has given an outsized importance to the former when it comes to analyzing determinants of specificity. As such, the contributions of regulatory interactions and ligand efficacy\(^2\) are not well-characterized.

In order to fill this gap in knowledge, we have turned towards the yeast *Saccharomyces cerevisiae*, also known as budding yeast. As a unicellular fungus, yeast has long been an invaluable tool for studying eukaryotic biology with longstanding techniques developed for bacteria. Besides its robustness and the possibility of growing it in large quantities, a key advantage of yeast lies in its propensity for genomic recombination. This feature facilitates the replacement of yeast genes with auxotrophic or antibiotic resistance markers, enabling the study of phenotypes linked to gene deletions. The importance of the yeast genome to cell biology led to its complete sequencing in 1996, the first among

\(^1\) Binding affinity refers to the ability of a ligand to physically associate and form non-covalent bonds with a receptor.

\(^2\) Ligand efficacy refers to the ability of a ligand to induce a conformational change in a receptor leading to its activation, i.e. stabilization of a receptor’s active state.
eukaryotes [27]. As with many other fungi, the life cycle of budding yeast is characterized by both haploid and diploid states which are linked by a sexual event consisting in the fusion of two haploid cells [28]. While mating is beneficial for mixing divergent DNA, it is a risky process. Since yeast is non-motile, mating requires growing towards the potential partner without division, resulting in a distorted cell affectionately called a “shmoo”. Shmooing is thus resource-expensive, and can result in failure to mate if the potential partner is either out of reach, or incompatible. Indeed, *S. cerevisiae* haploids can be one of two mating types, a and α. The delicate balance between sensitivity and specificity is managed by a chain of signaling events, known as the mating pathway, and at the top of this chain sit a pair of GPCRs called Ste2 (YFL026W) and Ste3 (YKL178C). These receptors are pheromone sensors; they detect the peptides α-factor and a-factor that are secreted by their respective mating type. It is through the activation of Ste2 or Ste3 receptors that a haploid cell can sense the presence of compatible mates in its proximity, and decide whether it should begin shmooing. This sensory input is localized and tuned to respond to an appropriate range of pheromone concentrations. Much research has focused on the mating pathway since it is composed of many hallmarks of signaling networks: a membrane receptor, a heterotrimeric G protein, a protein kinase cascade, a scaffolding protein, transcription factors, etc. Importantly, most of these components have direct homologs in humans and mice [29-31], with the notable exception of the membrane receptors. Still, investigations into Ste2 structure and regulation confirmed that, much like its counterparts in higher organisms, it acts as a guanine nucleotide-exchange factor (GEF), it undergoes ligand-induced endocytosis and it oligomerizes. Comparatively less is known about Ste3, but this is due to the difficulty
of synthesizing its ligand, a farnesylated peptide. On the other hand, α-factor, which does not need any modification to be active, lends itself well to artificial synthesis and laboratory experimentation with cells expressing Ste2.

Studies focusing on Ste2 signaling have revealed much about how this GPCR operates and which regions of the receptor are involved in each aspect of its function. The receptor’s binding region has been probed extensively using various ligands, including radio-labelled and fluorescent α-factor, α-factor analogs and the alternative agonist novobiocin [32-37]. This has led to a model whereby the 13 amino acid pheromone is bent around its center and each end interacts with specific residues of TM5 and TM6 of the receptor (for more details, an excellent review on the matter was written by Naider and Becker [38]). Likewise, sites involved in receptor activation have also been investigated by random and scanning mutagenesis [39-44]. Cross-linking and FRET (fluorescence resonance energy transfer) studies have shown that Ste2’s ability to oligomerize depends on its extracellular tail, TM1 and TM4 [45-49], while clathrin-mediated endocytosis has been shown to occur following the sequential phosphorylation and ubiquitination of the cytoplasmic tail [50]. The C-terminal tail of Ste2 is also known to interact with Sst2 (YLR452C), an RGS that downregulates the mating pathway response by inducing the GTPase activity of the G protein α subunit, Gpa1 (YHR005C) [11]. As can be seen, the S. cerevisiae α-factor receptor has been extensively studied for many years, with prolific results.
Yet, despite extensive knowledge of Ste2 functionality, few studies have examined the determinants of its specificity with respect to other yeast pheromones. The Ascomycota family, of which *S. cerevisiae* is a member, includes over 70 yeast species, each of which harbors unique Ste2 and α-factor homologs [28, 51]. Pheromones from one species do not typically induce a response in another, making the mating apparatus a key component of sexual isolation and speciation. A 1992 study first explored the ability of *Saccharomyces kluyveri* α-factor to induce a response in *S. cerevisiae* Ste2 mutants [52], while subsequent work by the same group used Ste2 chimeras to identify specificity-determining positions (SDPs) [53, 54]. However, no study has completed a full inversion of specificity in Ste2. Instead, the new variants were found to be promiscuous; they could still respond strongly to *S. cerevisiae* α-factor. Thus, the evolution of a new specificity was not previously demonstrated in Ste2 despite its well-characterized mode of action.

We sought to better understand how a GPCR can fully change ligand specificity by focusing on Ste2 in budding yeast. We envisioned a scenario in which a hybrid of two related yeast species, *S. cerevisiae* and *Kluyveromyces lactis* (hereafter abbreviated Scer and Klac), finds itself with mismatching pheromones and receptors (Ste2 and Klac α-factor). Occurrences of interspecies mating have been reported among food yeasts [55, 56] and pathogenic yeasts [57]. Although hybrids are usually sterile due to post-zygotic barriers [58], genomic re-modeling can preserve fertility and such an event is thought to be at the root of the Scer lineage [59]. For our hypothetical Scer-Klac hybrid, mating with other hybrids would be difficult since a cells would ignore α cells which would be perceived as foreign [60]. This predicament would be at least partially overcome by a
promiscuous variant of Ste2, but a full inversion of specificity would be necessary to establish a sexual barrier with Scer cells and undergo speciation. We hypothesized that such a switch in specificity would be facilitated through changes in ligand efficacy and in the regulation of Ste2 signaling rather than proceed exclusively through changes in ligand binding affinity.

In order to alter the ligand specificity of Ste2, we favored a directed evolution approach. Unlike engineering by design, directed evolution does not depend on prior information about a protein’s structure. This is ideal for a fungal GPCR like Ste2 which has no comprehensive structural data. Instead, directed evolution relies on a diverse library of random mutants and a selection process that targets a desired phenotype. This mimics the random changes and fitness requirements that characterize natural evolution. It has proven to be a powerful approach for obtaining highly performing enzymes, as well as for uncovering broad trends in protein evolution. This is because directed evolution provides the full evolutionary record from an ancestor to its descendant, including both the mutations that were selected and the order in which they appeared. This has been used to generate “maps” of possible evolutionary trajectories that revealed the importance of neutral mutations for obtaining novel protein functions [61-63] as well as the occurrence of generalist intermediates: proteins that can accomplish both their native function and the new, selected function [23, 64-67]. Thus, laboratory evolution is a powerful approach for understanding real evolution and was thus especially well-suited to our interests.
By performing the directed evolution of a receptor in its native context, i.e. Ste2 in yeast, rather than expressing a human GPCR in yeast, we reasoned that we would be able to uncover the contribution of pheromone efficacy, receptor internalization and Sst2 signal suppression on ligand specificity, none of which would be present in a heterologous expression system. In order to account for all these different processes, we chose to rely on a reporter of mating pathway activation [68]. This GFP-based reporter can be combined with fluorescence-activated cell sorting (FACS), a high-throughput method of selection. In this way, we sought to rapidly sample a large fraction of a library of Ste2 mutants based on their ability to signal, encompassing all aspects of their function.

Using this method, we were able to induce a full inversion of specificity in Ste2 towards the *Klac* pheromone. We achieved this in two steps. First, we used positive selection in order to generate a promiscuous Ste2 variant [69]. Our investigation into the molecular basis of promiscuity revealed the important role of network interactions in this process. Second, we completed our evolutionary scenario by restricting the specificity of a promiscuous Ste2 variant so that it could no longer respond to the native pheromone. This was done by combining both positive and negative selection rounds. This way, we revealed SDPs on both the receptor and its pheromone, as well as the importance of ligand efficacy on receptor discrimination. During both steps, we found that evolution can proceed through different ways; there are multiple different paths to the same destination. Here, I present the results of my work and I discuss its implications for evolutionary biology and GPCR pharmacology studies.
Chapter 2:

Evolution of a G-Protein Coupled Receptor Response by Mutations in Regulatory Network Interactions

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ABSTRACT

All cellular functions depend on the concerted action of multiple proteins organized in complex networks. To understand how selection acts on protein networks, we used the yeast mating receptor Ste2, a pheromone-activated G protein-coupled receptor, as a model system. In *Saccharomyces cerevisiae*, Ste2 is a hub in a network of interactions controlling both signal transduction and signal suppression. Through laboratory evolution, we obtained 21 mutant receptors sensitive to the pheromone of a related yeast species and investigated the molecular mechanisms behind this newfound sensitivity. While some mutants show enhanced binding affinity to the foreign pheromone, others only display weakened interactions with the network’s negative regulators. Importantly, the latter changes have a limited impact on overall pathway regulation, despite their considerable effect on sensitivity. Our results demonstrate that a new receptor-ligand pair can evolve through network-altering mutations independently of receptor-ligand binding, and suggest a potential role for such mutations in disease.
INTRODUCTION

Important cellular processes result from the concerted action of multiple proteins organized in complex networks. Studies in evolution have revealed how individual proteins can acquire new functions due to changes in their binding specificity or catalytic potential [70-74]. However, these characteristics alone often cannot explain the evolution of complex cellular functions, because network output does not solely depend on the function of an individual protein, but rather on the integrated function of multiple components with intricate regulatory relationships [75].

Past work has found evidence of network “re-wiring” in evolution from large-scale data [76-78], whereby proteins are conserved across organisms but the interactions between them differ, although the molecular basis of such re-wiring is not always evident. Studies focusing on well-characterized signaling systems in bacteria [79], yeast [80] and mammals [81, 82] have shed light on how changes in protein interactions can alter regulatory networks. Similarly, recent work has demonstrated that domain shuffling can extensively re-wire a signaling network by exchanging interaction motifs between proteins [3, 4, 83], a process which has been linked to the evolution of organism complexity [1]. Though much progress has been made, it remains unclear how selection acts on regulatory networks during the evolution of a new function.

To examine this question, we followed in real time the initial steps in the evolution of the response to a new ligand in the yeast pheromone receptor Ste2, a G protein-coupled receptor (GPCR). In *Saccharomyces cerevisiae* (hereafter abbreviated Scer) and in
related ascomycetes, the mating process involves the fusion of two haploid cells to produce a diploid daughter [84]. In one mating type, this process is triggered when the pheromone α-factor binds to its cognate receptor Ste2. This GPCR acts as a network hub, mediating both signal transduction and signal suppression through interactions with multiple protein partners (Figure II-1A). These interactions have been extensively studied and are found across eukaryotes [29-31]. For signal transduction, pheromone-bound Ste2 mediates the exchange of GDP for GTP in a heterotrimeric G protein. This triggers a mitogen-activated protein kinase (MAPK) cascade leading to the expression of mating-related genes. In addition, Ste2 contributes to signal suppression through its cytoplasmic domain. First, the receptor brings Sst2, a regulator of G-protein signaling (RGS), in proximity to its target, Gpa1α, to promote GTP hydrolysis and shut down signaling [11]. Second, Ste2 is internalized as homo-oligomers and degraded both constitutively and upon ligand binding, a process that involves sequential receptor phosphorylation, ubiquitination and the recruitment of the clathrin endocytic machinery [85-88]. Ste2’s central role in both network activation and signal suppression, along with its well-characterized and highly conserved regulatory interactions, make it an ideal target to investigate the contribution of network-altering mutations to the evolution of new function.
Figure II-1: The G protein-coupled receptor Ste2, a network hub in the yeast mating pathway, is used to study the contribution of network interactions in the evolution of a new response. A, Ste2 contributes to both signal transduction and signal suppression through a variety of physical interactions. B, Dose-response relationship of cells expressing GFP in response to mating pathway activation. Cells were treated with various concentrations of pheromone and incubated for 3 hours. GFP fluorescence was measured by flow cytometry. Wild-type Ste2 confers a weak, low-sensitivity response to Klac α-factor, a foreign pheromone. Error bars represent the s.e.m. C, Schematic view of our directed evolution method. A plasmid library of STE2 mutants generated by error-prone PCR was transformed in ste2Δ yeast. Cells were treated with 5 µM Klac α-factor and incubated for 3 hours. Fluorescence-activated cell sorting was used to select for highly-activating variants which were then plated. Individual colonies were screened to confirm the desired phenotype and sequenced.

We hypothesized that the evolution of a new function in the yeast mating pathway could occur through changes in the signaling hub Ste2. To test this, we mimicked an evolutionary scenario in which Scer cells were under selection pressure to respond to a
weak agonist, the pheromone of the related species *Kluyveromyces lactis* (hereafter abbreviated *Klac*). To focus on the receptor and its interactions, we introduced random mutations only in Ste2. We then used high-throughput selection to isolate cells that activated the mating pathway in response to *Klac* α-factor. We investigated the contribution of network-altering mutations by performing a detailed phenotypic analysis on a subset of selected variants.

Our analysis revealed two distinct evolutionary paths: a “classical” path involving improvements in binding affinity for the foreign agonist; and a “network-altering” path, wherein the interaction between the receptor and the RGS is no longer conducive to signal suppression due to a partial loss of the receptor’s cytoplasmic tail. Importantly, receptor truncations have only a limited effect on pathway regulation, suggesting that the partial loss of this interaction-rich region can be an acceptable evolutionary strategy, an observation supported by the large variability in cytoplasmic tail lengths found among Ste2 homologs. Together, these results point to a novel mechanism of network evolution, and suggest a possible link between RGS proteins and disease-causing GPCR mutations.

**RESULTS**

**Directed evolution of Ste2 yields diverse response profiles**

To characterize the mating response of *Scer* cells with different pheromones, we used a strain in which the promoter of the gene *FUS1* drives the expression of green fluorescent protein (GFP) [68]. We found that wild-type (WT) *Scer* cells’ response to *Klac* α-factor was weak, but detectable, with a lower sensitivity (higher EC50) and a lower maximum than the response to *Scer* α-factor (Figure II-1B). We also tested the α-factor pheromone.
of two more related species: *Naumovozyma castellii* (formerly *Saccharomyces castellii*) and *Candida glabrata* (abbreviated *Cgla*). However, the former elicited a response comparable to that of the native *Scer* pheromone while the response to the latter was negligible (data not shown). As we sought a weak, but measurable response, we proceeded to use *Klac* α-factor for our directed evolution experiment.

We used directed evolution to obtain variants of the pheromone receptor Ste2 that conferred a strong response to *Klac* α-factor (Figure II-1C). First, we transformed a *ste2Δ* yeast strain with a plasmid-based library of *STE2* mutants generated by error-prone PCR. We then used fluorescence-activated cell sorting (FACS) to select cells able to respond strongly to treatment with 5 µM *Klac* α-factor. After two iterative rounds of cell sorting followed by a screening step to isolate individual non-constitutive variants, we obtained 21 mutant receptors capable of responding strongly to *Klac* α-factor.

Sequencing of the selected Ste2 variants revealed a diversity of genotypes with one or more protein mutations (Supplementary Table II-1), and mutated sites spread throughout the entire receptor (Supplementary Figure II-1A). The mutant receptors were labeled according to their most severe protein mutation (S: substitution, T: truncation, F: frameshift) and numbered. Many of the mutated sites were recurrent within our set of selected variants, or had been implicated in receptor function in past studies [35-37, 40, 43, 48, 52, 86, 89-93]. We found that all selected receptors retained their ability to respond strongly to *Scer* pheromone, with most also displaying the ability to respond to *Cgla* α-factor.
(Supplementary Figure II-1B). These two features, a robust native response and the facile emergence of promiscuity in the function under selection, are thought to underlie the evolution of new protein functions in nature [64, 65, 94, 95].

<table>
<thead>
<tr>
<th>Name</th>
<th>Protein mutations</th>
<th>Scer EC50 (nM)</th>
<th>Klac EC50 (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>N/A</td>
<td>8.74 ± 2.2</td>
<td>726 ± 240</td>
</tr>
<tr>
<td>S1</td>
<td>I82N, N216D, Y266F</td>
<td>4.36 ± 0.42</td>
<td>19.1 ± 6.3</td>
</tr>
<tr>
<td>S2</td>
<td>V280I</td>
<td>5.90 ± 1.2</td>
<td>107 ± 2.5</td>
</tr>
<tr>
<td>S3</td>
<td>S267C,V280D, K358R, T414M</td>
<td>1.73 ± 0.096</td>
<td>6.24 ± 1.4</td>
</tr>
<tr>
<td>S4</td>
<td>N216S</td>
<td>8.45 ± 0.11</td>
<td>24.1 ± 7.6</td>
</tr>
<tr>
<td>S5</td>
<td>S267R, T282S</td>
<td>3.30 ± 0.19</td>
<td>86.6 ± 28</td>
</tr>
<tr>
<td>S6</td>
<td>M54V, A62T, M69L, G115R</td>
<td>7.81 ± 0.11</td>
<td>1500 ± 620</td>
</tr>
<tr>
<td>T1</td>
<td>Y30H, K358*</td>
<td>0.375 ± 0.14</td>
<td>80.4 ± 12</td>
</tr>
<tr>
<td>T2</td>
<td>T78M, A336D, K337P, S338E, S339*</td>
<td>0.909 ± 0.20</td>
<td>100 ± 8.9</td>
</tr>
<tr>
<td>T3</td>
<td>G237D, F312L, R350*</td>
<td>0.758 ± 0.0049</td>
<td>84.7 ± 20</td>
</tr>
<tr>
<td>F1</td>
<td>N25D, K202T, T309N, A397E, Fs401</td>
<td>0.649 ± 0.27</td>
<td>224 ± 1.8</td>
</tr>
</tbody>
</table>

Asterisks designate a premature stop codon while “Fs” designates a frameshift mutation. Values represent the mean ± s.e.m.

In order to characterize Ste2 mutants in detail and uncover potential changes in receptor-network interactions, we focused on a subset of 10 variants with sites mutated more than once and/or mutated sites known to affect Ste2 signaling such as V280 [40] or C-terminal lysines [89] (Table II-1). We first measured the dose-response relationship of each variant with either Klac or Scer α-factor in order to identify possible phenotypic clusters. As shown in Figure II-2 (left column), we visually grouped mutants into four clusters based on differences in their sensitivity (EC50), baseline response and maximum
response. Interestingly, the patterns uncovered with Scer pheromone were not found with Klac \( \alpha \)-factor, with the latter yielding more diverse dose-response relationships (Figure II-2, right column). This diversity was probably a consequence of our selection regime, wherein the single concentration of foreign pheromone used (5 \( \mu \)M) imposed no constraints on the strength of the response at other concentrations, making various sensitivities and Hill coefficients permissible.
**Figure II-2: Ste2 variants selected for their ability to confer a strong response to a foreign pheromone exhibit diverse response profiles.** Dose-response profiles of selected Ste2 variants using either the native or foreign pheromone. Variants were visually clustered according to the shape of their response to Scer α-factor. Error bars represent the standard error of the mean (s.e.m.)

**Binding affinity does not explain all acquired sensitivity**

Many of the selected Ste2 variants harboured mutations in the extracellular loops of the receptors or in regions that were previously implicated in ligand binding [32, 33, 37]. To differentiate between variants that had acquired a greater binding affinity for the foreign pheromone and those that had not, we measured receptor-ligand affinity in live cells using a fluorescently-labelled pheromone and flow cytometry. As the assay was done with live cells, we were able to simultaneously measure the amount of receptors at the cell surface for each variant ($B_{\text{max}}$). Cells were kept on ice during measurements to minimize the effects of ligand-induced receptor internalization, ensuring that $B_{\text{max}}$ values are ligand-independent.

We found that all variants tested displayed a strong binding affinity to Scer α-factor (Figure II-3A). Conversely, we observed important differences across variants when comparing $B_{\text{max}}$ values and Klac α-factor affinity (Figure II-3B). Half of the variants assayed displayed a stronger affinity for Klac α-factor than the wild-type (4 out of 10 when considering statistical significance), but had no increases in surface receptor expression. Of the remaining variants, 3 were highly expressed at the surface (2 were significant). Interestingly, 4 variants in total had dramatic changes in the receptor’s cytoplasmic tail resulting from either a premature stop codon or a frameshift mutation.
Among the mutant receptors showing a high binding affinity for *Klac* α-factor, variants S1, S2 and S4 showed an unchanged response sensitivity to *Scer* α-factor, but different sensitivities to *Klac* α-factor (Figure 2). For these, the ligand-specific effects of their mutations suggested that changes at their binding site specifically contributed to their phenotype. However, the presence of several low-affinity variants led us to conclude that a stronger receptor-ligand interaction was not the sole evolutionary path favoured by our directed evolution experiment. For such variants, we had to consider other possible mechanisms.
Figure II-3: Ligand binding affinity and surface receptor expression suggest independent mechanisms behind sensitivity to Klac α-factor. A. Binding constants of Ste2 variants for
NBD-labelled Scer pheromone. Values were obtained from a saturation assay in live cells using flow cytometry. None of the Ste2 variants displayed a significant difference to wild-type (Dunnett's Multiple Comparison Test). Values were also within the range of affinities previously reported for wild-type Ste2 and Scer α-factor [38]. Error bars represent the s.e.m. B, Binding constants for Klac α-factor (left) plotted alongside surface receptor expression (right). Binding constants were obtained from a competition assay using a range of concentrations of Klac α-factor and a constant concentration of 20 nM NBD-labelled Scer α-factor. Surface expression was measured from a saturation assay. A subset of Ste2 variants exhibit high binding affinity for the foreign pheromone, while another subset displays high surface receptor expression. Asterisks indicate statistically significant differences to wild-type (P < 0.05, Dunnett's Multiple Comparison Test). Error bars represent the s.e.m. C, Fungal Ste2 homologs were obtained from PSI-BLAST and transmembrane domain topologies were predicted by TMHMM [96]. The lengths of C-terminal tails were extrapolated from the predicted topologies and plotted as a frequency distribution. This wide distribution encompasses the lengths of our Ste2 tail truncation mutants (indicated by labels above the bars).

**GPCR tail length variability is found in natural evolution**

We were surprised by the prominence of C-terminal tail truncations among low-affinity variants due to the role of this region in numerous regulatory interactions and the apparent severity of this type of mutation. In total, we isolated five variants with premature stop codons with tail lengths of 39, 50, 58, 103 and 124 amino acids. This remarkable tolerance to truncations suggests that this region does not adopt a well defined structure [97] and this was confirmed with FoldIndex© [98], a predictor of disordered regions (Supplementary Figure II-2). To determine if cytoplasmic tail length variability is a natural trait of GPCRs, we examined the distribution of tail lengths in fungal Ste2 homologs. Through PSI-BLAST and the transmembrane domain predictor TMHMM [96], we retrieved 225 fungal GPCR and their cytoplasmic tail sequences. We found a wide distribution of tail lengths, ranging from less than 15 amino acids to about
240 amino acids, which encompassed those of our selected Ste2 variants (Figure II-3C). This confirmed that the truncated receptors selected in our study resemble Ste2 homologs found in nature and pointed to numerous truncation-elongation events in the evolutionary history of GPCRs.

We hypothesized that the partial loss of the cytoplasmic tail seen in some variants was a driving mutation behind *Klac* α-factor sensitivity. Notably, variants T1 and T2 contained a premature stop codon in their C-terminal tail and only one other mutation occurring outside of this region. We found that removing the latter mutation, as well as the sequence downstream of the stop codon to eliminate any possible read-through did not affect the overall properties of the truncated receptors (now called T1* and T2*) (Supplementary Table II-2), suggesting that their phenotype could be attributed to the truncation. To determine how tail truncations affected the function of the evolved receptors, we subsequently focused on these mutants.

**Partial tail truncations impair the Ste2-Sst2 interaction**

The receptor’s C-terminal tail has been linked to two aspects of negative pathway regulation: receptor internalization (also called receptor endocytosis) and Sst2 recruitment. We confirmed via receptor-GFP fusions that T1* and T2* were defective at internalizing, both with and without pheromone (Supplementary Figure II-3). We reasoned that this was the cause of their high $B_{\text{max}}$ values due to the accumulation of receptors at the cell surface. In principle, one might expect that higher surface receptor expression would enable the sensing of lower pheromone concentrations, resulting in a
more sensitive response. However, past studies have shown that defective endocytosis has only a minor effect on sensitivity to Scer α-factor [99] and this may only be apparent during long-term exposure to pheromone [100]. Likewise, impaired homooligomerization, a key feature of endocytosis, does not appear to greatly affect signaling although this has not been studied systematically [101]. Lastly, the impact of receptor overexpression is more controversial [11, 102-104].

To understand these effects, we constructed a mathematical model of a simplified Ste2 system (Supplementary Figure 4, Supplementary Note II-1). Our model confirmed that impaired receptor endocytosis results in the accumulation of receptors at the cell surface, but this was not predicted to shift the sensitivity of the response. Instead, our model predicted that the EC50 could be altered by unequal changes in the basal and induced rates of receptor internalization. This result was also observed in an analogous model focusing on the epidermal growth factor receptor (EGFR) [105]. However, based on simulations with many experimentally-derived parameter values, we found that Ste2 mutants solely defective in endocytosis would only show modest effects on sensitivity.

Our mathematical model led us to consider the receptor’s interaction with the RGS Sst2 as the main source of the sensitivity shift. Ste2 is thought to bring Sst2 in proximity of its membrane-anchored target, the GTPase subunit of the heterotrimeric G protein, through a physical interaction that enables efficient pathway deactivation [11]. This interaction is abolished in the RGS mutant Sst2^{Q304N}, and this confers a greater sensitivity to the native pheromone [106].
To experimentally test whether a greater sensitivity to *Klac* α-factor could result from receptor overexpression, impaired endocytosis and/or Sst2 recruitment, we measured *Klac* α-factor sensitivity under each scenario. First, we designed a gene construct in which wild-type receptor expression was driven by the strong promoter of the gene *ADH1* (pADH1). The $B_{\text{max}}$ value for pADH1-expressed Ste2 was 6.05 +/- 0.65 fold higher than that of Ste2 expressed from its endogenous promoter. Second, we obtained a Ste2 variant in which all C-terminal lysines involved in endocytosis were substituted to arginine, dubbed 7KtoR [89]. This variant is defective in endocytosis but not in Sst2 recruitment [11]. Third, we co-expressed the wild-type receptor with Sst2$^{Q304N}$. The resulting dose-response curves and their EC50 values are shown in Figure II-4A and II-4B respectively. Strikingly, we found that among the three scenarios, only Sst2$^{Q304N}$ conferred improved sensitivity to *Klac* α-factor. Since we allow GFP expression to proceed for 3 hours before making measurements, we considered the possibility that signaling under conditions of high receptor expression or low endocytosis was quashed by Sst2 over time. Therefore, we measured MAPK phosphorylation 30 minutes after *Klac* α-factor treatment by Western Blotting. Once again, only Sst2$^{Q304N}$ showed high MAPK phosphorylation levels (Supplementary Figure II-5).
Figure II-4: A decreased interaction with Sst2 explains the sensitivity shift of partially truncated receptors. A, Dose-response relationship of various Ste2 or Sst2 constructs with Klac α-factor. Only the variant Sst2^Q304N, which does not interact with the receptor, enables a stronger response to the foreign pheromone. B, Mating response sensitivity for the Ste2 or Sst2 constructs assayed in (A). EC50 values were derived from duplicate dose-response experiments. C, Sensitivity of the mating response conferred by Ste2 variants alone or in combination with Sst2^Q304N. The sensitivity was not additive for truncated receptors. D, Bimolecular fluorescence complementation of Venus fragments fused to Ste2 and Sst2. Venus fluorescence was measured by flow cytometry in duplicate experiments and normalized by the \( B_{\text{max}} \) values of the Ste2-C-
Venus variants. Venus fluorescence was lower when cells expressed Sst2 Q304N or truncated Ste2, suggesting that these mutations impair the interaction between the receptor and its RGS. Asterisks indicate statistically significant differences to wild-type (P < 0.05, Dunnett's Multiple Comparison Test). All error bars represent the s.e.m.

We proceeded to determine whether our truncated receptors were more sensitive to Klac α-factor due to an altered interaction with Sst2. We reasoned that if a partial tail truncation weakened the receptor’s interaction with Sst2, its effect would be equivalent to that caused by the Q304N mutation in Sst2 and, in consequences, the two mutations combined should not display an additive phenotype. We co-expressed truncated receptors or high affinity variants with Sst2Q304N and measured cells’ sensitivity to Klac pheromone. We found that the sensitivity conferred by Sst2Q304N was not improved when receptor truncations were co-expressed (Figure II-4C). Conversely, the high binding affinity variants S2 and S4 produced an even more sensitive response when co-expressed with Sst2Q304N.

While these results pointed to an altered interaction between truncated receptors and Sst2, we sought to determine if the interaction was in fact physically weaker rather than simply improper and to what extent. For this, we used bimolecular fluorescence complementation (BiFC) with Venus fluorescent protein [107, 108]. In this assay, Ste2 and Sst2 are fused to the C- and N-terminal halves of Venus respectively. Upon physical interaction, Venus is reconstituted and its fluorescence can be detected by flow cytometry and normalized to protein expression levels (Supplementary Table II-3). The Venus fluorescence for different construct combinations is presented in Figure 4D. We found that wild-type Ste2 and Sst2 produced significantly more fluorescence per Ste2 molecule.
than wild-type Ste2 and Sst2 Q304N, confirming the reduced physical interaction found by Ballon et al. Remarkably, the truncated receptors T1 and T2 also caused lower Venus fluorescence than wild-type, though higher than Sst2 Q304N. On the other hand, the two binding mutants S2 and S4 showed similar Venus fluorescence compared to wild-type. Together, these results suggest that the sensitivity of partial tail truncations to the foreign pheromone can be attributed to an impaired interaction with Sst2.

**Partial receptor truncations impair Sst2 activity**

We further investigated the effects of receptor truncations on Sst2 activity and pathway regulation. In the absence of a Ste2-Sst2 interaction, either through a point mutation in the RGS or by truncating the entire C-terminal tail of the receptor, signal down-regulation and recovery from pheromone-induced cell cycle arrest is deficient [11]. However, it was unclear if partial receptor tail truncations, such as those found in T1* and T2*, would have similar consequences. Past work suggests that the receptor’s third intracellular loop may also contribute to Sst2 function [43], possibly as an additional contact point which could offset the effects of partial truncations.

An important feature of normal Sst2 function is fast pathway deactivation as reflected by the dephosphorylation of Fus3, the pathway’s MAPK [106]. Since pathway activation causes cell cycle arrest, a rapid shut-off ensures that cells without a compatible mate are able to resume growth. We reasoned that if partial tail truncations affected Sst2 function, the dynamics of MAPK dephosphorylation, as well as the rates of growth recovery, would reveal this.
Figure II-5: MAPK phosphorylation dynamics and growth curves reveal the role of the receptor-RGS interaction in controlling both mating pathway activation and de-activation.

A-F, Levels of phosphorylated Fus3 after pheromone wash-off for different Ste2 variants. Following mating pathway activation with 3 µM pheromone cells, were washed in pheromone-free medium, incubated for the indicated time and lysed. Lysates were used for Western Blotting. Phosphorylated Fus3 levels were normalized to PGK levels and plotted. Green bar graphs indicate Scer α-factor, red indicated Klac α-factor. G-H, Growth curves of cells expressing different Ste2 or Sst2 variants. Following mating pathway activation with 3 µM pheromone, cells were washed in pheromone-free medium, incubated for the indicated time and culture density was measured by flow cytometry. Values were normalized to the initial cell concentration to yield a growth ratio. All error bars represent the s.e.m.

We measured the levels of phosphorylated Fus3 following pheromone wash-off, as was previously done by Dixit et al. Importantly, as we sought to determine if binding affinity influenced this process, we performed this assay under mild washing conditions. As previously reported, we confirmed that levels of phosphorylated Fus3 declined rapidly in wild-type cells (Supplementary Figure II-6A), while the expression of Sst2Q304N resulted in slower pathway deactivation (Supplementary Figure II-6B). We also observed that rapid pathway deactivation was preserved for the high affinity variants S2 and S4, whether Scer or Klac pheromones were used to activate the pathway (Figure II-5A and B). In contrast, pathway regulation was more complex for the truncated receptors T1* and T2*. When treated with Scer α-factor, these variants exhibited slow Fus3 de-phosphorylation (Figure II-5C) as predicted, further indicating that their partial tail truncations impaired normal Sst2 function. However, this effect was not observed when Klac α-factor was used to activate the pathway (Figure 5D). Remarkably, cells expressing Sst2Q304N and treated with Klac α-factor showed a similar result (Supplementary Figure II-6C and D).
The different rates of pathway de-activation could not be explained by the initial levels of phosphorylated Fus3, as these were all comparable for each pheromone (Supplementary Figure II-6E and F) which de-activated rapidly in all cases. Instead, these results pointed to a pheromone-specific effect, whereby a partial Sst2 function could still down-regulate Fus3 phosphorylation provided that the receptor was activated by a weak ligand. We reasoned that if a truncated receptor were activated more potently by Klac pheromone, its deactivation would be slower. To test this, we generated hybrid mutants by truncating the high binding affinity mutants S2 and S4 to the same extent as T1*, which we called S2-T1 and S4-T1. As expected, these hybrid mutants displayed slow Fus3 de-phosphorylation even when Klac pheromone was used, although this effect was less pronounced than with Scer pheromone (Figure II-5E and F). Remarkably, these results were mirrored in growth assays where cell density was measured for 7 hours following pheromone treatment and wash-off (Figure II-5G and H). Rapid de-phosphorylation correlated with rapid growth, while slow de-phosphorylation matched slow growth.

Taken together, these experiments suggest that Sst2 plays two distinct roles. First, Sst2 creates a threshold of receptor activation needed to activate the pathway, with partial Ste2 truncations enabling a response to a weak agonist by lowering this threshold. Second, Sst2 also contributes to pathway deactivation and this depends on both its interaction with the receptor and the affinity of the ligand (Figure II-6). Importantly, neither an altered Ste2-Sst2 interaction nor a high affinity ligand alone can disrupt pathway deactivation. In the case of T1* and T2* treated with Klac α-factor, the interaction with
Sst2 is impaired, thereby lowering the activation threshold that it provides, but with no consequences on deactivation, Sst2’s second function. In this way, truncated receptors confer a strong response to *Klac* pheromone while preserving normal pathway deactivation and growth recovery.

Figure II-6: Pathway regulation depends on both ligand-receptor binding and a receptor-RGS interaction. Schematic map of the Ste2 evolutionary landscape. A stronger ligand-receptor interaction or a weaker receptor-RGS interaction can each promote mating response sensitivity to *Klac* pheromone, while a combination of the two is accompanied with slower pathway deactivation.
Mutants can mediate the formation of mating projections

An additional function of Ste2’s C-terminal tail consists in regulating the proper formation of mating projections, also known as shmoos. Mating projections are a critical step in the fusion of haploid yeast cells, the end goal of mating pathway activation [84]. While full C-terminal truncations strongly affect shmoo orientation toward potential mates, the impact of partial truncations is less severe and they permit mating [60, 109]. Recent work demonstrated that Sst2’s RGS activity, but not its interaction with Ste2, is required for proper mating orientation [110]. To confirm this, we tested the ability of our truncated receptors to enable the formation of shmoos. Like their high-affinity counterparts, truncation mutants enabled the formation of typical mating projections based on morphology and frequency when treated with either pheromone (Supplementary Figure II-7). In contrast, only a few cells expressing wild-type Ste2 could shmoo when treated with Klac α-factor, and the shape of their mating projections was atypical. Together with GFP expression and MAPK phosphorylation, this data further confirms that mutant receptors confer a strong and biologically meaningful response to a foreign pheromone.
DISCUSSION

In this study, we investigated the role that mutations in a network hub can have in the evolution of a protein network. Our findings demonstrate that a GPCR can evolve the ability to respond to a weak agonist through at least two distinct mechanisms. The first is classical, and consists of an altered interaction between the receptor and the ligand reflected by an enhanced binding affinity. The second, which sheds light on an alternate evolutionary path, involves a change in the relationship between the receptor and its regulatory network. Specifically, we isolated multiple mutant Ste2 receptors with a truncated cytoplasmic tail, a region involved in protein-protein interactions. These truncated receptors exhibited various changes compared to wild type Ste2, such as a higher surface expression and defective endocytosis, but their sensitivity to Klac α-factor was attributable to an impaired interaction with Sst2, a signal suppressor of G protein activity. Strikingly, these changes had a minimal impact on key aspects of pathway regulation. Our results thus demonstrate that it is possible to evolve a new receptor-ligand response by altering regulatory network interactions rather than the receptor’s binding site. Furthermore, the prominence of truncated receptors in our selection, as well as the extensive cytoplasmic tail length variability that we observed in natural Ste2 homologs, suggest that evolutionary events that shorten or extend the cytoplasmic tail are rather common and may contribute to adaptive functional changes.

In a pioneering study performed over two decades ago, Lorraine Marsh used laboratory evolution to obtain Ste2 variants that could confer a mating response to the pheromone of *Saccharomyces kluyveri* [52]. In this way, she found various mutations affecting the
receptor’s ability to discriminate between the two ligands. However, due to a limited understanding of Ste2 signaling at the time, it was not possible to conclude on the precise molecular mechanisms behind the new phenotype. In the decades that followed, many more studies used mutagenesis and selection to characterize Ste2, often by focusing on a single region of the receptor and its hypothesized function. In this way, different Ste2 variants revealed the amino acid residues involved in pheromone binding [33, 35], signal transduction [35, 91, 92], internalization [85-87], oligomerization [46, 48] and signal regulation [11, 43, 46, 90]. While these studies led to a greater understanding of the mechanisms behind the many facets of Ste2 signaling, their combined role in the evolution of new function remained unclear. Our work addresses this gap by shedding light on the molecular mechanisms through which a GPCR network can be linked to a foreign ligand.

As expected, our selection yielded several Ste2 variants with mutations affecting the receptor’s binding site as revealed by their significantly enhanced binding affinity to Klac α-factor. Strikingly, all five high-affinity variants share the same three mutated sites: N216, V280 and S267. Mutations at the first two sites were previously shown to enable a pathway response to a weak agonist as well as an antagonist [36, 40]. Both sites were also involved in suppressing a loss-of-function mutation at a known pheromone-binding residue, Y266 [36]. Although S267 was not previously linked to pheromone binding, it is adjacent to Y266 and thus may also contribute to binding. The apparent broad specificity that arises when these residues are mutated, which we further confirmed with Klac and Cgla pheromones, initially suggested that such residues were “gatekeepers”, ensuring the
specificity of the Ste2-α-factor interaction. However, our results show that wild-type Ste2 binding is inherently promiscuous. Instead, the RGS Sst2 is required to impose a signaling threshold that is more stringent than binding specificity, allowing cells to discriminate between strong or weak ligands. Moreover, this threshold depends on a strong interaction between Sst2 and the receptor, as Sst2’s presence alone is also insufficient to lock away the promiscuous potential of Ste2.

Despite the role of Ste2’s cytoplasmic tail in signaling regulation, our data support the view that partially truncated receptors retain key aspects of pathway regulation. First, these variants did not result in constitutive signaling, indicating that overall pathway responsiveness was preserved. Second, the frequency and the morphology of mating projections in cells expressing truncated receptors were undistinguishable from those of wild-type cells, in agreement with a recent study that found that truncated variants can mediate mating [60]. Third, pathway deactivation proceeded normally in truncation mutants, provided that the receptor’s interaction with the pheromone was weak. When cells expressed receptors combining a truncation with a binding affinity mutation, Fus3 dephosphorylation was slow with either pheromone. This suggests an interesting paradigm: if rapid pathway deactivation is to be preserved, then receptors can evolve either a stronger interaction with the ligand or a weaker interaction with Sst2, but not both. Since pathway activation causes cell cycle arrest, hybrid mutants may be disadvantaged unless they can mate efficiently, leading to additional selection pressure.
To conclude, our work highlights the prominent role of a receptor-network interaction in the evolution of a new ligand response. This scenario is of particular relevance in *S. cerevisiae* where interspecies mating has been a major evolutionary driver [59], but its significance can be extended to other GPCR networks due to the underlying conservation that exists in this large family [15, 111]. Through partial C-terminal truncations, the yeast GPCR Ste2 can acquire foreign pheromone sensitivity at little cost to signaling regulation. This has interesting implications in the study of GPCR evolution, where the highly variable length of the cytoplasmic tail has not been thoroughly examined. Our work suggests that this variability may not be random, but could instead be linked to network re-wiring events. Furthermore, the demonstrated importance of the RGS-tail interaction on response sensitivity and ligand discrimination may be relevant in GPCR-linked diseases. Alternative splice variants of GPCRs leading to truncated receptors are well documented, but little is known about their consequences beyond altered receptor trafficking [112-114]. If such splice variants are unable to interact with regulatory factors, particularly RGS proteins, hypersensitivity and a lack of ligand discrimination may play a role in their phenotype.
METHODS

Mating pathway activation assays

Cells from three independent colonies were treated with one or a series of concentrations of pheromone and placed in a 30°C shaking incubator. After 3 hours, protein synthesis was inhibited by treating cells with 10 µg/mL cycloheximide. The intensity of the fluorescence in the 525/50 nm range was measured by analytical flow cytometry with a MACSQuant VYB (Miltenyi Biotec) with a 588 nm laser. Cells without receptors were used to subtract basal cell fluorescence from other samples to reveal the net GFP fluorescence. For dose-response assays, the data were fitted with the “log(agonist) vs. response – Variable slope (four parameters)” model in Prism (GraphPad). All experiments included a wild-type Ste2 control. The fluorescence intensity was normalized to the maximum intensity of the wild-type control and multiplied by 100. EC50 values represent the mean of two experiments normalized to the wild-type value of each experiment.

Mutagenesis and selection

For mutagenesis, the full-length wild-type STE2 open reading frame (ORF) was amplified from pRS-STE2 by error-prone PCR using the GeneMorph II kit (Agilent). PCR conditions (500 ng of template DNA, 20 cycles) were selected to yield a mean mutation rate of 4.0 DNA mutations per ORF. The resulting amplimers were ligated in pRS-pSTE2 and amplified in E. coli DH5α to generate a plasmid library of approximately 50 000 Ste2 mutants. The library was transformed in the yeast strain RB001, yielding 20 000 colonies. For selection, yeast cells were treated with 5 µM Klac and incubated for 3 hours in a 30°C shaker. Cell sorting was done in a FACSARia (BD) while gating for
high GFP fluorescence. In the first round, 19 000 events were sorted. In the second round, which was done using the cells recovered from the first round, 11 000 events were sorted. A total of 282 colonies recovered from both rounds were screened for their ability to activate the mating pathway better than wild-type. Mutant STE2 genes were extracted from the most promising colonies, transformed in naïve cells to confirm that they conferred the phenotype, and sequenced.

**Pheromone binding assays**

To measure binding affinities and binding site levels, we used the NBD-labelled pheromone binding assay [115]. Cells from two independent colonies were treated with either varying concentrations of NBD-labelled Scer (saturation binding assays) or varying concentrations of Klac pheromone mixed with 20 mM NBD-Scer (competition binding assays) and left on ice for 10 min with occasional mixing. Samples were processed by flow cytometry. For saturation binding, the data were fitted to the “One site – Total and nonspecific binding” model in Prism (GraphPad), and cells without receptors were used to account for non-specific binding. This model uses the equation $Y = \frac{B_{\text{max}}[L]}{K_d + [L]} + N[L] + \text{Background}$, where $Y$ is the mean fluorescence, $[L]$ is the concentration of labelled ligand and $N$ is a proportionality constant for nonspecific binding. For competition binding, the background-subtracted data were fitted to the “One site – Fit Ki” model with the appropriate constraints. This model uses the equation $K_i = \frac{IC_{50}}{1 + \frac{[L]}{K_D}}$. All experiments included a wild-type Ste2 control. $K_D$, $B_{\text{max}}$ and $K_i$ values represent the mean of two experiments normalized to the wild-type values of each experiment.
Predicting the lengths of C-terminal tails in Ste2 homologs

To identify the predicted disordered region, the amino acid sequence of *Scer* Ste2 was queried in FoldIndex© [98]. PSI-BLAST for three iterations and with default parameters [116]. We restricted the search to matches of fungal origin, excluded *Scer* matches and discarded partial sequences. We submitted the resulting dataset to the transmembrane domain predictors TMHMM [96] and extracted the predicted topology of sequences with 7 transmembrane domains. As TMHMM also predicts the orientation of the helices, we discarded sequences for which the C-terminal tail was extracellular. From the resulting topologies, we measured the length of the cytosolic tail by defining its N-terminal boundary as the last amino acid of the seventh transmembrane domain, and its C-terminal boundary as the stop codon. The frequency distribution of the resulting amino acid lengths was plotted in Prism (Graphpad).

Bimolecular fluorescence complementation

To measure the relative strength of the physical interaction between Ste2 variants and Sst2 variants, we used bimolecular fluorescence complementation with Venus fluorescent protein [107, 108]. N- and C-terminal Venus fragments were generated by dividing the protein at amino acids 172-173. C-Venus was fused to Ste2 variants and N-Venus to Sst2 variants. Venus fluorescence was measured by flow cytometry as for GFP and normalized to the $B_{\text{max}}$ of each receptor construct to account for receptor expression levels. $B_{\text{max}}$ values were obtained with NBD-*Scer* as described above.
Western blotting of MAPK deactivation

Activation and deactivation of the mating pathway was performed as described in [106]. Briefly, cells were treated with 3 µM pheromone and incubated in a 30°C shaker for 30 minutes. The pheromone was washed off with fresh growth medium and cell aliquots were taken immediately after wash-off or following 10 and 20 minutes incubations. Cell lysates were resolved in a 10% SDS-PAGE gel and transferred to a PVDF membrane using a BioRad Trans-Blot Turbo. Membranes were blocked overnight with Licor Odyssey TBS-formulated blocking buffer. Blotting of Fus3-P was done with a primary anti-p44/42 MAPK antibody (Cell Signaling Technology, #4370) diluted by 1:2000, followed by the secondary antibody IRDYE 800 (Licor, #926-32211). Blotting of total Fus3 was done with a primary anti-Fus3 antibody (Santa Cruz Biotechnology, #sc6773) diluted by 1:5000, followed by the secondary antibody IRDYE 800 (Licor, #926-32214). Blotting of the loading control phosphoglycerate kinase (PGK) was done with a primary anti-PGK antibody (Invitrogen, #459250) diluted by 1:5000, followed by the secondary antibody IRDYE 680LT (Licor, #926-68020). All secondary antibodies were diluted by 1:10 000. Bands were visualized with a Licor Odyssey CLx infrared imaging system (Licor). Band intensity was extracted with Image Studio Lite (Licor). The intensities of Fus3-P bands were normalized to that of the PGK loading control. The experiment was performed in duplicates.

Cell growth recovery assay

To measure recovery from cell cycle arrest, cells were first treated with 500 nM pheromone and incubated in a 30°C shaker for 30 minutes. The pheromone was then
washed off with fresh growth medium. Culture aliquots were taken immediately after wash-off (time zero) and subsequently every hour for 7 hours. Aliquots were used to measure culture density by flow cytometry. Culture densities were normalized to time zero to obtain growth factors.

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**Author contributions**

R.B.D.R and S.G.P designed study; R.B.D.R. performed experiments and simulations; A.T. provided guidance in simulations; R.B.D.R., A.T., B.C. and S.G.P. discussed results. R.B.D.R. and S.G.P. wrote the manuscript with input and commentaries from all authors.
Chapter 3:

The directed evolution of ligand specificity in a GPCR and the unequal contributions of efficacy and affinity

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ABSTRACT

G protein-coupled receptors (GPCRs) must discriminate between hundreds of related signal molecules. In order to better understand how GPCR specificity can arise from a common promiscuous ancestor, we used laboratory evolution to invert the specificity of the *Saccharomyces cerevisiae* mating receptor Ste2. This GPCR normally responds weakly to the pheromone of the related species *Kluyveromyces lactis*, though we previously showed that mutation N216S is sufficient to make this receptor promiscuous. Here, we found that three additional substitutions, A265T, Y266F and P290Q, can act together to confer a novel specificity for *K. lactis* pheromone. Unlike wild-type Ste2, this new variant does not rely on differences in binding affinity to discriminate against its non-preferred ligand. Instead, the mutation P290Q is critical for suppressing the efficacy of the native pheromone. These two alternative methods of ligand discrimination were mapped to specific amino acid positions on the peptide pheromones. Our work demonstrates that changes in ligand efficacy can drive changes in GPCR specificity, thus obviating the need for extensive binding pocket re-modeling.
INTRODUCTION

Communications within and between cells are an essential feature of life. As organisms evolved, the complexity of cell signaling networks has increased dramatically [1]. Protein receptors today must distinguish between countless signaling molecules, many of them displaying similar structures. This is especially true for G protein-coupled receptors (GPCRs), the large family of seven-transmembrane receptors involved in neurotransmission, chemokine recognition, vision and olfactory sensing. Changes in GPCR specificity can have dramatic consequences in phenotype, causing disease [16, 117] or major evolutionary shifts [118]. Due to their pharmacological importance, much research has been devoted to understanding how GPCRs interact with their cognate ligands and how their binding specificity can be altered. High-resolution crystal structures of GPCR-ligand complexes have been invaluable for revealing the precise electrostatic interactions that are involved in binding [15, 119]. However, much less is known about how specificity can be altered. Phylogenetic analyses have been used to identify specificity-determining positions (SDPs) which can then be validated in the laboratory [25, 26], but this approach is limited to GPCRs for which sufficient structural and sequence data are available. Alternatively, experimental evolution, which combines random mutagenesis and high-throughput selection of novel specificities, can identify SDPs without prior knowledge of GPCR structure or sequence homology.

We sought to further our understanding of how ligand specificity can change by focusing on the pheromone receptor Ste2 in the yeast *Saccharomyces cerevisiae*. Like other ascomycetes, *S. cerevisiae* senses short peptide pheromones from nearby compatible
mates through the use of GPCRs. Upward of 70 yeast species are known to harbor unique receptor-pheromone pairs that act as major determinants of sexual compatibility [28, 51]. This impressive diversity has common evolutionary roots, providing fertile grounds for studying how ligand discrimination arose with each speciation event. Furthermore, the *S. cerevisiae* lineage is thought to have originated through a rare instance of interspecies hybridization [59], a scenario which could lead to a hybrid with a “mismatched” receptor and pheromone pair. However, how the GPCR becomes responsive to the new ligand but irresponsive to its former ligand remains unclear. We hypothesized that changes in ligand specificity may not require extensive remodeling of the receptor’s binding pocket, but may instead proceed from changes in signal regulation and/or small differences in the receptor’s structure.

We previously used an experimental evolution approach to understand how *S. cerevisiae* (hereafter abbreviated Scer) can become responsive to the α-factor pheromone peptide of a related yeast species, *Kluyveromyces lactis* (*Klac*) [69]. This foreign ligand triggers only a weak response with wild-type (WT) Ste2, but we found that single point mutations in the receptor could increase pheromone potency to high levels. Several Ste2 variants achieved this without displaying greater binding affinity for *Klac* α-factor, but had instead facilitated pathway activation by shedding a regulatory region. However, in all cases, the new Ste2 variants still responded strongly to the native Scer pheromone. Though our work revealed different ways that Ste2 can respond to a foreign ligand, it left open-ended the question of how ligand discrimination arises from a broad-specificity receptor.
Here, we aimed to gain further insights on the evolution of ligand discrimination in Ste2. For this, we used directed evolution in order to obtain a variant which exhibits a strong preference for Klac pheromone. We investigated the effects of the mutations selected on sensitivity and binding affinity. Remarkably, we found that binding affinity did not play a significant role in discrimination against the native pheromone for our variant. Instead, the ability of each ligand to induce receptor activation, i.e. their efficacy, appears to have become the major determinant of specificity.

RESULTS

*Iterative rounds of mutations and selection yield Klac pheromone-preferring variants*

Our previous work uncovered several broad-sensitivity Ste2 variants that were responsive to Klac pheromone [69]. In order to select a starting point for the directed evolution of a ligand-discriminating variant, we focused on the high-affinity mutations N216S, V280I and S267C since we sought a strong interaction with both pheromones as a baseline. We also reasoned that a highly sensitive receptor would be more likely to remain functional despite extensive mutagenesis, and we previously showed that N216S or V280I alone is sufficient to enable a strong response to Klac pheromone without impairing receptor expression and trafficking, while S267C has a milder effect on sensitivity. Here, we found that combining N216S and V280I greatly increased sensitivity to Klac α-factor, while a triple mutant including S267C was not significantly improved (Figure III-1A). As we were concerned about the effect of adding unnecessary mutations on overall protein
stability, we decided to use Ste2 N216S V280I as our initial variant from which we might obtain a *Klac* pheromone-specific receptor.

**Figure III-1:** The directed evolution of a broad-specificity variant of the GPCR Ste2 to obtain a ligand-discriminating variant. **A,** Mating response of cells expressing various broad-specificity Ste2 mutants in the presence of different concentrations of *Klac* pheromone. We selected Ste2 N216S V280I as the starting point of our directed evolution experiment due to its high sensitivity and its small number of mutations. Error bars represent the standard error of the mean (s.e.m.) **B,** Schematic representation of the iterative process underlying directed evolution. An initial Ste2 ORF is amplified using error-prone polymerase and the resulting amplimers are cloned in a plasmid vector. Yeast cells expressing the mutant Ste2 library are sorted based on their strong response to *Klac* α-factor and their weak response to *Scer* α-factor, as measured from a GFP reporter of mating. Sorted candidates are then screened to confirm their response profile. Further rounds of random mutagenesis and selection can be performed on promising Ste2 variants.
Directed evolution consists of an iterative process combining multiple rounds of mutagenesis and selection (Figure III-1B). We introduced random mutations in the entire open reading frame (ORF) of Ste2 N216S V280I and generated a library of ~100 000 unique mutants. This library was transformed in a ste2Δ yeast strain with a GFP reporter of mating pathway activation, yielding ~150 000 colony-forming units. Following pheromone treatment, we used fluorescence-activated cell sorting (FACS) to select variants according to their GFP levels. In order to obtain variants with a preference for Klac pheromone, we performed a series of positive (+) and negative (-) sorts. In a + sort, cells were treated with Klac α-factor and those expressing high levels of GFP were selected. In a – sort, cells were treated with Scer α-factor and those expressing little or no GFP were selected. We experimented with various combinations of + and – sorts and found that alternating the two worked best (Supplementary Figure III-1). Following a +--+ selection routine, we screened 184 variants individually for their ability to respond to either pheromone (Fig III-2A). Among these, we identified two variants, 3S6-2 and 3S6-4, with a small preference for Klac α-factor over the native pheromone. Strikingly, both variants shared a common non-seed mutation: P290L.

We confirmed that P290L was responsible for the inverted specificity of 3S6-2 and 3S6-4 by introducing it in a WT or N216S background (Figure III-2B). Interestingly, we found that P290L did not invert specificity on its own. Instead, the sensitizing mutation N216S was necessary to observe a preference for Klac α-factor, although this was difficult to ascertain due to the weakness of overall signaling in Ste2 P290L. The addition of P290L was accompanied by reduced surface expression levels (data not shown). Combining P290L to the sensitizing mutations N216S, S267C and V280I enhanced overall signaling.
Figure III-2: Identifying mutations that confer a new ligand specificity from a sorted library of Ste2 variants. A, Screening of promising candidates following three rounds of sorting from a library of random mutants based on Ste2 N216S V280I. The mating response was measured for each candidate following treatment with 1 µM of each pheromone. The response values are shown in a scatter plot where the diagonal indicates equal Klac and Scer pheromone-induced activation. Preference for Klac pheromone was confirmed in two variants, 3S6-2 and 3S6-4. B, The Ste2 mutation P290L confers Klac pheromone preference in the presence of N216S and other sensitizing mutations, but also reduces overall receptor activity. The mating response of each Ste2 variant to either pheromone is shown in bar plots derived from duplicate experiments. Error bars represent the standard error of the mean (s.e.m.) C, Screening of promising candidates following seven rounds of sorting from a library of random mutants based on Ste2 N216S S267C V280I P290L. The mating response is shown in a scatter plot similar to the one in (A). Preference for Klac pheromone was confirmed in variant 4Q7-1. D, The Ste2 mutation A265T further suppresses the response to Scer pheromone in the presence of N216S and P290L. The mating response of each Ste2 variant is shown in bar plots similar to the one in (B).

In order to identify additional mutations promoting Klac pheromone specificity, we performed a second round of mutagenesis and selection on the quadruple mutant Ste2 N216S S267C V280I P290L. This time, we did not find a suitable variant until the seventh round of sorting, one that followed a +++- selection pattern. During our initial screen of this sorted library, we identified variant 4Q7-1 which showed a high Klac-to-Scer response ratio (Figure III-2C). However, following plasmid extraction and transformation into new cells, this variant exhibited a much lower ratio. We suspect that this discrepancy was due to a cell-specific genomic mutation enhancing overall mating pathway response. Despite this, we found that one of the mutations in this variant, A265T, improved Klac α-factor sensitivity dramatically but did not invert ligand specificity on its own or with N216S (Figure III-2D). Instead, this mutation enhanced
selectivity slightly when combined with both N216S and P290L by suppressing the response to *Scer* α-factor.

*A combination of ligand-discriminating and sensitizing mutations results in the optimal variant*

Although random mutagenesis and selection successfully identified P290L as crucial for enabling a preference for *Klac* pheromone, its detrimental effect on overall signaling was problematic. In order to obtain a better variant, we reasoned that the site P290 was likely more important than the mutation itself, and so we proceeded to test all other amino acid substitutions at this site, an approach known as site saturation mutagenesis (Figure III-3A). These substitutions were introduced in a Ste2 N216S background due to the apparent cooperative effect of this mutation with P290L. The resulting variants were then assayed for their sensitivity to saturating concentrations of either *Klac* or *Scer* pheromones and compared to WT Ste2 (Figure III-3B). We observed a broad range of phenotypes in P290 mutants, suggesting that this site can tune both receptor activity and specificity. Several substitutions, such as those involving aromatic and charged residues, resulted in an inversion of specificity but also a lower overall response. In that regard, P290Q proved to be a superior alternative to P290L. In fact, no mutation was as detrimental to overall receptor activity as P290L. A non-saturating concentration of pheromone yielded broadly similar results (Supplementary Figure III-2). Remarkably, we found that the proline of TM7 is highly conserved among known Ste2 orthologs (Supplementary Figure III-3).
Figure III-3: Site saturation mutagenesis identifies mutation P290Q as a superior alternative to P290L. A, The amino acid residue 290, a proline in WT Ste2, was changed to every other natural amino acid in a Ste2 N216S background by using site-directed mutagenesis. B, Mutations at site P290 lead to a diverse set of response profiles. The mating response was measured for each variant following treatment with 5 µM of each pheromone. The response values are shown in a scatter plot where the diagonal indicates equal Klac and Scer pheromone-induced activation. Variants are labeled according to the amino acid present at site P290, where variant “P” represents Ste2 N216S and “WT” represents WT Ste2. C, The mutation P290Q can be combined with other mutations to yield a receptor with inverted specificity. The mating response of each Ste2 variant to either pheromone is shown in bar plots derived from duplicate experiments. Error bars represent the standard error of the mean (s.e.m.)

On its own, mutation P290Q appears to act largely by suppressing the receptor’s response to Scer pheromone (Figure III-3C). This effect is greater in the presence of N216S, suggesting that the latter mutation is not simply sensitizing. Rather, its effect on receptor function is dependent on the amino acid present at position 290. Although Ste2 N216S
P290Q is able to clearly discriminate between native and foreign pheromones, we attempted to improve its low activity without impairing specificity. First, we found that adding mutation A265T resulted in even greater suppression of the *Scer* α-factor response. Although this caused a slight loss of overall receptor sensitivity, we were able to counter this effect with the sensitizing mutation Y266F [69, 120]. As we were unsure whether A265T and Y266F contributed usefully to our desired phenotype, we performed a detailed analysis of each variant’s response profile by measuring dose-response curves and EC50 values. This revealed that A265T reduced the maximum response to *Scer* α-factor when added to Ste2 N216S P290Q (Figure III-4A), while the response to *Klac* α-factor was unaffected (Figure III-4B). Conversely, Y266F had the opposite effect on the maximum response to each pheromone when added to Ste2 N216S P290Q A265T. Likewise, EC50 values showed that Y266F re-balanced the desensitizing effect of A265T (Fig III-4C). Thus, both mutations were beneficial and the resulting Ste2 variant harboring mutations N216S P290Q, A265T and Y266F (hereafter abbreviated to Ste2 K-Switch) displayed a full inversion of specificity for the *Klac* and *Scer* pheromones compared to the WT.
Figure III-4: The Ste2 variant harboring mutations N216S, P290Q, A265T and Y266F discriminates between Scer and Klac pheromones through differences in ligand efficacy. A-B, Dose-response curves showing the effects of adding sensitizing mutations (N216S and Y266F) and discriminating mutations (P290Q and A265T) to the mating response profile of cells treated with either Scer or Klac pheromones. C, Mating response sensitivity for the Ste2 variants assayed in (A) and (B). EC50 values were derived from duplicate dose-response experiments. D, Binding constants and surface expression levels of Ste2 variants harboring one or multiple mutations identified in this study. The binding affinity for Scer α-factor and the levels of surface expression were obtained from duplicate saturation binding assays. The binding affinity for Klac α-factor was obtained from duplicate competition binding assays. All variants could bind to Scer pheromone with similar affinity (nonparametric Kruskal-Wallis test), suggesting that this was not the basis of ligand discrimination observed at the mating response level. Error bars represent the standard error of the mean (s.e.m.)
Differences in binding affinity are not necessary to acquire novel specificity

GPCR activation is a multi-step process involving at the very least a ligand binding step that is distinct from subsequent conformational changes in receptor structure. We wondered if the ability of Ste2 K-Switch to discriminate between the *Scer* and *Klac* pheromones could be explained by their relative binding affinity. Through the use of a fluorescently-labelled pheromone, we measured the binding affinity and surface expression levels of our Ste2 variants and made several observations. First, the affinity for *Scer* α-factor was consistently high (Figure III-4D) and there was no significant difference between the variants analyzed (one-way ANOVA). This suggested that P290Q and A265T do not cause weaker *Scer* pheromone binding, but rather that they alter the ability of each pheromone to activate the receptor, a property known as efficacy. Second, affinity to *Klac* α-factor was much improved by the mutation N216S, and only marginally by P290Q. Third, and perhaps most unsurprisingly, we found that variants harboring multiple mutations showed lower surface expression, most likely due to destabilizing effects. However, the strong mating response observed in these suggested that their reduced receptor levels were still adequate. We also confirmed that overexpression of Ste2 K-Switch did not affect its response profile (Supplementary Figure III-4).

*Klac* and *Scer* pheromone variants reveal that positions 2 and 12 of α-factor determine receptor specificity

The pheromone peptides of *Klac* and *Scer* are both 13-amino acid long and share a similar sequence (Figure III-5A). We sought to identify the amino acids responsible for
the opposite specificities of WT Ste2 and Ste2 K-Switch by using pheromone variants focusing on the most significant biochemical differences between the two peptides, positions 2, 5 and 12. Interestingly, we found that changing position 2 of the *Scer* pheromone to the *Klac* equivalent (His to Ser) decreased its efficacy with WT Ste2 while the opposite effect was observed when using a *Klac* peptide where position 2 was changed to the *Scer* equivalent (Ser to His) (Fig III-5B). For Ste2 K-Switch, changing position 12 of *Scer* pheromone to the *Klac* equivalent (Met to Ile) enhanced the response dramatically, while the reverse substitution (Ile to Met) in the *Klac* pheromone reduced its activity (Figure III-5C). Remarkably, position 12 substitutions did not affect WT signaling, while the same was true for position 2 and Ste2 K-Switch. Finally, substitutions at position 5 had no effect alone, or in combination with the other substitutions (Supplementary Figure III-5). These results suggest that position 2 and 12 are each important for ligand discrimination in WT Ste2 and Ste2 K-Switch respectively.

Binding assays revealed that changes at positions 2 and 12 contribute to ligand discrimination in distinct ways. Substitutions at position 2 affected binding affinity to WT Ste2 by either decreasing it (Ser) or by increasing it (His) significantly (Figure III-5D). On the other hand, changing position 2 or position 12 had no effect on the binding affinity to K-Switch (Figure III-5E). This further supports the notion that Ste2 K-Switch does not distinguish between *Klac* and *Scer* on the basis of binding affinity, but rather does so through differences in efficacy.
Figure III-5: Position 2 and position 12 of the peptide pheromones determine the ligand specificity of WT Ste2 and Ste2 K-Switch. A, The primary structures of the main pheromone variants used in this study. The WT pheromones are labeled Scer and Klac and their dissimilarities are emphasized by boxes. The other pheromone variants harbor single amino acid substitutions with respect to the WT sequences, which are highlighted by their different colours, and are named accordingly. B-C, The mating response of WT Ste2 (B) and Ste2 K-Switch (C) to each pheromone variant is shown in bar plots derived from duplicate experiments. Position 2 controls the WT response, while position 12 controls the response in the Klac-specific variant. D-E, Binding constants of WT Ste2 (D) and Ste2 K-Switch (E) to each pheromone variant. Binding affinity values were obtained from at least two competition binding assays. Asterisks denote statistically significant differences between values (***: P < 0.001) based on a Bonferroni-corrected multiple comparison test. WT can distinguish between Scer and Klac based on binding affinity and this is strongly linked to the amino acid at position 2 of each peptide, while Ste2 K-Switch cannot distinguish between any pheromone through binding affinity. Error bars represent the standard error of the mean (s.e.m.)
DISCUSSION

In the present study, we used a laboratory evolution approach to completely invert the specificity of a GPCR relative to its WT state. The Ste2 mating receptor contributes to the sexual isolation of the yeast *Scer* through its incompatibility with the pheromone of the related species *Klac*. Through a combination of random mutagenesis, high-throughput selection and site-saturation mutagenesis, we identified a set of four mutations that render Ste2 fully responsive to *Klac* α-factor but also incompatible with its original cognate ligand. Importantly, we have found that a GPCR can evolve to discriminate between related ligands in at least two distinct ways. Unlike WT Ste2, the *Klac*-specific Ste2 variant does not distinguish between pheromones based on binding affinity. Rather, the new mutations specifically reduce the ability of *Scer* α-factor to trigger pathway signaling, i.e. its efficacy. The difference in ligand recognition between the WT and the mutant is linked to two amino acid residues on the peptide pheromones. These results show that a novel GPCR-ligand specificity can arise without changes in binding affinity, while the efficacy-suppressing mutations identified here point to novel SDPs in both the yeast mating receptor and its pheromone.

Laboratory evolution has proven to be a powerful approach for visualizing the evolutionary path of proteins in real-time [69, 121, 122]. Studies using this method have shown that in order to alter the specificity of a protein, be it enzyme or receptor, it is preferable to go through a generalist stage where it can promiscuously interact with both the new and the old substrate or ligand [23, 64-67]. This can then be followed by a re-specialization step, where the old activity is largely abolished. This evolutionary path is
sometimes called “convex”, due to its shape on a map of relative activity and is characterized by a weak negative trade-off between each specificity [95]. The alternatives, where a protein switches specificity with a progressive loss of the native activity (direct or straight path) or where it goes through an inactive stage (concave path), are thought to be less likely due to the need to maintain a strong activity for at least one substrate/ligand. In agreement with this, ancestral gene resurrection studies have found that promiscuity is a recurrent feature of ancestral proteins, supporting the view that the convex path is favored in real evolution [123, 124]. In our own experiment, we uncovered all three convex, concave and direct trajectories (Figure III-6). The variant Ste2 N216S is a clear generalist intermediate, and so its occurrence is part of a convex path. However, mutations at site P290 can lead to different routes. P290L makes the path to a specialist concave, while P290Q can either continue on the convex route to specificity, or form a direct route, depending on whether it occurs before or after N216S. Although random mutations were necessary to identify a functionally significant site with little prior knowledge of Ste2’s structure (P290), it was saturation mutagenesis that enabled us to find the optimal amino acid residue at this site (Q). Our strict selection strategy involving non-overlapping sorting gates may explain why we found P290L first, but it is possible that P290Q would have been preferable in nature due to its compromise between functionality and specificity. This highlights the importance of the selection strategy in determining what directed evolution will yield: less stringent negative selection might have uncovered alternative substitutions at P290. Likewise, the convex, direct and concave paths may all exist in nature but their likelihood is determined by the effect of the gene mutations on overall fitness, i.e. selection pressure. For Ste2, it is
unclear how important efficient mating really is since yeast can exist in diploid form for many generations.

Figure III-6: Ste2 can evolve a new ligand specificity via different evolutionary paths. A scatter plot of the relative mating response of the Ste2 variants used in this study reveals the Ste2 specificity landscape. The different ways in which an inversion of specificity can occur are indicated by arrows and labeled by the shape of their trajectory. Our study uncovered all three possible evolutionary intermediates: broadly-activated (N216S, convex), broadly inactivated (P290L, concave) and balanced specificity (P290Q, direct). Error bars represent the standard error of the mean (s.e.m.)
A primary aim of our study was to determine how ligand discrimination arises in a promiscuous GPCR. We hypothesized that although the ligand-receptor interface would play a major role, it would not be limited to changes in binding affinity. Since the binding pocket of a GPCR involves interactions with multiple helices, we reasoned that it was unlikely to undergo significant re-modeling in order to exclude its native ligand. Instead, as past studies have shown, a new specificity does not have to lie very far; a small number of mutations is sufficient [66, 122]. Our findings support this view, as N216S and P290Q together led to reasonable ligand discrimination and activity with little change to binding affinity. Indeed, we found that WT Ste2 and Ste2 K-Switch distinguish between pheromones in very different ways. While N216S rendered the Ste2 binding pocket permissive to both *Klac* and *Scer* pheromones, its combination with P290Q and A265T did not restrict *Scer* α-factor binding, but instead reduced its efficacy specifically. This stands in contrast to WT Ste2, which relies at least partly on binding affinity to ensure a weak response to related pheromones. While previous work in GPCRs demonstrated that ligand efficacy is an important contributor to specificity alongside binding affinity [26], our results with Ste2 K-Switch indicate that it can be a sole contributor under certain conditions of selection. These conditions include a need for rapid change and the absence of negative pressure against antagonism. In our experiment, *Scer* α-factor became a competitive antagonist for *Klac* α-factor. In nature however, there would likely be additional selection pressure against antagonism which would favor geographic isolation, since competition for receptors would weaken signaling wherever *Scer* cells are present [125, 126]. And so, the specificity of WT Ste2 is the result of millions of years of sexual isolation and receptor-pheromone co-evolution in a diverse environment. Advances in
library design, high-throughput methods of selection and \textit{in silico} evolution could eventually be used to reveal the broader range of mutations that accompany multi-ligand discrimination in a controlled setting.

Due to a lack of comprehensive 3D structural data for Ste2 and other fungal GPCRs, we can only speculate on what drives the suppression of \textit{Scer} efficacy among our variants. For N216S, past work showed that another mutation at site, N216D, can counter the effects of the loss of function mutations Y266C and F204S, each of which affects either ligand binding or receptor activation, making this site relevant to both processes [40]. Likewise, A265T is adjacent to Y266, a crucial residue in Ste2 function which is thought to interact with the pheromone’s N-terminus and take part in the subsequent conformational change that accompanies receptor activation [38]. But the most important mutation that we found, P290Q, is perhaps also the most puzzling. Prolines are known to interrupt helical structures, and a NMR analysis of Ste2’s seventh transmembrane domain suggests that P290 is no exception [127]. Prolines are well-tolerated throughout the seventh transmembrane domain of Ste2, suggesting that this helix does not need to be continuous for proper signaling [128]. Our results support this (e.g. P290N), but many other substitutions at this site do change function dramatically by inverting specificity. This is especially surprising when considering the apparent lack of involvement of P290 in \textit{Scer} pheromone binding or receptor activation as probed in past studies [32-34, 40].

Our work suggests that P290 plays a significant role in establishing ligand discrimination, even if it does not contact the pheromones directly. Furthermore, this role appears to be linked to positions 2 and 12 of the pheromones. Specifically, we found that position 2
substitutions affected binding and efficacy with the WT receptor, while position 12 substitutions affected only efficacy with Ste2 K-Switch. The precise function of each of α-factor’s amino acid residues has been probed using a variety of peptide analogs and mutants, with some studies suggesting that the pheromone harbors distinct signaling and binding domains at the N-terminus and C-terminus respectively [38], while other studies showed significant overlap between these two roles [129, 130]. While our results support the latter view, the divergence in the field highlights the need for further studies using systematic substitutions beyond alanine-scanning, as was done recently by Stainbrook et al. [131], as well as exploring the diversity of yeast pheromones and their ability to create sexual barriers between related species.

In conclusion, our work demonstrates that the evolution of a novel GPCR ligand specificity can proceed from a promiscuous variant through changes in ligand efficacy rather than binding affinity. Furthermore, our work establishes a novel relationship between the proline of Ste2’s seventh transmembrane domain and position 2 and 12 of bound pheromones. These results may be especially relevant for GPCRs in higher eukaryotes with peptide ligands (chemokine receptors, opioid receptors, neuropeptide receptors, etc.) or peptide antagonists. Lastly, while we benefited from the relatedness of Scer and Klac mating apparatus, the ascomycete family includes many more species with additional receptor-pheromone homologs, providing further opportunities to improve our understanding of peptide discrimination in GPCRs.
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Author contributions

R.B.D.R and S.G.P designed study; R.B.D.R. performed experiments; R.B.D.R., B.C. and S.G.P. discussed results. R.B.D.R. wrote the manuscript with input and commentaries from all authors.
METHODS

Strains and growth conditions

The S. cerevisiae strains used in this study were derived from strain W303 and are listed in Supplementary Table III-1. For all assays, yeast strains were first transformed with plasmids by the lithium acetate / polyethylene glycol method [132] and grown on selective synthetic defined (SD) plates. Transformed colonies were picked and grown overnight in selective liquid SD medium in a 30°C shaking incubator. Cultures were then diluted to an OD = 0.1 and grown to exponential phase (OD = 0.5 to 0.8) before all treatments. Liquid SD medium was prepared from 6.74 g/L of yeast nitrogen base with ammonium sulfate without amino acids (BioShop), 1 g/L of the appropriate amino acid drop-out mix (BioShop) and 2% v/v glucose. Solid SD medium was prepared by adding 20 g/L of agar to liquid SD.

Plasmid propagation and construction

The plasmids and oligonucleotides used in this study are listed in Supplementary Table III-2 and Supplementary Table 3 respectively. Plasmid propagation in Escherichia coli DH5α was done with Luria Bertani (LB) medium supplemented with 50 µg/mL carbenicillin, and cultures were grown at 37°C. For plasmid construction, promoters were amplified from yeast genomic DNA (Invitrogen) and cloned at the PspOMI and XhoI sites in a pRS313 backbone. Ste2 ORFs were cloned at AarI sites.
**Peptide preparations**

Peptides were synthesized externally (Biomatik) to at least 95% purity. Peptides were dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM.

**Mating pathway activation assays**

Transformed RB001 cells from two independent colonies were treated with one or a series of concentrations of pheromone and placed in a 30°C shaking incubator. After 3 hours, protein synthesis was inhibited by treating cells with 10 µg/mL cycloheximide. The intensity of fluorescence in the 525/50 nm range was measured by analytical flow cytometry with a MACSQuant VYB (Miltenyi Biotec) equipped with a 588 nm laser. Cells without receptors were used to subtract basal cell fluorescence from other samples to reveal the net GFP fluorescence. For dose-response assays, the data were fitted with the “log(agonist) vs. response – Variable slope (four parameters)” model in Prism (GraphPad). All experiments included a WT Ste2 control. The fluorescence intensity was normalized to the maximum intensity of the WT control and multiplied by 100. EC50 values represent the mean of two experiments normalized to the WT value of each experiment. All Ste2 variants were under the control of the STE2 promoter.

**Mutagenesis and selection**

For site-directed mutagenesis, Ste2 ORFs were amplified using complementary mutagenic primers and Turbo polymerase (Agilent). For saturation mutagenesis of site 290, codons used are listed in Supplementary Table III-4. For random mutagenesis, the full-length open reading frame of Ste2 N216S V280I was amplified by error-prone PCR.
using the GeneMorph II kit (Agilent). PCR conditions (500 ng of template DNA, 20 cycles) were selected to yield a mean mutation rate of 4.0 DNA mutations per ORF [133]. The resulting amplimers were ligated in pRS-pSTE2 and amplified in *E. coli* DH5α to generate a mutant library. This library was transformed in the yeast strain RB001. For selection, cells were treated with 5 µM pheromone, incubated for 3 hours in a 30°C shaker and sonicated briefly. Cell sorting was done in a FACS-Aria (BD). For intermediate selection rounds, cells were washed in growth medium before being incubated in a 30°C shaker overnight for sorting the following day. For final selection rounds, cells were plated on solid SD medium. Colonies recovered this way were screened for their ability to activate the mating pathway better in response to either pheromone. Mutant *STE2* plasmids were extracted from the most promising colonies by treating saturated yeast cultures with zymolyase overnight at 4°C, followed by a standard plasmid miniprep protocol (Qiagen). Plasmids were then amplified in *E. coli*, transformed in naïve yeast cells to confirm that they conferred the phenotype, and sequenced.

**Pheromone binding assays**

To measure binding affinities and binding site levels, we used the NBD-labelled pheromone binding assay [115]. Transformed RB002 cells were treated with either varying concentrations of NBD-labelled *S. cerevisiae* alpha-factor (saturation binding assays) or varying concentrations of *Klak* pheromone mixed with 20 mM NBD-*S. cerevisiae* (competition binding assays) and left on ice for 10 min. Samples were processed by flow cytometry. For saturation binding, the data were fitted to the “One site – Total and nonspecific binding” model in Prism (GraphPad), and cells without receptors were used to account

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for non-specific binding. This model uses the equation $Y = B_{\text{max}}[L]/(K_d + [L]) + N[L] + \text{Background}$, where $Y$ is the mean fluorescence, $[L]$ is the concentration of labelled ligand and $N$ is a proportionality constant for nonspecific binding. For competition binding, the background-subtracted data were fitted to the “One site – Fit Ki” model with the appropriate constraints. This model uses the equation $K_i = IC_{50}/(1 + [L]/K_D)$. All experiments included a WT control and an empty vector negative control. $K_D$, $B_{\text{max}}$ and $K_i$ values were averaged from at least two experiments and normalized to the WT values of each experiment. For affinity values, Ste2 variants were under the control of the ADH1 promoter with the exception of Ste2 N216S P290Q, Ste2 N216S P290Q A265T and N216S P290Q A265T Y266F, which were under the control of the GPD promoter. For $B_{\text{max}}$ values, all variants were under the control of the ADH1 promoter.
Chapter 4:

GENERAL DISCUSSION AND FUTURE DIRECTIONS

In cells, a constant flow of information is managed by large networks of proteins and small molecules. The complexity of such networks allows organisms to cope with the wide diversity of situations that can be encountered in their environment. Understanding how this complexity has evolved has been a major challenge for biologists, one justified by the importance of cell signaling in disease, as well as the potential benefits of engineering proteins to make useful synthetic pathways. This thesis was undertaken with the specific aim of understanding a fundamental aspect of cell signaling evolution: how signaling networks can respond to new stimuli. For this, we focused on the GPCR family due to its size and the broad variety of ligands that can interact with these receptors. Despite this focus, the questions addressed and the conclusions drawn from our results are not strictly dependent on the unique features of this receptor class.

Whether they are located in a rich ecosystem or within a complex organism, GPCRs are capable of discerning between closely related molecules in order to trigger a suitable signaling response. Our work aimed to develop a better understanding of how ligand-receptor specificity can change GPCRs by focusing on the mating receptor Ste2. We originally hypothesized that the ligand binding pocket of this receptor was not likely to be the sole region involved in the evolution of new specificity because its extensive remodeling may require a large number of mutations, delaying adaptation by thousands of generations. Instead, we posited that other aspects of GPCR function might contribute to altering specificity whenever possible. One can divide a GPCR’s specificity into three
“layers” before an extracellular signal is allowed to be transduced. First, there is binding affinity, characterized by the fine interactions that provide a surface complementary to the ligand’s own structure. This enables strong and precise binding. A second layer of specificity can then be found at the level of ligand efficacy. A ligand may bind strongly, but fail to induce a conformational change necessary to result in the receptor’s active state, making that ligand an antagonist. Lastly, a third layer of specificity occurs at the level of signaling regulation. Interactions between the receptor and modulators of signaling can impose constraints that help the system discriminate between partial and full agonists. This can take the form of a threshold of receptor activation necessary to trigger downstream steps in a signaling pathway. Studies have examined all three layers of specificity for various GPCRs, but none as thoroughly as for the yeast pheromone receptor Ste2.

Our study took advantage of decades of accumulated insights on Ste2 function to unravel the relative contribution of binding affinity, efficacy and network regulation in the evolution of a new specificity. In two steps, we mimicked an evolutionary process whereby Ste2 was under selection pressure to respond to a foreign pheromone but not to its native ligand. By doing so, we uncovered the existence of alternative evolutionary paths provided by the three layers of specificity discussed above. In the first step of our experiment, we obtained promiscuous receptor variants with a reduced ability to interact with a negative regulator of signaling. This change constituted an evolutionary path that was fully independent from changes in ligand binding affinity or ligand efficacy. In the second step, we obtained a Ste2 variant, Ste2 K-Switch, with a fully inverted specificity
for *Klac* α-factor. We found that ligand efficacy constituted the primary mechanism of ligand discrimination in Ste2 K-Switch, while binding affinity played a major role for the wild-type specificity against the foreign peptide. The ability of Ste2 to change specificity in such diverse ways can be attributed to the complexity of its structure and of the signaling network that surrounds it. These features thus facilitate Ste2’s ability to change and adapt, i.e. its evolvability. The concept of evolvability, wherein successful organisms harbor proteins with an inherent ability to adapt, was first proposed for enzymes [65, 134]. Our work extends this notion to GPCRs.

*The Klac pheromone-specific Ste2 receptor: Ste2 K-Switch*

Over the course of our directed evolution experiment, we experimented with over 150 Ste2 variants, culminating in the new receptor Ste2 K-Switch. Although this variant was technically designed, the four amino acid substitutions it contains were all found through random mutagenesis and selection. As we searched for the right combination of mutations that would lead to a novel specificity, we did not formally set a definition of what constitutes a ligand-discriminating receptor as we felt this would be too arbitrary and difficult to justify. After all, a ligand’s potency is a continuum; it can range from being fully inert, to partial agonism and finally to “full” agonism, which implies a known response ceiling. In the case of WT Ste2, *Klac* α-factor is a partial agonist, with a response plateau of approximately 50% that of *Scer* α-factor at saturating concentrations (Figure III-4A). We were satisfied to see that the Ste2 K-Switch response in the presence of *Klac* pheromone was over 90% that of the wild-type response, while *Scer* pheromone led to a response plateau below 40% (Figure III-4B). Thus, the response profile of Ste2
K-Switch is an approximate “mirror image” of the WT receptor. However, a key difference between the two lies in the EC50 values of the pheromones that are being discriminated against. For the WT receptor, Klac α-factor has an EC50 of ~470 nM, indicating poor sensitivity to the foreign pheromone. Conversely, the EC50 of Scer α-factor with Ste2 K-Switch is ~7 nM, which is very close to its value with WT Ste2 of ~6 nM. Thus, in strict terms, Ste2 K-Switch is equally sensitive to either pheromone, giving it a lower discriminating ability at low concentrations. The reason for this may lie with binding affinity, since the mathematical model we developed in Chapter II showed that this property has a strong effect on sensitivity, but not on maximum signaling. It is not known what pheromone concentration range haploid cells encounter in nature, although high concentrations would be expected to work in Ste2 K-Switch’s favor for the purpose of ligand discrimination.

The low surface expression of Ste2 K-Switch constitutes an additional flaw of our variant. It is no surprise that some mutations, despite their positive effect on overall function, can have destabilizing effects on protein structure, thus jeopardizing proper folding and where applicable, transport to the membrane [121]. The combination of the two substitutions N216S and P290Q seems to have an especially strong negative effect on receptor expression. Directed evolution studies have shown that “stabilizing” rounds, consisting in selecting stable variants before focusing on the desired phenotype, can prove beneficial down the road by enabling combinations of destabilizing mutations to occur [61, 135]. We did not pursue this approach as we were primarily interested in the minimal set of mutations necessary to achieve a new specificity, although some
stabilizing Ste2 mutations have been described [136]. Furthermore, mating pathway responsiveness is very robust to low receptor levels as we demonstrated with variants S3 and T3 in Chapter II and this was reported previously as well [128]. While there must be a threshold below which Ste2 expression directly affects pathway activation, we do not appear to have reached it, even with Ste2 K-Switch which has a strong activity in the presence of its preferred pheromone. This robustness may be due to the high sensitivity of Gpa1 to changes in Ste2 conformation. Still, it is possible that low receptor expression could have negative consequences beyond signaling strength. The location of activated Ste2 receptors is thought to help establish cell polarity during mating so that shmooing can proceed towards the optimal mating partner [109, 137, 138], while another study has proposed that Ste2 acts as an adhesion molecule during cell-cell fusion [139]. It is not clear if the former process depends more on the fraction of occupied receptors, as is the case for signaling [125, 126], while the latter may suffer from weaker adhesion without an adequate number of receptors. These questions and others could be investigated using mating assays involving Scer cells expressing Ste2 K-Switch or Klac α-factor, or even WT Klac cells. While we were primarily interested in the evolutionary path taken from Ste2 to Ste2 K-Switch rather than the new receptor itself, we now find ourselves with a novel tool to interrogate mating efficiency under unique parameters.

The contribution of network interactions

In Chapter II, we demonstrated that the network of regulatory interactions surrounding Ste2 can provide alternative evolutionary routes when it is under selection pressure to respond to a new ligand. Since we identified several Ste2 variants with truncations in the
cytoplasmic tail, we focused our study primarily on the regulatory processes that take place in this region of the receptor. The first was receptor endocytosis, which was impaired in mutants, resulting in heightened levels of receptor surface expression. The second was Sst2-mediated downregulation of signaling, which was also weakened, resulting in greater sensitivity to Klac α-factor.

At least four of the variants selected after a single round of mutagenesis and selection showed a significantly enhanced binding affinity for Klac α-factor. Many of these harboured mutations occurring at sites that were previously linked to aspects of Ste2 ligand binding and signaling, while other sites were recurrent within our own study (N216, M218, G237, S267 and V280). The rapid emergence and frequent recurrence of such sites point to their likely importance in evolution. Although we did not focus on these variants as we felt that they were part of an “expected” evolutionary route, this set of high-affinity promiscuous receptors proved rather useful for the second step of our directed evolution experiment. As our starting point in Chapter III, we sought variants that could interact with both pheromones equally well to establish a baseline from which ligand discrimination would arise. Likewise, other variants with broad specificity-inducing mutations were useful for countering the signal-suppressing effects of P290 substitutions. Thus, our second study used mutations from variants S1, S2, S3 and S4 from our first study in order to obtain Ste2 K-Switch.

The irrelevance of receptor endocytosis for short-term sensitivity was contrary to our expectations. We originally reasoned that higher expression of the receptor on the cell
surface would lead to a stronger signal, even if the ligand was a weaker inducer/binder. This proved incorrect, as we demonstrated by modeling and experimentally. Subsequent work confirmed that receptor occupancy, not the absolute number of bound receptors, is what determines signaling strength and sensitivity [125, 126]. This suggests that non-signaling receptors play a negative role in signal transduction. Interestingly, our mathematical model suggested that unequal changes in endocytic rates (constitutive vs. ligand-induced) could affect sensitivity, but that this was not likely to be significant since it comes at the cost of signaling strength. Impairing endocytosis by shedding parts of the tail region may not meet this criterion if it leads to a reduction in both endocytic rates. It would be interesting to investigate this further by blocking one type of endocytosis but not the other. Unfortunately, most of the known set of proteins and tail sites involved in Ste2 internalization are involved in both constitutive and ligand-induced processes, with the possible exception of the α-arrestins Ldb19, Rod1 and Rog3 [100]. As such, the selective impairment of one endocytic process has not been demonstrated.

One of the major findings of our directed evolution experiment was the crucial role that the RGS Sst2 plays in ligand specificity. Previous work investigating Sst2 function focused on its ability to accelerate the GTPase activity of Gpa1 [140]. This shuts down pathway signaling and promotes recovery from pathway activation and cell-cycle arrest [141]. Deletion of Sst2 was also shown to enhance sensitivity to Scer α-factor [102]. Mathematical modeling revealed that this serves to “align” the dose-response sensitivity to receptor occupancy [75]. From these results, a model emerged whereby Sst2 contributes to a negative feedback loop and provides a noise “filter” by adding a
threshold of receptor activation necessary to trigger the mating pathway. Our results extend this model by adding a new role to the Sst2 filter. Through our experiments with Sst2Q304N and Klac α-factor, we demonstrated that wild-type Ste2 and Gpa1 are not sufficiently discriminating on their own. Rather, Ste2 must interact with Sst2 in order to exclude the activity of partial agonists. Although our work focused on mutations in Ste2 in order to alter specificity, this finding showed that another protein in the pathway can also be mutated, resulting in a new response. Furthermore, the fact that the interaction itself is necessary, while Sst2’s mere presence is not, was especially surprising since it gives great significance to the mediators of this interaction: the DEP domain of Sst2 and the C-terminal tail of Ste2. The role of the DEP domain in membrane localization has been shown previously for various proteins, including RGS proteins [142]. In one example analogous to Ste2 and Sst2, the M3 muscarinic receptor interacts with the DEP domain of RGS7, although this takes place through the third intracellular loop of the GPCR, not the cytoplasmic tail [12]. It is not currently known whether this system also benefits from a specificity filter provided by this interaction.

The newfound role of the C-terminal tail in Ste2 ligand specificity is especially interesting due to the apparent lack of major structures in this region. Our simple analysis with FoldIndex© found that the tail is most likely disordered, although in silico methods may over-predict disorder in terminal regions [143]. Extensive work undertaken by Becker and Naider demonstrated that the structure of the tail may depend on its surrounding environment. In their studies, the structure of the seventh transmembrane domain and part of the connecting cytoplasmic tail was investigated by NMR under
different solvent conditions [127, 144, 145]. Their results suggest that the C-terminal tail harbors short helices that are dependent on 1) proximity to the seventh transmembrane domain; and 2) an organic solvent which may simulate the interphase between the cytoplasm and the cell membrane. These helices may then act as interacting surfaces for the ubiquitinases and protein kinases that promote receptor endocytosis. The differences in rates between constitutive and ligand-induced endocytosis may thus be explained by a structure that is constantly in flux depending on conformational changes in the core receptor. Unfortunately, the end of the tail, beyond amino acid 372, was not investigated by this group. Our Ste2 mutant F1, along with a sensitivity assay conducted with several partially truncated variants (data not shown), suggest that at least part of the Sst2-interacting region may lie downstream of amino acid 400. Advances in structural biology methods for membrane proteins may eventually tell us whether this region is structured or disordered, or both.

The apparent disorder or “loose structure” of the C-terminal tail of Ste2 may allow it to act as a reservoir of interaction motifs and thus, a driver of network evolution for this protein. Previous studies have shown that disordered regions, like non-coding DNA, are not devoid of function. In fact, an abundance of protein-protein interaction motifs have been identified in disordered regions [146, 147]. While each individual motif does not necessarily provide a strong contact, a multitude of them does. As such, disordered regions are thought to act as incubators of sub-optimal interaction domains, a place where the trial and error of evolution can flourish. With high redundancy, the consequences of losing a single motif are not drastic. Likewise, a missense mutation is not problematic
because there is no global structure to perturb. If robustness to mutations is an inherent property of disordered regions, it must be especially true of C-terminal disordered regions, where a nonsense substitution can also be tolerated. Our first directed evolution experiment uncovered several mutants with premature stop codons occurring at different points along the C-terminal tail. Based on the high degree of variability in the length of the cytoplasmic tail among fungal Ste2 homologs, we posit that tail truncation and tail extension events may be quite common in evolution. These events would significantly affect how Ste2 and other GPCRs can interact with protein networks, since interaction motifs would be lost and gained each time. For functions that are especially important to maintain, redundancy throughout the tail would limit the impact of truncations. This may be the case for receptor internalization. The Ste2 tail harbours seven lysines that can be ubiquitinated, but only one ubiquitin moiety is necessary and sufficient to trigger endocytosis [89]. While phosphorylation of the tail is necessary to enable ubiquitination [85], there are 33 serines and threonines in this region and only a subset of these from different parts of the tail is sufficient to enable endocytosis [148]. An additional level of redundancy also appears to be provided by a small NPFX_{1,2,D}-like motif, GPFAD, at position 392. This motif interacts with Sla1p, an adaptor for the clathrin-based endocytic machinery [86]. Together, these sites may help ensure that receptor endocytosis can persist despite random mutations affecting either the ubiquitination/phosphorylation sites or the GPFAD motif. This is supported by the observation that receptors truncated at amino acid 345 or receptors lacking C-terminal lysines can still be internalized, albeit slowly [85]. It is worth noting that the tail of Scer Ste2 is rather long compared to other
Ascomycetes (Figure II-3) and may thus harbor more redundancy than most. It is unclear whether this benefits this species in particular and, if so, for what reason.

*The contribution of ligand efficacy*

In Chapter III, we showed that Ste2 K-Switch acquired specificity for *Klac α*-factor in a radically different way than WT Ste2 acquired its specificity for *Scer α*-factor. In the former, binding affinity plays no role in suppressing the potency of the *Scer* peptide, leaving efficacy as the sole contributor to ligand discrimination. This outcome reinforces the notion that affinity and efficacy are distinct properties. However, they are also interdependent, with GPCR activation thought to proceed as a series of transitions from an inactive state to intermediates and finally one of a variety of possible active states [149]. Each of these states may have their own affinities for the ligand and their own signaling ability, reflecting the complex interactions and conformational changes that occur at a molecular level. Since our measurements were taken over a significant period of time, they correspond to the “apparent” affinity and signaling ability of each pheromone-receptor pair. A closer investigation of GPCR activation dynamics may reveal more subtle differences between WT Ste2 and Ste2 K-Switch. For signaling, this can be done by FRET between the G protein α and β subunits [150]. For ligand binding, methods are scarcer, although NBD-Scer could potentially be used to reveal receptor binding steps based on the environment-sensitive properties of NBD [151].

The novel way in which Ste2 K-Switch acquired its discrimination ability is likely related to the use of laboratory evolution to obtain it. Although we attempted to maximize the
number of mutant alleles generated and screened, our study remains limited by its small scale compared to the millions of years of evolution that led to wild-type Ste2 and its binding pocket. A limited set of mutagenesis rounds and a strict selection strategy presumably favor small changes in structure with big consequences on function, hence why the evolutionary trajectories we identified change specificity in 2-4 mutations. With respect to real evolution, the conditions of our study may simulate natural conditions where rapid change is needed. For yeast, this might take the form of a random hybridization event leading to an inability to mate. Alternatively, the advent of a random mutation such as P290Q may precede a hybridization event and actually promote it by enhancing the response to Klac α-factor. In this case, our study shows that a sensitizing mutation like N216S would mostly suffice to complete the transition to Klac pheromone specificity.

Since Ste2 K-Switch is unable to discern between pheromones via binding affinity, this implies that Scer α-factor is now an antagonist. Antagonism can be problematic, since two pheromones are competing for the same binding site but only one can trigger a response, thus resulting in reduced signaling when both are present. While it is not known how much of a fitness cost this would impose, we can speculate on the various ways that cells expressing Ste2 K-Switch might cope with this issue. For one, antagonism itself might provide the solution: mutant cells that are in close proximity to WT Scer cells would be unresponsive and mate less, thus favoring cells that are farther away where mating is beneficial. Alternatively, since yeast can sense pheromones spatially, shmooing might be enhanced towards pure Klac α-factor-producing populations, resulting in
progressive spatial and eventually geographical isolation. Such scenarios could be verified through \textit{in silico} simulations of mixed yeast populations. Another solution to antagonism would be co-evolution of the pheromone with the receptor. Mutations in the \textit{Klac} peptide sequence, which we did not explore extensively, could enhance its binding affinity with Ste2 K-Switch, out-competing \textit{Scer} \(\alpha\)-factor for binding sites. A large library of pheromone variants would be necessary to test this and we resorted to synthetic peptides in our studies. However, mating pathway activation from GPI-anchored pheromones was previously demonstrated in \cite{152}. This approach could theoretically be combined with a library of mutant pheromone alleles to investigate receptor-ligand co-evolution with biosynthetic peptides.

\textit{Studying ligand specificity and sensitivity in other GPCRs}

It is our hope that our findings may spur further research in GPCR evolution and function. Specifically, the principles uncovered in this thesis pertaining to the alternative ways that Ste2 specificity can change are likely generalizable to other GPCRs in higher eukaryotes since Ste2 signaling and mating pathway architecture are not unique to yeast. Likewise, the notion that efficacy alone can change specificity may apply to other membrane and cytoplasmic receptors where agonists can be turned into competitive antagonists. This has important implications in health and protein engineering, where binding affinity is often a primary focus at the expense of other aspects of receptor function. Our work suggests that in-depth studies of receptor specificity must take into account ligand efficacy and network contributions in order to fully understand the ability to discriminate between ligands. For the latter especially, this means studying a receptor
in its native context in the presence of its interaction partners. For GPCRs, yeast strains engineered with mammalian RGS and G proteins could provide a compromise between biological relevance and cell robustness.

Additionally, our findings concerning the importance of the C-terminal tail on Ste2 sensitivity and specificity could potentially open a new area of study in GPCR biology. Cytoplasmic tail length variability has not been extensively studied in higher eukaryotes, although reported examples of protein-protein interactions occurring at the tail are numerous [112, 153, 154]. A few studies did report potentially significant changes in tail length. For instance, the gonadotrophin-releasing hormone receptor shows taxon-specific tail lengths, with mammals possessing a uniquely tailless receptor [155, 156]. The reasons behind this variability are unclear, although our work suggests that interactions with signaling proteins should be a focal point for further research. Analyzing post-stop DNA sequences may also reveal the presence of vestigial interaction motifs. Tail length variability can also occur within an organism through alternative splicing, resulting in differences in receptor trafficking and desensitization between GPCR isoforms [114, 157], as well as important clinical effects [158]. We believe that ligand specificity should be examined carefully in such cases. This may uncover novel roles of the cytoplasmic tail in disease.
Supplementary Figure II-1: The Ste2 variants selected show a diversity of mutations, a robust native activity and a concomitant increase in promiscuity. A, Snake plot of the G protein-coupled receptor Ste2. Mutated amino acid residues are
highlighted according to the legend in the top right. B, GFP fluorescence was measured in cells treated with 5 µM pheromone, incubated for 3 hours and analyzed by flow cytometry. All variants retained their ability to response to Scer α-factor, and many variants acquired the ability to respond to Cgla α-factor, which was not a criterion for selection. Error bars represent the s.e.m.
Supplementary Figure II-2: The Ste2 cytoplasmic tail is disordered. The predicted disordered region of Ste2 was obtained from FoldIndex© [98]. The blue-shaded area indicates the cytoplasmic tail region, amino acids highlighted in yellow are predicted to be unfolded and boxed residues indicate the premature STOP sites of our truncation mutants. The predicted disordered region encompasses most of the cytoplasmic tail.
Supplementary Figure II-3: Truncated receptors show defective internalization.

Cells expressing Ste2-GFP fusions were visualized by fluorescence microscopy before and after treatment with 5 µM Scer pheromone. The top two rows show fluorescence and the bottom two rows show differential interference contrast (DIC). While the WT receptor internalizes upon addition of pheromone (GFP signal moves from the plasma membrane to endosomes), the GFP signal of the truncated receptors remains largely at the plasma membrane after pheromone induction. Scale bar in bottom right represents 25 µm.
Supplementary Figure II-4: A simplified model of the Ste2 signaling network explains the mixed effects of receptor endocytosis on pathway sensitivity. A, Free Ste2 receptors (R) bind to their cognate pheromone ligand (L), forming a receptor-ligand complex (RL). Both are internalized and degraded (dotted proteins). Only non-internalized receptors can signal by promoting GFP expression from a mating-responsive promoter. GFP is degraded at a basal rate. Reactions and their rate constants are indicated by labeled arrows. B, Conceptual view of the bound-unbound receptor equilibrium. The equilibrium is dictated by both binding and endocytosis kinetics. C, Theoretical effects of altering endocytosis rates on sensitivity and maximum response. Fold-changes are...
applied on wild-type values. Shaded red area represents lower rates. D. Simulated dose-response relationships with experimentally-derived endocytosis rates. Lower rates of endocytosis do not greatly improve the response to *Klac* pheromone without losing sensitivity.
Supplementary Figure II-5: MAPK phosphorylation confirms the ability of Sst2^{Q304N} to enable a strong response to Klac α-factor with WT Ste2. Pathway activation was measured in cells treated with 3 μM pheromone, incubated for 30 min and lysed for Western Blotting. Phosphorylated Fus3 levels were normalized to PGK levels and plotted. The asterisk indicates a statistically significant difference to wild-type (P < 0.05, Bonferroni-Corrected t-test). Error bars represent the s.e.m.
Supplementary Figure II-6: Cells expressing wild-type Sst2 and Sst2\textsuperscript{Q304N} show different rates of Fus3 de-phosphorylation. A-D, Levels of phosphorylated Fus3 following pathway deactivation, normalized to time zero. Pheromone-treated cells were washed once, resuspended in pheromone-free medium, incubated for the indicated time and lysed. E-F, Levels of phosphorylated Fus3 following pathway activation with either
Scer α-factor (A) or Klac α-factor. Cells were treated with 3 µM pheromone, incubated for 30 min and lysed. Lysates were used for Western Blotting. Phosphorylated Fus3 levels were normalized to PGK levels and plotted. Green bar graphs indicate Scer α-factor, red indicated Klac α-factor. Variants assayed are indicated in bold. Error bars represent the s.e.m.
**Supplementary Figure II-7: Mating projections of cells expressing Ste2 variants are typical with both pheromones.** Mating-competent cells expressing Ste2 variants were visualized by differential interference contrast microscopy before and after treatment with 5 µM pheromone. Cells expressing wild-type receptor form normal mating projections in response to Scer α-factor, while Klac α-factor induces abnormal shmoo formation. Cells expressing mutant receptors shmoo normally with either pheromone. We observed no shmoo in the absence of pheromone for any variant. Scale bar in bottom right represents 25 µm.
**Supplementary Figure II-8: Full Western blots of Figure 5 (mating pathway deactivation).** Uncropped Western blots of mating pathway deactivation featured in Figure 5 using *Scer* (left blots) or *Klac* pheromone (right blots). Green bands represent phosphorylated Fus3 (top four blots) or total Fus3 (bottom four) while red bands represent either the loading control PGK or the molecular weight marker bands.
Supplementary Figure II-9: Full Western blots of Supplementary Figure 6 (mating pathway deactivation). Uncropped Western blots of mating pathway deactivation featured in Supplementary Figure 6 using *Scer* (left blots) or *Klac* pheromone (right blots). Green bands represent phosphorylated Fus3 (top four blots) or total Fus3 (bottom four) while red bands represent either the loading control PGK or the molecular weight marker bands.
Supplementary Figure II-10: Full Western blots of Supplementary Figures 5 and 6 (initial mating pathway activation). Uncropped Western blots of initial mating pathway activation featured in Supplementary Figures 5 and 6 using *Scer* or *Klac* pheromone. Green bands represent phosphorylated Fus3 (top four blots) or total Fus3 (bottom four) while red bands represent either the loading control PGK or the molecular weight marker bands.
### Supplementary Table II-1: Ste2 variants isolated following directed evolution.

<table>
<thead>
<tr>
<th>Name</th>
<th>Selection round</th>
<th>Mutations</th>
</tr>
</thead>
<tbody>
<tr>
<td>S1</td>
<td>1</td>
<td>I82N, N216D, Y266F</td>
</tr>
<tr>
<td>S2</td>
<td>1</td>
<td>V280I</td>
</tr>
<tr>
<td>S3</td>
<td>2</td>
<td>S267C, V280D, K358R, T414M</td>
</tr>
<tr>
<td>S4</td>
<td>2</td>
<td>N216S</td>
</tr>
<tr>
<td>S5</td>
<td>2</td>
<td>S267R, T282S</td>
</tr>
<tr>
<td>S6</td>
<td>2</td>
<td>M54V, A62T, M69L, G115R</td>
</tr>
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<td>I92M, R233K, F244L, T414M</td>
</tr>
<tr>
<td>S8</td>
<td>0(^a)</td>
<td>T155A, M218V, L255F</td>
</tr>
<tr>
<td>S9</td>
<td>0(^a)</td>
<td>A229V, E373V, A416T</td>
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<tr>
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<td>2</td>
<td>S73L, T282A, E361K</td>
</tr>
<tr>
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<td>A5T, S47R, L222M, V407D, K422Q</td>
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<td>2</td>
<td>Q21H, L226F, V257L</td>
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<td>T1</td>
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<td>Y30H, K358*</td>
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<td>T3</td>
<td>2</td>
<td>G237D, F312L, R350*</td>
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<tr>
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</tr>
<tr>
<td>F1</td>
<td>2</td>
<td>N25D, K202T, T309N, A397E, Fs401</td>
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\(^a\): Mutants isolated prior to FACS rounds. These were obtained from a random selection of unsorted mutants followed by a screen for mating pathway activation in the presence of \(Klac\) pheromone.

Mutations in **bold** affect sites previously implicated in Ste2 function.

### Supplementary Table II-2: Artificial truncation mutants and their properties

<table>
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<th>Mutant</th>
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<th>T1(^*)</th>
<th>T2</th>
<th>T2(^*)</th>
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<tbody>
<tr>
<td>Scer EC50</td>
<td>0.375 ± 0.14</td>
<td>0.174 ± 0.026</td>
<td>0.909 ± 0.20</td>
<td>0.751 ± 0.071</td>
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<tr>
<td>Klac EC50</td>
<td>80.4 ± 12</td>
<td>45.7 ± 11</td>
<td>100 ± 8.9</td>
<td>61.6 ± 5.7</td>
</tr>
<tr>
<td>Scer (K_D)</td>
<td>5.20 ± 1.0</td>
<td>5.19 ± 0.85</td>
<td>4.23 ± 0.43</td>
<td>5.84 ± 1.4</td>
</tr>
<tr>
<td>Klac (K_i)</td>
<td>517 ± 58</td>
<td>418 ± 9</td>
<td>420 ± 49</td>
<td>480 ± 100</td>
</tr>
<tr>
<td>(B_{max})</td>
<td>7.97 ± 0.83</td>
<td>13.2 ± 1.3</td>
<td>11.6 ± 1.4</td>
<td>16.8 ± 3.3</td>
</tr>
</tbody>
</table>

Values represent the mean ± the standard error of the mean (s.e.m.)
Supplementary Table II-3: $B_{\text{max}}$ values of Ste2-C-Venus variants

<table>
<thead>
<tr>
<th>Ste2 variant</th>
<th>Mean $B_{\text{max}}$ ± s.e.m.</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT</td>
<td>1 ± 0.26</td>
</tr>
<tr>
<td>T1</td>
<td>3.19 ± 1.0</td>
</tr>
<tr>
<td>T2</td>
<td>4.52 ± 2.2</td>
</tr>
<tr>
<td>S2</td>
<td>0.738 ± 0.10</td>
</tr>
<tr>
<td>S4</td>
<td>0.805 ± 0.25</td>
</tr>
</tbody>
</table>

Supplementary Table II-4: Model parameters and numerical values used in fits and simulations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Wild-type value</th>
<th>Source</th>
<th>Alternative value</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{R_p}$</td>
<td>3.12 nM min$^{-1}$</td>
<td>[90, 159, 160], our binding data$^a$</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>$k_{on}$</td>
<td>0.19 nM$^{-1}$ min$^{-1}$</td>
<td>[150], our binding data$^b$</td>
<td>0.0022 nM$^{-1}$ min$^{-1}$ (Klac pheromone) Binding assays$^b$</td>
<td></td>
</tr>
<tr>
<td>$k_{off}$</td>
<td>0.6 min$^{-1}$</td>
<td>[150]</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>$k_{Re}$</td>
<td>0.0156 min$^{-1}$</td>
<td>[159]</td>
<td>0.005 min$^{-1}$ Binding assays$^c$</td>
<td></td>
</tr>
<tr>
<td>$k_{RL_e}$</td>
<td>0.0693 min$^{-1}$</td>
<td>[159]</td>
<td>0.023 min$^{-1}$ Binding assays$^d$</td>
<td></td>
</tr>
<tr>
<td>$k_{Rs}$</td>
<td>9.05·10$^{-6}$ min$^{-1}$</td>
<td>Median fit to dose-response$^e$</td>
<td>N/A</td>
<td></td>
</tr>
<tr>
<td>$k_{RL_s}$</td>
<td>1.42·10$^{-4}$ min$^{-1}$</td>
<td>Median fit to dose-response$^e$</td>
<td>1.70·10$^{-5}$ min$^{-1}$ (Klac pheromone) Median fit to dose-response$^e$</td>
<td></td>
</tr>
<tr>
<td>$\tau$</td>
<td>148 min</td>
<td>[68]</td>
<td>N/A</td>
<td></td>
</tr>
</tbody>
</table>

$^a$: Wild-type cells harbor 4900 STE2 molecules, corresponding roughly to a concentration of 200 nM for a cellular volume of 42 fL. The synthesis rate is calculated from equation (9), $B_{\text{max}}$ values from our binding assays and the wild-type value of $k_{Re}$.

$^b$: The binding rate is calculated using the $k_{off}$ of Yi et al. and our measured $K_D$ values.

$^c$: The basal endocytosis rate is calculated using $B_{\text{max}}$ values from our binding assays and the wild-type value of $k_{R_p}$ which is assumed to be constant.

$^d$: The alternative induced endocytosis rate is calculated by preserving the ratio between basal and induced rates.

$^e$: The signaling ability of a receptor-ligand pair was obtained by fitting Equation (9) to dose response data.
### Supplementary Table II-5: Yeast strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>SΦ992</td>
<td>W303 MATa, STE2, SST2, BAR1, FAR1, MFA2, his3, trp1, leu2, ura3</td>
<td>[68]</td>
</tr>
<tr>
<td>CB008</td>
<td>SΦ992 bar1::NatR, far1Δ</td>
<td>[68]</td>
</tr>
<tr>
<td>CB009</td>
<td>CB008 mfa2::pFUS1-GFP</td>
<td>[68]</td>
</tr>
<tr>
<td>RB001</td>
<td>CB009 ste2::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>RB002</td>
<td>CB008 ste2::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>RB003</td>
<td>RB001 sst2::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>RB004</td>
<td>RB002 sst2::URA3</td>
<td>This study</td>
</tr>
<tr>
<td>RB005</td>
<td>SΦ992 ste2::TRP1</td>
<td>This study</td>
</tr>
<tr>
<td>RB006</td>
<td>RB005 sst2::URA3</td>
<td>This study</td>
</tr>
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</table>

### Supplementary Table II-6: Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
<th>Purpose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ste2KO_Trp3</td>
<td>AGGTGGTTTCTACCACCTACCTACG AGATGTTTATTATGTAAGGAAGTT TAAATATTATCAAATAAGAAAGAT ACCATTTTTTTTTATAGAGAA GTGCACTCTGATGAGCACCTGAATC TAGTGTAACCTTATAACCGAAGGT CACGAAATTTCTTTCAAAAACCG TAAATTTGTAGTTATCACATTAT ATCCCT</td>
<td>Deletion of STE2 with TRP1 marker</td>
</tr>
<tr>
<td>Ste2KO_Trp5</td>
<td>AAAAAAGCTTTTCTACATATTCAAG ATTTTTTTCTGTGGGTGGAATACTA TTTAAGGAGTGCTATTAGTATCTTA TTTGACTCTCAAAAGCAATACGATAC CTTTCTTTTTTCACCTGCTTGGCTA TAATTTAATTTGTATCTTAAAAAT GCACCGTTAAAGAAACCATATCAAG AATCAAACCCGTCGTTTTGAGAGA AATC</td>
<td></td>
</tr>
<tr>
<td>pSTE2_PspOMI_5</td>
<td>TACTGCAGGGGCCCATCCAAT</td>
<td>Cloning of STE2 promoter with flanking PspOMI and XhoI sites</td>
</tr>
<tr>
<td>pSTE2_XhoI_3</td>
<td>TGG TAA TCC TCG AGT TTT GAT TCT TGG ATA TG</td>
<td></td>
</tr>
<tr>
<td>STE2_AarI_5</td>
<td>GCC ATG AAC ACC TGC AAC ACC CTA TGT CTG ATG CGG CTC CTT C</td>
<td>Cloning and mutagenesis of STE2 ORF with flanking AarI sites</td>
</tr>
<tr>
<td>STE2_AarI_3</td>
<td>GTTACAGGCACTGCAACATCGCT CATAAATTATTAT TATC</td>
<td></td>
</tr>
<tr>
<td>Name</td>
<td>Sequence</td>
<td>Description</td>
</tr>
<tr>
<td>---------------------------</td>
<td>--------------------------------------------------------------------------</td>
<td>------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pSTE2_AarIfix_3</td>
<td>TTATAATTATAGCCAGAGCAGTG AAAAGAAAAGGTATCGT</td>
<td>Removing AarI site in STE2 promoter</td>
</tr>
<tr>
<td>pSTE2_AarIfix_5</td>
<td>ACGATACCTTTTCTTTTCACGTGCT CTGGCTATAATTATAA</td>
<td></td>
</tr>
<tr>
<td>T1_AarI_3</td>
<td>GTTACA GGC ACC TGC AAC ATC GCT CAT AAA TTA TTA TTA TCT TCA G</td>
<td>Truncation of STE2 to make T1*</td>
</tr>
<tr>
<td>T2_AarI_3</td>
<td>GTT ACA GGC ACC TGC AAC ATC GCT TAC TCA GGA TCA TCG TTG TTG ATA CTA TCA</td>
<td>Truncation of STE2 to make T2*</td>
</tr>
<tr>
<td>STE2_AarI_fuse_3</td>
<td>GTTACAGGCACCTGCAACATCGCT CACCTCCTAAATTATTATTATCTTTC</td>
<td>Cloning of STE2 without a STOP codon</td>
</tr>
<tr>
<td>STE2_AarI_fuse358_3</td>
<td>GTTACAGGCACCTGCAACATCGCT CACCTCCATCCGATGTGTGTTTTCTT</td>
<td>Cloning of T1 mutant without a STOP codon</td>
</tr>
<tr>
<td>STE2_AarI_fuse350_3</td>
<td>GTT ACA GGC ACC TGC AAC ATC GCT CAC CTC CCT CAG GAT CAT CGT TGT T</td>
<td>Cloning of T2 mutant without a STOP codon</td>
</tr>
<tr>
<td>SST2_KO_URA_5</td>
<td>GAATTTGTGTCTGTCTCTGTTATATT TATACCGGTGAATGAAATCTAATAT CCCAGCTCTGTCTTTACTTCATC GTCCTTTAACTTTTGAGGGTTTACT GTCGTACGTTCCTTCCTAGGTTTGTGCC ACGCAGTATCTGAGGGCGTTTATTAGG TTCAATT TTGTAAT TAAAGATAGA GTGTGTAAGGTGTCTAGACACCTGCAA CATG</td>
<td>Deletion of SST2 with URA3 marker</td>
</tr>
<tr>
<td>SST2_KO_URA_3</td>
<td>AAATGGGCTTTATTTAATCATATTAT AGTTGATATAACAAATCACACCTC CGCTTTAATTCAGTATAGTATT TACAGTCCTATAGGTATCAGAC ATATAGGTTAGGAACCTAAAGA AAAAAAAAAAGGACTGTTTGTGCCA TTGTACCTGAAAGATGATGAAGACT CTCAATGAAATCGATGAATTTCGAG CTCGTT</td>
<td></td>
</tr>
<tr>
<td>SST2_BamHI_3</td>
<td>TGG TAA TCG GAT CCT TAG CAC TTT TCT TGG ATT TC</td>
<td>Cloning of SST2 with flanking PspOMI and BamHI sites</td>
</tr>
<tr>
<td>SST2_PspOMI_5</td>
<td>TAC TGC AGG GGC CCT GTC TAC TTC AAA TTC GAA C</td>
<td>Site-directed mutagenesis of SST2</td>
</tr>
<tr>
<td>SST2_Q304N_SDM_3</td>
<td>TCA GTA CAG TCC ATT ATC CAA TTC CAT ATA GCC TTT GTT GTA A</td>
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Supplementary Table II-7: Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
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</thead>
<tbody>
<tr>
<td>pRS313</td>
<td>CEN HIS3 vector</td>
</tr>
<tr>
<td>pRS315</td>
<td>CEN LEU2 vector</td>
</tr>
<tr>
<td>pRS-PSTE2</td>
<td>CEN HIS3 STE2 promoter (P&lt;sup&gt;STE2&lt;/sup&gt;)</td>
</tr>
<tr>
<td>pRS-STE2</td>
<td>CEN HIS3 P&lt;sup&gt;STE2&lt;/sup&gt; STE2</td>
</tr>
<tr>
<td>pRS-PADH1</td>
<td>CEN HIS3 ADH1 promoter (P&lt;sup&gt;ADH1&lt;/sup&gt;)</td>
</tr>
<tr>
<td>pRS-PADH1-STE2</td>
<td>CEN HIS3 PADH1&lt;sub&gt;1&lt;/sub&gt; STE2</td>
</tr>
<tr>
<td>pRS-PSTE2-GFP</td>
<td>CEN HIS3 P&lt;sup&gt;STE2&lt;/sup&gt; GFP</td>
</tr>
<tr>
<td>pRS-PSTE2-CVen</td>
<td>CEN HIS3 P&lt;sup&gt;STE2&lt;/sup&gt; C-Venus fragment</td>
</tr>
<tr>
<td>pRS-PSTE2-7KtoR</td>
<td>CEN HIS3 P&lt;sup&gt;STE2&lt;/sup&gt; STE2 7KtoR variant</td>
</tr>
<tr>
<td>pRS-SST2</td>
<td>CEN LEU2 P&lt;sup&gt;SST2&lt;/sup&gt; SST2</td>
</tr>
<tr>
<td>pRS-SST2 Q304N</td>
<td>CEN LEU2 P&lt;sup&gt;SST2&lt;/sup&gt; SST2&lt;sup&gt;Q304N&lt;/sup&gt;</td>
</tr>
<tr>
<td>pRS-PADH1-SST2-NVen</td>
<td>CEN LEU2 P&lt;sup&gt;SST2&lt;/sup&gt; SST2 N-Venus fragment</td>
</tr>
<tr>
<td>pRS-PADH1-SST2 Q304N-NVen</td>
<td>CEN LEU2 P&lt;sup&gt;SST2&lt;/sup&gt; SST2&lt;sup&gt;Q304N&lt;/sup&gt; N-Venus fragment</td>
</tr>
</tbody>
</table>

Supplementary Table II-8: α-factor pheromones and their peptide sequences.

<table>
<thead>
<tr>
<th>Species</th>
<th>Peptide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saccharomyces cerevisiae</td>
<td>WHWLQLKPGQPMY</td>
</tr>
<tr>
<td>Naumovozyma castellii</td>
<td>WHWLRLDPPGQPLY</td>
</tr>
<tr>
<td>Kluyveromyces lactis</td>
<td>WSWITLRPGQPIF</td>
</tr>
<tr>
<td>Candida glabrata</td>
<td>WHWVRLRKGQGLF</td>
</tr>
</tbody>
</table>
SUPPLEMENTARY NOTE

Supplementary Note II-1: Mathematical model of Ste2 signaling and endocytosis

We investigated the contribution of receptor internalization on mating pathway sensitivity to explain our dose-response data with endocytosis-defective mutants. Ste2 endocytosis involves two distinct processes: constitutive (slow) and ligand-induced (fast). We postulated that the two processes might not contribute equally to sensitivity due to the differences in their rates. However, we faced difficulties in trying to untangle the two phenomena, as the same key amino acid residues operate in both. As such, we resorted to mathematical modeling in order to reveal the contribution of each process.

Our model describes a simplified Ste2 signalling pathway (Supplementary Fig. 3A). In this pathway, a free receptor and ligand associate according to classical single-site kinetics, forming a receptor-ligand complex. Free and bound receptors are internalized at different rates, and the MAP kinase cascade is “black-boxed”, such that free and bound receptors signal by directly promoting the expression of GFP through a mating-responsive promoter. GFP is then degraded and diluted by cell division over time. For simplicity and to focus on receptor internalization, we did not include the RGS Sst2. The effects of Sst2 on sensitivity and maximum response were modeled previously [102].

From this model, we obtained the following simplified equation describing the concentration of GFP at steady-state (see derivation below).
As expected, the relationship between GFP and ligand has the form of a Hill equation resulting in a sigmoidal dose-response relationship. As the equation demonstrates, the sensitivity of the response is determined by the product of the dissociation constant $K_D$ and the ratio of the endocytosis rate constants, $k_{Re}$ and $k_{RLe}$. This ratio implies that the two endocytic rates contribute to sensitivity in an opposing manner; slower basal endocytosis is predicted to improve sensitivity, while slower induced endocytosis has the reverse effect. Conceptually, this is because basal and induced endocytosis have opposite consequences: the former reduces the pool of free receptors while the latter reduces the pool of bound receptors (Supplementary Fig. 3B). This parallels the outcomes of ligand binding and dissociation respectively. As such, the difference between $k_{Re}$ and $k_{RLe}$ modifies the equilibrium constant of the response, thereby controlling sensitivity, much like the difference between $k_{on}$ and $k_{off}$.

Furthermore, the amount of receptors on the cell surface is given by the ratio $k_{Rp} / k_{Re}$ and is predicted to control the magnitude of the response but not the sensitivity. Although our data confirms the lack of a relationship between receptor overexpression and sensitivity, we observed no effect on magnitude (Figure 4A). This may be because the G protein is limiting in the Ste2 signaling process [161, 162].

We proceeded to perform time simulations in MATLAB (Mathworks) to test whether altering individual endocytic rates could improve mating pathway sensitivity to *Klac* pheromone. Parameter values were obtained from the literature or derived from our data.
Simulations began 1000 minutes prior to pheromone addition to ensure steady-state concentrations of free receptor and GFP. Following pheromone addition, the concentration of GFP was extracted after 3 hours to construct a dose-response. In this way, we first examine the effects of altering the rates of receptor endocytosis on response strength and sensitivity (Supplementary Fig. 3C). While lowering both rates has the potential to increase response strength, sensitivity moves in the opposite direction. This is reflected when lowering both rates to the same extent as our truncation mutants (Supplementary Fig. 3D). While the lower rate of basal endocytosis improves sensitivity, the lower rate of induced endocytosis has the opposite effect: lowering both leads to greater response strength, but a largely unchanged sensitivity. These simulations help to explain why a global impairment in receptor endocytosis has only marginal effects on sensitivity.

**Derivation of steady-state equation**

The steady-state equation of GFP concentration was derived from a system of three ordinary differential equations (ODEs) describing changes in the concentration of GFP, free receptor \( R \) and bound receptor \( RL \). The concentration of ligand \( L \) was assumed to be constant throughout (in excess). Furthermore, the model assumes that all chemical species are spatially homogeneous and that changes in concentration are continuous and not significantly affected by discrete molecular events.

\[
\frac{d[GFP]}{dt} = k_{RLs}[RL] + k_{Rs}[R] - \frac{[GFP]}{\tau} \quad (1)
\]

\[
\frac{d[RL]}{dt} = k_{on}[R][L] - k_{off}[RL] - k_{RLe}[RL] \quad (2)
\]
\[
\frac{d[R]}{dt} = k_{Rp} - k_{on}[R][L] + k_{off}[RL] - k_{Re}[R] \tag{3}
\]

For a more meaningful derivation, we introduce a redundant ODE describing the change in \(R_T\), the total number of surface receptors such that \(R_T = R + RL\):

\[
\frac{d[R_T]}{dt} = k_{Rp} - k_{Re}[R] - k_{RLe}[RL] \tag{4}
\]

At steady state, the net change in concentration of a species is zero, yielding steady-state concentrations (subscript SS):

\[
GFP_{SS} = \tau((k_{RLS} - k_{Rs})RL_{SS} + k_{Rs} \cdot R_{TSS}) \tag{5}
\]

\[
RL_{SS} = \frac{R_{TSS}[L]}{[L]+(k_{off}+k_{RLe})/k_{on}} \tag{6}
\]

\[
R_{TSS} = \frac{k_{RP}/k_{Re}}{1+k_{RLe}^{-k_{Re}}[L]/[L]+k_{D}+k_{RLe}/k_{on}} \tag{7}
\]

Substituting the steady-states expressions for free and bound receptors into the equation for \(GFP_{SS}\), we obtain:

\[
GFP_{SS} = \tau \frac{k_{RP}}{k_{Re}} \left((k_{RLS} \frac{k_{Re}}{k_{RLe}} - k_{RS}) \frac{[L]}{[L]+\frac{k_{off}+k_{RLe}}{k_{on}} \frac{k_{Re}}{k_{RLe}}} + k_{RS}\right) \tag{8}
\]
We can approximate $k_{\text{off}} + k_{\text{RLe}} \approx k_{\text{off}}$, such that the dissociation constant $K_D = k_{\text{off}} / k_{\text{on}}$ can be used, yielding equation 9. This approximation is reasonable at wild-type values of $k_{\text{off}}$ and $k_{\text{RLe}}$, and mutants are expected to have lower values of $k_{\text{RLe}}$. 
SUPPLEMENTARY METHODS

Strains and growth conditions

The *S. cerevisiae* strains used in this study were derived from strain W303 and are listed in Supplementary Table 5. Gene deletions were done by standard homologous recombination. For all assays, yeast strains were first transformed with plasmids by the lithium acetate / polyethylene glycol method [132] and grown on selective synthetic defined (SD) plates. Transformed colonies were picked and grown overnight in selective liquid SD medium in a 30°C shaking incubator. Cultures were then diluted to an OD = 0.1 and grown to exponential phase (OD = 0.5 to 0.8) before all treatments. Liquid SD medium was prepared from 6.74 g/L of yeast nitrogen base with ammonium sulfate without amino acids (BioShop), 1 g/L of the appropriate amino acid drop-out mix (BioShop) and 2% v/v glucose. Solid SD medium was prepared by adding 20 g/L of agar to liquid SD.

Plasmid propagation and construction

Plasmid propagation in *Escherichia coli* DH5α was done with Luria Bertani (LB) medium supplemented with 50 µg/mL carbenicillin, and cultures were grown at 37°C. For yeast expression of Ste2 and Sst2, the promoters and ORFs were amplified from yeast genomic DNA (Invitrogen). For Ste2, the promoter and ORF were cloned separately into a variant of the pRS313 vector with designed AarI sites [4]. The promoter was cloned first at the PspOMI and XhoI sites, and its endogenous AarI recognition site was removed by substituting C for G at position -69, generating pRS-PSTE2. The STE2 ORF was subsequently cloned at the AarI sites, generating pRS-STE2. pRS-PADH1 and
pRS-PADH1-STE2 were cloned as described for pRS-PSTE2 and pRS-STE2 respectively, though no AarI recognition site was present in the promoter. The ORF of the endocytosis-defective Ste2 mutant 7KtoR was obtained by gene synthesis (Integrated DNA Technologies) and cloned into pRS-PSTE2 at the AarI sites, generating pRS-PSTE2-7KtoR.

For the expression of Sst2, the SST2 promoter and ORF were cloned in a single step at the PspOMI and BamHI sites of pRS315, generating pRS-SST2. The mutation Q304N was added with the Quickchange II site-directed mutagenesis kit (Agilent).

For receptor-GFP or fusions, the GFP ORF was first cloned into pRS-pSTE2 at the BamHI site to generate pRS-PSTE2-GFP. This vector was used to accept wild-type or mutant STE2 ORFs lacking STOP codons at the AarI sites. Similar steps were performed to fuse receptors to C-Venus fragments and Sst2 to N-Venus fragments.

All oligonucleotides and plasmids used in this study are listed in Supplementary Tables 6 and 7 respectively.

**Peptide preparations**

Peptides were synthesized externally (Biomatik) and dissolved in dimethyl sulfoxide (DMSO) to a stock concentration of 10 mM. For microscopy, peptides were dissolved in water to preserve good optical performance. Peptides are listed in Supplementary Table II-8.
Live cell imaging

For shmooing experiments, yeast cells were fixed with 100 µg/mL concavalin A in a 96-well glass bottom Sensoplate (Greiner). The cells were treated with 5 µM of pheromone and incubated at 30°C for 2 hours. Multiple fields from duplicate samples were imaged using a TCS SP8 confocal microscope (Leica). For visualizing endocytosis, cells expressing GFP-tagged Ste2 receptors were loaded in a CellASIC Onix microfluidic plate (Merck Millipore) and treated with 5 µM of pheromone. A single field from duplicate samples were imaged at intervals up to 45 minutes after treatment.
Appendix II: Supplementary information for Chapter III

SUPPLEMENTARY FIGURES

Supplementary Figure III-1: Alternating positive and selection works best to obtain functional discriminating Ste2 variants. Example data from a library of mutants generated using Ste2 N216S V280I. Sorting rounds involving either positive or negative selection were conducted in different combinations (top). Functional screening of the
variants selected show that multiple negative selection rounds produce libraries where most mutant receptors are inactive. The mating response was measured for each candidate following treatment with 1 µM of each pheromone. The response values are shown in a scatter plot where the diagonal indicates equal $K_{lac}$ and $S_{cer}$ pheromone-induced activation.
Supplementary Figure III-2: The mating response of P290 mutants treated with a non-saturating concentration of pheromone. Mutations at site P290 lead to a diverse set of response profiles when treated with 50 nM of each pheromone. The response values are shown in a scatter plot where the diagonal indicates equal $K_{lac}$ and $S_{cer}$ pheromone-induced activation. Variants are labeled according to the amino acid present at site P290, where variant “P” represents Ste2 N216S and “WT” represents wild-type Ste2. Error bars represent the standard error of the mean (s.e.m.)
Supplementary Figure III-3: The proline of TM7 is highly conserved among Ste2 orthologs. Multiple sequence alignment of Ste2 orthologs. The proline of TM7, corresponding to position 290 in the Scer sequence and located at position 355 on the alignment, is part of a highly conserved motif. The alignment was obtained using Clustal Omega [163].
Supplementary Figure III-4: The overexpression of Ste2 K-Switch does not affect its response profile. A, Different promoters alter the surface expression levels of Ste2 K-Switch. Surface expression was measured in a saturation binding assay with NBD-Scer. The endogenous Ste2 promoter results in undetectable levels of receptor while the promoters of the genes ADH1 and GPD enhance expression to detectable levels. B, Different promoters do not alter the response profile of Ste2 K-Switch. Dose-response curves show the negligible effects of enhancing expression for this Ste2 variant. Error bars represent the standard error of the mean (s.e.m.)
Supplementary Figure III-5: Amino acid substitutions at positions 5 and 13 show no
effect on ligand discrimination for WT Ste2 and Ste2 K-Switch. A, The primary
structures of additional pheromone variants used in this study are shown. These Scer α-
factor variants harbor single amino acid substitutions with respect to the WT sequences,
which are highlighted by their different colours, and are named accordingly. B-C, The mating response of WT Ste2 (B) and Ste2 K-Switch (C) to select pheromone variants is shown in histograms derived from duplicate experiments. The substitution Q5T alone has no effect on efficacy, while combinations with H2S and M12I show no apparent synergy. The conservative substitution Y13F also has no effect. Error bars represent the standard error of the mean (s.e.m.)
SUPPLEMENTARY TABLES

Supplementary Table III-1: Yeast strains used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Origin</th>
</tr>
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<tbody>
<tr>
<td>SΦ992</td>
<td>W303 MATa, STE2, SST2, BAR1, FAR1, MFA2, his3, trp1, leu2, ura3</td>
<td>[68]</td>
</tr>
<tr>
<td>CB008</td>
<td>SΦ992 bar1::NatR, far1Δ</td>
<td>[68]</td>
</tr>
<tr>
<td>CB009</td>
<td>CB008 mfa2::pFUS1-GFP</td>
<td>[68]</td>
</tr>
<tr>
<td>RB001</td>
<td>CB009 ste2::TRP1</td>
<td>[69]</td>
</tr>
<tr>
<td>RB002</td>
<td>CB008 ste2::TRP1</td>
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</table>

Supplementary Table III-2: Plasmids used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Description</th>
<th>Origin</th>
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</thead>
<tbody>
<tr>
<td>pRS313</td>
<td>CEN HIS3 vector</td>
<td>[69]</td>
</tr>
<tr>
<td>pRS-STE2</td>
<td>CEN HIS3 STE2 promoter (P&lt;sub&gt;STE2&lt;/sub&gt;)</td>
<td>[69]</td>
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<tr>
<td>pRS-PADH1</td>
<td>CEN HIS3 ADH1 promoter (P&lt;sub&gt;ADH1&lt;/sub&gt;)</td>
<td>[69]</td>
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<tr>
<td>pRS-PGPD</td>
<td>CEN HIS3 GPD promoter (P&lt;sub&gt;GPD&lt;/sub&gt;)</td>
<td>This study</td>
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</table>

Supplementary Table III-3: Oligonucleotides used in this study

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence</th>
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<tr>
<td>STE2_AarI_5</td>
<td>GCC ATG AAC ACC TGC AAC ACC CTA TGT CTG ATG CGG CTC C TT C</td>
<td>Random mutagenesis of STE2 ORF with flanking AarI sites</td>
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<tr>
<td>STE2_AarI_3</td>
<td>GTTACAGGCACCTGCAACATCG CTCATAAATTATTATTC</td>
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<tr>
<td>SDM_Ste2_280I_5</td>
<td>GAAACGATGTCTTGACTACTATT GCAACATTACTTGCTGTA</td>
<td>Change position 280 in STE2 to I</td>
</tr>
<tr>
<td>SDM_Ste2_280I_3</td>
<td>TACAGCAAGTAATGTTGCAATA GTAGTCAAGACATCTGTTCC</td>
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</tr>
<tr>
<td>SDM_Ste2_267C_5</td>
<td>TAATATTCATCCTCGCATCTGT TTGAAAACCAAGGGA</td>
<td>Change position 267 in STE2 to C</td>
</tr>
<tr>
<td>SDM_Ste2_267C_3</td>
<td>TTGCCTGTTGTTGTTGCAACAGT ATGCGAGGATGAATATTA</td>
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<tr>
<td>SDM_Ste2_290X_5</td>
<td>TACTTGCTGTATTTGCTTTANNNTTATCATCAATGTGGGCCA</td>
<td>Change position 290 in STE2 to X (see Supplementary Table 4 for NNN)</td>
</tr>
<tr>
<td>SDM_Ste2_290X_3</td>
<td>TGGGCCACATTGATGATAANNN TAAAGACAATACAGCAAGTA</td>
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<tr>
<td>SDM_Ste2_265T_5</td>
<td>CAT CGA TAA TAT TCA TCC TCG CAT ACA GTT TGA AAC CAA AC</td>
<td>Change position 265 in STE2 to T</td>
</tr>
<tr>
<td>SDM_Ste2_265T_3</td>
<td>GTT TGG TTT CAA ACT GTA TGC GAG GAT GAA TAT TAT CGA TG</td>
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</tr>
<tr>
<td>Amino acid</td>
<td>Codon</td>
<td>Amino acid</td>
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<tr>
<td>P</td>
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<tr>
<td>D</td>
<td>GAT</td>
<td>H</td>
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REFERENCES


156. Silver, M.R., et al., *Cloning and characterization of a functional type II gonadotropin-releasing hormone receptor with a lengthy carboxy-terminal tail*


