Construction of multicellular yeast networks using the communication toolkit with variable specificity and attenuation

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Abstract

The key next step in synthetic biology is to extend cellular network engineering to the multicellular level by utilizing cell-cell communication for information processing. To facilitate the implementation of multicellular networks in the most commonly used eukaryotic chassis, *Saccharomyces cerevisiae*, we developed the yeast communication toolkit (YCTK). This toolkit is based on the fungal mating pathway and contains five pheromone-inducible promoters (response parts), eleven pheromones (α -factors; sender parts), eleven pheromone receptors (Ste2; receiver parts), as well as five Bar1 proteases (suppressor parts). All YCTK parts were thoroughly characterized and are compatible with the commonly used yeast Golden Gate cloning standard. We demonstrated the application of the YCTK by implementing several different logic gate-like population networks. Furthermore, we

used this toolkit to investigate the pheromone-receptor promiscuity patterns among different yeast species. This toolkit extends currently available resources for construction of complex multicellular eukaryotic networks with varying degrees of promiscuity and attenuation.

Introduction

Directed cell-cell communication was one of the key developments in the evolution of complex multicellular organisms¹, but also unicellular pro- and eukaryotic organisms evolved cell-cell communication systems to synchronize various population behaviors²⁻⁴. Besides being central to many biological phenomena, cell-cell communication is being increasingly employed in synthetic biology as a tool for engineering multicellular networks⁵. The majority of currently established synthetic multicellular networks were implemented in prokaryotes using bacterial quorum sensing communication systems, mainly based on well-characterized homoserine lactone signaling⁶. Examples of these networks include pattern formation^{7,8}, programmed population dynamics like oscillations⁹ or stabilizations¹⁰, and multicellular computing^{11,12}.

Eukaryotic organisms also possess cell-cell communication systems that bear valuable potential for synthetic biological applications, but only recently this potential began to be utilized^{13,14}. The mating pathway of the budding yeast *S. cerevisiae* is one of the most intensely studied eukaryotic communication and signaling pathways⁴. Similar to all ascomycetes, *S. cerevisiae* can exist as two haploid mating types (*MATa* and *MATα*) that communicate by secreting either of the two chemically different peptide pheromones, **a**-factor or α -factor, respectively. Most yeast species carry multiple different copies of the α -factor gene, which can itself encode several,

sometimes varying, copies of the mature α -factor^{15,16}. Prior to the secretion of the mature α -factor, this pre-pro- α -factor is processed by the proteases Kexin 2 (Kex2), dipeptidyl aminopeptidase (Ste13), and Kexin 1 (Kex1). Due to its chemical properties, the α -factor is less studied but it is also specifically processed and actively secreted¹⁷. The pheromones stimulate G-protein-coupled receptors (GPCR) in the opposite mating type, Ste2 in *MATa* cells and Ste3 in *MAT α* cells. Upon binding of the pheromones to the GPCRs, the downstream mating MAP kinase signaling pathway is activated, leading to the expression of specific genes required for the physiological changes, including cell cycle arrest and directed cell growth ('shmooing')¹⁸. In addition, *MATa* cells are secreting the barrier aspartyl-peptidase Bar1, which degrades the α -factor¹⁹.

The mating pathway of *S. cerevisiae* has been already used in a number of synthetic biology applications, for instance to create clinical or metabolic biosensors^{20–24} and to implement artificially controlled behaviors^{22,25,26}. To further advance the implementation of multicellular networks based on the mating pathway, we develop here the yeast communication toolkit (YCTK) that contains a set of pheromone-inducible promoters, α -factors, Ste2 GPCRs, and Bar1 proteases from different yeast species.

The parts were designed to be fully compatible with the commonly used Golden Gate cloning standard of the yeast toolkit (YTK)²⁷. All parts were characterized for their functionality, sensitivity as well as their promiscuity, which enables construction of multicellular networks with varying degrees of defined crosstalk. With this comprehensive toolkit, that is publicly available on Addgene, we provide parts that exhibit different unique characteristics and enable rapid and easy construction of sophisticated multicellular communication networks in *S. cerevisiae*. We further

analyze the relationship between observed recognition of non-cognate α -factors and evolutionary distance of Ste2 receptors and Bar1 proteases as well as identity of α -factors. Finally, as a proof of concept we use YCTK to implement several logic gate-like population networks.

Results

Overview of the YCTK design. In order to engineer multicellular yeast communication networks, our toolkit was designed to contain a set of well-characterized response, sender, receiver, and suppressor parts (Fig. 1). As response parts, we selected seven promoters of *S. cerevisiae* genes that are known to be regulated in response to the pheromone-induced activation of the mating signaling pathway²⁸. *mfa1* genes that encode α -factors and their corresponding Ste2 GPCRs from several different *Saccharomycotina* species were selected as sender and receiver parts, respectively. When multiple α -factor genes were present in genome, we selected the gene encoding for highest number of, in some cases slightly different, α -factors. Finally, as suppressor parts we chose Bar1 peptidases from the same species. For facilitated part assembly, all parts were designed to be fully compatible with the Golden Gate cloning standard of the yeast toolkit (YTK)²⁷, including removal of recognition sites of *Bsa*I, *Bsm*BI and *Not*I endonucleases as well as codon optimization. As a notable difference to the YTK where the stop codon is included in the terminator part which adds amino acids at the C-terminus of the protein²⁷, we rather included the stop codon downstream of the coding sequences of the *mfa1* and *ste2* genes. This was done because α -factors are post-translationally processed, and the C-terminal region of the Ste2 receptor is of importance for its function^{29,30}. However, this difference still allows the incorporation of our parts into

100 the existing toolbox. All assembled devices in our study were chromosomally
101 integrated.
102

Toolkit

response	sender	receiver	suppressor

pheromone-inducible promoter	α -factor (pheromone)	Ste2 (GPCR)	Bar1 (protease)
P_{DIG2}	α_{Ca} -factor	Ste2 _{Ca}	Bar1 _{Ca}
P_{FUS1}	α_{Ec} -factor	Ste2 _{Ec}	Bar1 _{Ec}
P_{FUS3}	α_{Ka} -factor	Ste2 _{Ka}	Bar1 _{Kn}
P_{MSG5}	α_{Kn} -factor	Ste2 _{Kn}	Bar1 _{Kl}
P_{PRM1}	α_{Kl} -factor	Ste2 _{Kl}	Bar1 _{Sc}
P_{YPS1}	α_{Lf} -factor	Ste2 _{Lf}	
P_{SST2}	α_{Lm} -factor	Ste2 _{Lm}	
	α_{Lt} -factor	Ste2 _{Lt}	
	α_{Sc} -factor	Ste2 _{Sc}	
	α_{Tp} -factor	Ste2 _{Tp}	
	α_{Vp} -factor	Ste2 _{Vp}	

103
104 **Fig. 1:** The toolkit composition for yeast-based multicellular networks. The toolbox consists of 7 response parts
105 (pheromone-inducible promoters), 11 sender parts (α -factors), 11 receiver parts (Ste2 receptors), and 5
106 suppressor parts (Bar1 proteases) originating as indicated from *Candida albicans* (Ca), *Eremothecium*
107 *cymbalariae* (Ec), *Kazachstania africana* (Ka), *Kazachstania naganishii* (Kn), *Kluyveromyces lactis* (Kl),
108 *Lachancea fermentati* (Lf), *Lachancea mirantina* (Lm), *Lachancea thermotolerans* (Lt), *Saccharomyces*
109 *cerevisiae* (Sc), *Tetrapispora phaffii* (Tp), or *Vanderwaltozyma polyspora* (Vp). The parts highlighted in grey
110 showed low activity.

111
112 **Characterization of response parts.** The set of pheromone-inducible promoters of
113 *S. cerevisiae* genes was selected to cover different expression strengths and
114 profiles, based on RNA sequencing data for *S. cerevisiae* MATa cells stimulated with
115 different doses of α -factor²⁸. To characterize response characteristics of these
116 promoters controlling expression of fluorescent reporter Venus, we constitutively

expressed *S. cerevisiae* Ste2, therefore decoupling it from the native positive feedback loop that might affect promoter response profiles²⁰ (Supplementary Fig. 1 a,b). To allow comparison with previous promoter characterization in the YTK, we utilized the constitutive promoter P_{RPL18B} as benchmark²⁷. We observed that selected promoters displayed different basal and maximal expression levels, as well as different fold changes in expression (Fig. 2, Supplementary Fig. 1c, and Supplementary Table 1). Although the constitutive promoter RPL18B also exhibited increased promoter activity, most likely an artifact due to increase in size of the α -factor stimulated cells, this increase was much weaker compared to the other promoters. Sensitivity to α -factor has also shown several-fold difference, with lowest EC_{50} observed for *SST2* promoter and highest EC_{50} for *MSG5* promoter, consistent with previous observations²⁸ (Supplementary Table 1).

Fig. 2: Characterization of response parts. Responses of indicated pheromone-inducible promoters upon stimulation with different doses of α -factor for 3 h, measured using flow cytometry. Each point reflects the mean of three biological replicates, with error bars indicating standard deviation. The grey lines are sigmoidal regression curve fits. See also Supplementary Table 1 and Supplementary Fig. 1c.

Selection of sender, receiver and suppressor parts. For the selection of other parts for our toolkit, we analyzed genomes of a number of *Saccharomycotina* species. Our selection was primarily based on availability of identified *mfa1*-like genes. Different from previous studies that generated chimeric pre-pro- α -factors^{13,24}, we selected natural sequences encoding pre-pro- α -factors that are likely to be processed by the *S. cerevisiae* Kex2 protease, which recognizes arginine-arginine (RR) and lysine-arginine (KR) motifs³¹, and by Ste13 that recognizes repetitive x-alanine and x-proline sequences³². Species with the pre-pro- α -factors lacking these motifs were sorted out. Some of the selected *mfa1*-like genes encode multiple copies of mature α -factors with slightly different sequences (Supplementary Table 2). For our final selection, we chose 10 *Saccharomycetaceae*³³ species with different degrees of relatedness as well as *Candida albicans* that belongs to the *Debaryomycetaceae*³³ as an outgroup (Supplementary Table 3). The overall phylogeny of receiver-sender pairs, which is based on the sequence alignment of Ste2 proteins concatenated with their respective α -factors (see Materials and Methods), as well as their evolutionary distances were congruent with a recently published whole-genome phylogenetic tree³³ (Fig. 3a, Supplementary Fig. 2a). As suppressor parts, we utilized Bar1 proteins annotated by protein alignments as (hypothetical) barrier proteases and contained the export signal sequence³⁴ in five of the selected species. The phylogeny and the evolutionary distances of these suppressor-sender pairs was also congruent to the receiver-sender pairs and with the whole-genome phylogeny (Fig. 3b, Supplementary Fig. 2b).

Fig. 3: Evolutionary distance of the receiver-sender and suppressor-sender pairs of the selected species. **a** Evolutionary distance of Ste2-pheromone sequences, displayed as a heatmap based on the alignment of concatenated Ste2- α -factor protein sequences. **b** Evolutionary distance of Bar1-pheromone sequences, displayed as a heatmap based on the alignment of concatenated Bar1- α -factor protein sequences.

Expression and characterization of sender and receiver parts. The alignments of the mature α -factors are displayed in Supplementary Fig. 3a. The N-terminal WXWL motif is conserved in all α -factors of the *Saccharomycetaceae*, and most species also contain the C-terminal GXP motif³⁵. The α -factors of *C. albicans* have no apparent homologies to the other α -factors. The successful expression of codon-optimized *mfa1*-like genes from chromosomally-integrated constructs (Supplementary Fig. 4a, b), as well as their processing and secretion were confirmed by MALDI-TOF (Supplementary Table 4).

Regions that are crucial for connecting to the mating pathway of *S. cerevisiae*³⁶ are conserved in all selected Ste2 receptors (Supplementary Fig. 3b, c). To confirm their expression and functionality, codon-optimized *ste2* genes were expressed from chromosomally-integrated constructs, along with a pheromone-inducible reporter construct and stimulated with synthetic cognate α -factor (Supplementary Fig. 5a, b,

178 c, Supplementary Table 5). Dose responses measured for each of the Ste2-
 179 expressing strains stimulated with synthetic cognate α -factor showed similar fold
 180 changes in pathway activation but large differences in EC_{50} (Fig. 4, Supplementary
 181 Table 6). Different values of EC_{50} are likely to reflect differences in receptor-
 182 pheromone interactions, and they could be used for engineering regulatory
 183 interactions with different thresholds.
 184 The functionality of heterologously produced α -factors and Ste2 receptors was
 185 further confirmed by co-cultivating cognate sender and receiver strains and
 186 measuring the receiver response (Supplementary Fig. 6a, b, c).

Fig. 4: Experimental characterization of receiver parts. Dose-responses of indicated Ste2 receptors towards their cognate α -factors. Each measurement point shows fluorescence of P_{FUS1} -Venus reporter in $\sim 50,000$ single-cell events measured by flow cytometry. Grey curves are fitted sigmoidal regression curves.

Sender-receiver crosstalk. Besides response characteristics of cognate sender-receiver parts, another important parameter for the multicellular network design is the ability of Ste2 receptors to be stimulated by non-cognate α -factors. To elucidate such crosstalk, we first stimulated each receiver strain with the same (high) level of every synthetic α -factor (Fig. 5a, Supplementary Fig. 5d, Supplementary Table 7). The Ste2 receptors of *C. albicans*, most distantly related species, and *K. africana* were exclusively stimulated by the cognate α -factor, whereas all other receptors

199 exhibited clear promiscuity towards non-cognate α -factors. To further quantify this
 200 promiscuity, dose responses were measured for all synthetic α -factors (Fig. 5b) as
 201 well as in co-cultures with the α -factor-producing strains (Supplementary Fig. 6d, e,
 202 Supplementary Table 8). Although all Ste2 receptors exhibited highest sensitivity
 203 towards their cognate α -factors, several promiscuous receptors showed nearly
 204 identical responses for one or several non-cognate α -factors.

single-cell events each. A similar heatmap was generated with the data of the co-culture experiment (Supplementary Fig. 6d). **b** Heatmaps of representative dose-responses for P_{FUS1} -Venus reporter controlled by indicated Ste2 receptors towards indicated non-cognate α -factors, with ~50,000 single-cell events measured by flow cytometry.

Suppressor part expression and characterization. In order to characterize activity of Bar1 proteases for signal suppression in the YCTK, we co-cultivated strains with chromosomally-expressed Bar1 with the Ste2-expressing strains stimulated with the cognate α -factor, and measured the reduction of stimulation (Fig. 6a, Supplementary Fig. 7, Supplementary Table 9, Supplementary Table 10). The barrier proteases of *C. albicans* and *E. cymbalariae* were specific to their cognate α -factors, whereas the remaining three barrier proteases were more promiscuous. Notably, despite only limited number of identified functional proteases, they could be used to suppress almost all sender parts in our toolkit. Our analysis of promiscuity of barrier proteases did not support previously proposed co-evolution of Ste2 and Bar1 to recognize similar amino acid sequences of the α -factor³⁷, since no apparent correlation between receptor and protease promiscuity could be observed (Fig. 6b, Supplementary Fig. 8, Supplementary Fig. 9).

Fig. 6: Experimental characterization of suppressor parts. **a** Heatmap displays fold reduction of stimulation of indicated Ste2 strains carrying P_{FUS1} -Venus reporter in presence of Bar1-producing strain, in comparison to the

negative control (no Bar1 strain). Each square is the mean of three biological replicates measured by flow cytometry of ~25,000 single-cell events. The fold reduction was computed by normalizing mean fluorescence intensity as a result of Bar1 activity to the fluorescence intensity without Bar1. **b** Cross-activation of Ste2 receptors plotted against the activity of Bar1, for all pheromones (Supplementary Fig. 8). Each point represents the mean of three biological replicates. The relative activation/reduction was computed by normalizing the fold activation/reduction of the cognate pairs to 1.

Crosstalk between sender and receiver or suppressor parts and their

evolutionary relatedness. We further more generally investigated whether the

observed crosstalk between sender, receiver and suppressor parts could be

accounted for by the evolutionary relatedness of *Saccharomycetaceae* species.

Surprisingly, this was not the case, since the receptor cross-activation by α -factors

exhibited only a weak correlation with the evolutionary distance of separating any

non-cognate receptor-pheromone pair from the corresponding cognate pairs (Fig.

7a, Spearman's $r = -0.22$, $p = 0.02$). Neither did we observe a clear trend when

comparing cross-activation with the α -factor sequence identities (Fig. 7b,

Supplementary Fig. 10a). Similarly, neither significant correlation between the Bar1

specificity and relatedness of Bar1 sequences (Spearman's $R = -0.069$, $p = 0.77$) or α -

factor sequence identities (Fig. 7c,d, Supplementary Fig. 10b) could be seen.

Interestingly, we noticed that if an α -factor from species A could activate a non-

cognate receptor from species B, the reciprocal pairing of receptor from species A

with α -factor from species B almost always showed no activation (Supplementary

Fig. 11). Whether this apparent anticorrelation is related to the mode of α -factor

recognition by the receptor or whether it is the result of natural selection remains to

be investigated.

Fig. 7: Relation between receptor and protease crosstalk and evolutionary distance and α -factor identity. **a** Rank order correlation between cross-activation of Ste2 receptors by non-cognate α -factors and the evolutionary distance (branch length) of the receptor-pheromone pair from the corresponding cognate pair. Purple line shows the correlation for binned distance ranks. **b**, Correlation between receptor activation and the sequence identity of the α -factor to the receptor's cognate pheromone. **c** Rank order correlation between cross-reduction of Bar1 proteases of non-cognate α -factors and the evolutionary distance of protease-pheromone pair from the corresponding cognate pair. Purple line shows the correlation for binned distance ranks. **d**, Correlation between the protease efficiency and the sequence identity of the α -factor to the protease's cognate pheromone. The fluorescence intensities of activated P_{FUS1} -Venus reporter were normalized to activation/reduction by/of the cognate α -factor.

Construction of logic-like population networks using YCTK parts. As a proof of principle for the implementation of population networks based on the YCTK, we

aimed to generate several population networks that exhibit the logics of various Boolean gates. Differently from regular cellular logic gates, the output of such logic-like networks depends on the population composition rather than on external input signals³⁸.

Simple buffer-like logic networks were implemented by co-culturing the α -factor-producing and the Ste2-expressing strains (Supplementary Fig. 12a, b). To establish an OR gate-like population network consisting of three strains, we made use of the promiscuity of Ste2 receptors. Both sender strains produced unique α -factors, while the receiver cells expressed Ste2 receptor that could be stimulated by either of the two α -factors (Fig. 8a). The receiver cells indeed displayed the OR gate characteristics (Fig. 8b), although the response was not fully digital as exposure to both sender strains resulted in somewhat higher induction than to individual senders.

To implement an AND gate-like population network, we initially constructed the networks where Ste2 receptor in the receiver cells was constitutively expressed (Supplementary Fig. 12c, d). The response of our initial design (AND 1.0) was only partly consistent with the AND gate logic, but through a subsequent design-build-test-learn cycle we implemented several versions with altered strength of Ste2 promoters (Supplementary Fig. 12e-h), one of which (AND V2.3) exhibited markedly improved response characteristics (Fig. 8c, d).

To demonstrate application of the suppressor parts in multicellular networks, we designed and built a three-strain XOR gate-like population network, where each sender strain expresses a unique α -factor as well as a barrier protease suppressing the α -factors of the other sender strain (Fig. 8e). Both α -factors can stimulate Ste2 receptor of the receiver cells. Depending on the population composition, the receiver cells should thus display XOR gate logics (Fig. 8f). Our initial design used weak

promoters for the expression of the sender parts, which did not result in the anticipated outputs (XOR V1.0, Supplementary Fig. 12i-j). However, a second version (XOR V2.0) with stronger promoters for the pheromone genes showed much improved receiver output and displayed the XOR gate characteristics. With this proof of principle we showed that the YCTK in combination with the YTK²⁷ allows rapid generation of different multicellular yeast networks for various applications.

Fig.8: Logic-like population networks constructed using YCTK parts. Design of population networks, their output for different population compositions, and resulting activity of P_{FUS1} -Venus reporter are shown. **a,b** OR gate-like population network. **c,d** AND gate-like population network (AND V2.3). **e,f** XOR gate-like population network (XOR V 2.0). For each network, three biological replicates were analyzed, with ~25,000 single receiver cells measured in each experiment using flow cytometry.

Discussion

Here, we present yeast communication toolkit YCTK that is based on the yeast mating pathway. The YCTK utilizes the standardized cloning system based on the most commonly used yeast golden gate toolkit (YTK)²⁷, and it contains a set of well characterized response (promoters), sender (α -factors), receiver (Ste2 receptors), and suppressor (Bar1 proteases) parts (Fig. 1). By expanding the range and enabling easy integration of already available sender and receiver parts¹³, as well as

synthetic promoters controlled by the mating pathway^{20,39–42}, YCTK extend its application possibilities for engineering complex multicellular networks^{43,44}. In addition to orthogonal pheromone receptors that were used in these previous studies^{13,21,38,43,45–47}, the YCTK utilizes characterized crosstalk between individual sender-receiver parts. The observed extent of crosstalk between the mating systems of different *Saccharomyces* species was surprising, since the receptor specificity is expected to serve as a species barrier⁴⁸ and undesired stimulation of the mating pathway might negatively affect fitness⁴⁹. The crosstalk could not be simply accounted for by the currently available working model of α -factor recognition^{35,50–53}, and understanding of its molecular basis requires further analysis. Nevertheless, only weak correlation between Ste2 activation by non-cognate α -factors and the evolutionary separation between cognate and non-cognate receptor-pheromone pairs suggests that signaling specificity is determined in a lineage specific way by a relatively small number of amino acid sites in both the pheromone and the receptor, such that the overall sequence divergence carries little information about compatibility. In one specific case, Ste2_{Ka} receptor appears to be selective for the isoleucine residue at position 14, which is unique in its cognate α -factor. This may highlight the importance of the C-terminal region of the pheromones for recognition by Ste2³⁵. Promiscuity was also observed for Bar1 barrier proteases, but with different pheromone preferences and again without apparent correlation with evolutionary distance of Bar1 sequences or identities between α -factor sequences. This indicates that also specificity of Bar1 recognition is determined by a small number of residues, and that the receptors and barrier proteases of the same species do not apparently recognize similar motives of the α -factors, as previously proposed⁵⁴. The ecological implications of such promiscuity, which might enable

cross-recognition and/or -quenching of pheromone signaling by different
Saccharomycotina species inhabiting the same niche, as well as of the apparent
anticorrelation between promiscuity of receptors and pheromones from individual
species, remain to be investigated.

Finally, to demonstrate applicability of this toolkit, we engineered several population-
based logic gates where population composition functions as the input signal, which
make use of the various features of the YCTK. The promiscuity of the receiver parts
was employed in the implementation of an OR gate-like multicellular network. The
realization of the AND gate-like population network illustrated that complex designs
can be realized and optimized using YCTK parts. Finally, as proof of principle for
application of the suppressor parts, we implemented a functional XOR gate-like
population network. Although our designs of the population networks were limited to
three sub-populations, functionality of these networks confirms that all YCTK parts
could be utilized for network construction, suggesting that the YCTK is similarly
applicable for construction of more complex communication networks with diverse
future applications, including multicellular biosensors and metabolically cooperating
multicellular cell factories^{20,22,43,55–60}. In principle, the entire toolkit could also be
transferred to other eukaryotic chassis organisms, such as mammalian cells, for
example to be used for tissue engineering⁶¹.

Material and Methods

Strains and plasmids. All yeast strains and plasmids are listed in Supplementary
Table 12-23. The strains are derived from SEY6210A and the background strain of
this study is yNK159 (*MATa leu2-3,112 ura3-52 his3Δ200 trp1Δ901 lys2-801 suc2Δ9*
LYS2::rttAS2 mfa2Δ::hphNT1 mfa1Δ::klTRP1 bar1Δ::kanMX ste2Δ::natNT

far1Δ::URA3). The plasmids harbor either toolkit parts, the constructs utilized for part characterization or the gates and were sequence-verified. These were subsequently chromosomally integrated into yNK159-derived strains and PCR-verified. The plasmids were constructed using YCTK and YTK toolkit parts²⁷. For plasmid construction and propagation, the *E. coli* strain NEB Turbo was used.

Genetic parts. The genetic sequences from the parts were obtained from databases and the genes were codon-optimized for an expression in *S. cerevisiae*. The α -factor and Ste2 genes contain stop codons as part of their gene sequence in the collection, as the conservation of their C-terminal sequences is essential for their functionality. The Bar1 genes are lacking a stop codon, which is part of the terminator part. The parts of the YCTK are fully compatible with the YTK from the Dueber Lab²⁷.

Media and growth conditions. Media components for synthetic dropout media supplemented with appropriate amino acids for yeast growth were obtained from Formedium and Roth. To obtain solid media, 2 % agar was added to synthetic dropout media. For strain construction and selection synthetic dropout media (SD) was utilized, while all experiments were performed using synthetic complete media (supplemented with all amino acids) and supplemented with 2 μ M casein. Yeast cells were grown in 2x YPD during preparation for competent cells⁶². *E. coli* cells were cultured in Luria Broth (LB) media supplemented with ampicillin (100 μ g/mL), kanamycin (50 μ g/mL), or chloramphenicol (34 μ g/mL). Yeast cells were always grown at 30°C. SD dropout/complete liquid media or plates were utilized to grow cells. After genomic integration, the cells were grown on plates for 2-4 days. Liquid overnight cultures were prepared in 24-well plates with 1.5 mL

SD dropout or SD complete media and inoculated from single colonies on plates and cultivated for 12-16 hours prior inoculation for experimental procedures. Day cultures for experiments were inoculated into 24-well plates to an OD of 0.05 into 2.25 mL SD complete media and grown under shaking conditions at 30°C for 4 hours. Afterwards, the cultures were either induced with synthetic α -factors or mixed with other cultures (1:1 ratio) into 96-well plates to a total volume of 300 μ L. The cells were measured 3 hours after transfer onto the 96-well plates using flow cytometry. To quantify α -factors using LC-MS/MS, the different α -factor-producing strains were inoculated into 2.25 mL SD complete media to an OD of 0.05 into 24-well plates and grown for 7 hours at 200 rpm at 30 °C. For the yeast gate-like population networks, the different populations were directly mixed and cultured in 24-well plates as described above for 4 h and then transferred to 96-well plates and further cultured for 3 h before measuring.

Cloning. PCR was performed using the high-fidelity polymerase PrimeSTAR GXL DNA (Clontech). Golden Gate assembly was executed using the NEB Golden Gate Assembly Kit, T7 DNA ligase, which was purchased from New England Biolabs, as well as required restriction enzymes from New England Biolabs. Synthetic α -factor was purchased from Sigma-Aldrich (*S. cerevisiae*) or synthesized by BioCat (remaining ones) and stock solutions were stored in synthetic complete media supplemented with 2 μ M casein, 20% DMF and 0.1% TFA. Primers were obtained from Eurofins Genomics, synthetic DNA were purchased from Twist Bioscience. The microtiter plates for the experiments were obtained from Greiner BioOne. All plasmids were constructed using Golden Gate assembly and utilizing parts of the YTK and YCTK collections²⁷. We used the NEB Golden Gate Kit (*Bsal*-HFv2) for

Level 1 assemblies and for L0/L2 assemblies 0.5 μ L *BsmBI/Esp3I*, 1 μ L T4 DNA Ligase Buffer and 0.5 μ L T7 DNA ligase as reaction mixture. 20 fmol vector DNA fragments and 100 fmol of each insert part were added to the reaction mixture and added up with H₂O to 10 μ L total reaction volume. The following thermocycle protocol was used: 50 cycles of 37 °C/42°C – 2 min, 16°C – 5 min, followed by 37°C/60°C – 60 min (when entry vectors were constructed, 60 min step was removed) and 80°C - 10 min. Reaction mixture was dialyzed and transformed by electroporation into NEB Turbo *E. coli* cells⁶³.

Peptide Mass Spectrometry. The peptide containing samples were desalted with C18-SPE, the eluates were concentrated using a vacuum dryer and then analyzed with MALDI-MS. Samples were prepared directly onto a MALDI-Plate by mixing with a matrix composed of 3 mg ml⁻¹ α -cyano-4-hydroxycinnamic acid in 80% (v/v) acetonitrile and 0.3% (v/v) trifluoroacetic acid (Dried-Droplet Crystallization). MALDI-TOF analysis was performed with a 4800 Proteomics Analyzer (Applied Biosystems/MDS Sciex, Forster City, CA, USA) using the 4800 Series Explorer software (positive-ion reflector mode in a mass range from 800 to 2500 Da). The data were calibrated externally using a peptide mixture for mass calibration from Bruker (Bremen, Germany) spotted onto the same MALDI target.

Data acquisition and analysis. Most of the data were obtained by flow cytometry with the Fortessa flow cytometer from BD Biosciences and the FACS DIVA software was used for data acquisition. The *S. cerevisiae* cells were expressing fluorescence reporters that were detected using different lasers and filters. We measured cells expressing Venus, mTurquoise2, or mRuby. Since we measured many samples at

one time point, the cells were measured from 96-well plates using the high throughput sampler (HTS). The cells were gated using DIVA and FlowJo (BD Bioscience). Within DIVA, cells were gated while measuring, to exclude events hitting the upper detector limits and events smaller than average yeast cells while data processing for the experiments was performed using the FlowJo software. FlowJo was further used for raw data visualization. The mean fluorescence of the yeast cells was then obtained based on the applied gates and further used to plot figures using MATLAB. To plot the flow cytometry results, different approaches were taken. Partly, flow cytometry histograms were exported from FlowJo. For the inducible promoter dose-response characterization, a MATLAB script was used. For the fitted sigmoidal α -factor dose-response curves and EC_{50} computation, a MATLAB script from Ritchie Smith was adapted. To compute the EC_{50} and the Hill coefficient, in MATLAB, a four-parameter logistic nonlinear regression model for sigmoidal curves as well as curve fitting were utilized in the form⁶⁴

$$R = a + \frac{(b - a)}{(1 + \frac{c}{X})^d}$$

where a is the background activity, b is the maximal response level, c is the EC_{50} , X is the α -factor concentration, and d is the Hill coefficient.

To generate the heatmaps, a heatmap script from Ameya Deoras was customized. The supplementary crosstalk plots were generated using a R script by A. Anders. The script plotted the recorded flow cytometry data as histograms. Additionally, a color code indicated the $\log(\text{par.mean})$ of the mating pathway activity, which was obtained by calculating the logarithm of the mean fluorescence intensities. For the Bar1 crosstalk plot, the cytometer data was plotted as histograms. Here, the color

code indicated the fold reduction ($\log(\text{fold.chang})$) of the mating pathway activity as a result of α -factor degradation by Bar1. The fold change was calculated by dividing the $\log(\text{par.mean})$ fluorescence intensities obtained in the absence of Bar1 by the mean fluorescence intensities in the presence of Bar1. All figures were further revised using Adobe Illustrator after plotting with FlowJo, MATLAB, or R. All data underlying the Figures and Tables of this publication are provided within Supplementary Data.

Phylogenetic Analysis. For protein phylogenies we aligned the sequences of Bar1 and Ste2 separately from various species using muscle 3.8.31 using default parameters and curated the alignments manually. We used RaxML 8.2.10 to infer the phylogenies, using the PROTGAMMAAUTO command to infer the best fit substitution model (LG+emiperical frequencies in all cases). For the evolutionary distance calculations for sender-receiver and sender-suppressor pairs, we also built two concatenated alignments: one for Ste2, and the pheromones, and another for Bar1 and the pheromones. We recalculated the branch-lengths for Ste2 and Bar1 trees with these alignments using RaxML. To calculate the evolutionary distance of a non-cognate from a cognate interaction, e.g. receptor from species A and pheromone for species B, we calculated the branch-length separating species A from species B on our concatenation trees. This measurement of distance combines the difference between the pheromones and the receptors into one metric. We note that the distance calculated in this way is the same for two reciprocal pairs (i.e. pheromone from species A with receptor from species B and pheromone from species B with receptor from species A). Statistical analysis was carried out using

scipy 1.5.0 in a python 3 environment. Ranks were calculated using the
scipy.stats.mstats.rankdata function, which averages the ranks of tied values.
The pheromone identities (amino acid sequences) were computed using Geneious
Alignment. The alignment type was “Global alignment with free end gaps” and the
Blosum62 cost matrix were utilized with 12 gap open penalty, 3 gap extension
penalty and 2 refinement iterations.

Data Availability

The underlying data of this study are available upon request by the corresponding
authors. The toolkit plasmids are deposited with Addgene.

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Competing Interests. All authors declare that they have no competing interests.

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