Supplementary Materials

Supplementary Table 1. PCR mutagenesis of Ste2p extracellular regions

Region of interest	**Primer pair for mutagenesis**	**Mutagenized region of *STE2*** (nucleotide no.)[a]	**Mutagenized region of *STE2*** (amino acid no.)	**Plasmid vector for homologous recombination**	**Restriction site used for linearization**
N-terminal	ON403/ON668	-148 - 237	M1 - P79	pMD1349	*Hpa*I
EC1	ON864/ON947	238 - 477	I80 - F159	pMD1450	*Xba*I
EC2	ON948/0N949	492 - 696	M165 - S232	pMD1230	*Nhe*I
EC3	ON868/ON869	736 - 897	I246 - A299	pMD1230	*Kpn*I

[a]Nucleotides are numbered from the first position of the initiation codon

Supplementary Table 2: Sequences of clones initially recovered from the N-terminal, EC1, EC2, and EC3 libraries

Library	Clone name	Mutations[a]
N-Terminal	NR1	F12L, S34T
	NR2	T16S, Q51R
	NR3	I53F
	NR4	WT (S22S)
	NR5	T27S, L44F
	NR6	S47R
	NR7	S47R
	NR8	N46I
	NR9	S34F, T35S, Q51L, A63A
	NR10	WT
	NR11	N32N, F55S
	NR12	V49A, Q51H
	NS1	Q51K, T65A
	NS2	WT
	NS3	Q51K, T65A
	NS4	Q51K, T65A
	NS5	T37S, Q51R
	NS6	V49A, Q51H
	NS7	Q51H, G60G
	NS8	S9C, T48S, Q51H
	NS9	Q51H
	NS10	G31G, T50T, Q51H, I53F
	NS11	V49A, Q51H
	NS12	Q51K, T65A
EC1	2SEC101	V86I, F116L, Y128H, I133V, V139A, I142I
	2SEC102	F99L, P117S
	2SEC103	D124G, L137P
	2SEC104	L93STOP, L113P, R122S, S145S
	2SEC105	L102S, V136A, A140A
	2SEC106	I192T, Y101C, F119V
	2SEC107	F99L, V125V, I134S
	2SEC108	Y98N, K100N, L103Q, S108P, A112A
	2SEC109	A96A, F99L, S107S
	2SEC110	Y101C, I134I
	2SEC111	Y98C, Y101C
	2SEC112	A96A, F99I
	2SEC114	F99L
	2SEC115	A96A, F99L, S107S
	2SEC116	F99S
	2SEC117	Y98C, Y101C
	2SEC118	F99L
	2SEC119	I91I, Y101C, F148S
	2SEC120	Y98C, Y101C
	ECL104S	L113P
	ECL106S	Y128H
EC2	2SEC201	N194S, D195G, S197G, T208S, I209F
	2SEC202	V196D, N205D, S207G
	2SEC203	L211L, F220I

	2SEC204	I190N, D201V, Y203F, S207G, I209V, S214S, S219S
	2SEC205	WT
	2SEC206	G188S, D195V, A198A, T208A
	2SEC207	WT
	2SEC208	V178D, V183V
	2SEC209	WT (V178V, V196V)
	2SEC210	T199A, T208A, A212A
	2SEC211	S214T
	2SEC212	I215T, M218T
	2SEC213	I175S, V178G, T192T, K202R
	2SEC214	I190N, D201V, Y203F, S207G, I209V, S214S, S219S
	2SEC215	I227V
	2SEC216	WT
	2SEC217	V186V, D201Y, F204I, S207G, M218T
	2SEC218	S207I
	2SEC219	N205H
	2SEC220	N205Y
	ECL202S	Q200L, F204L, T208A, I215M
	ECL204S	Q200L, F204L, T208A, I215M
	ECL205S	F204L
	ECL206S	V178A, N205D
EC3	EC3-1R	D275E, V280V
	EC3-2R	F262Y, P270P, D275E, T282S
	EC3-3R	I260L, F262Y, I2632V, D275G
	EC3-4R	D275E, T278A
	EC3-5R	G273S, T279T, V280V, A281A
	EC3-6R	G273S, L291L
	EC3-7R	G273S
	EC3-8R	D275E, V280V, A281T
	EC3-9R	K269N, G273S
	EC3-10R	D275E
	EC3-11R	L277S, W295*
	EC3-12R	S259S, I263S, T274I, L289L
	EC3-13S	V257V, S267C, D275G
	EC3-14S	Q272R, D275E, A281A, A285A
	EC3-15S	G273G, D275E
	EC3-16S	D275E
	EC3-17S	G273G, D275E
	EC3-18S	T274T, D275E
	EC3-19S	Q272P, T278A, V280V, L287L, S293S
	EC3-20S	D275E
	EC3-21S	T274S, D275E, L277P
	EC3-22S	D275E
	EC3-23S	K269R, D275E
	EC3-24S	WT

[a] Underlined mutations are translationally silent

Supplementary Table 3. Effects of receptor mutations that alter the emission spectrum of [Lys^7(NBD),Nle^{12}] agonist on the binding and emission spectrum of the antagonist DTyr^3[Lys^7(NBD),Nle^{12}] α-factor.

Mutation	Rel. B_{max} Ratio[a] (FL1/FL2)	FL1		Rel. Constitutive Activity
		Rel. K_d[b]	Rel. B_{max}[c]	
WT	**0.3[a]**	**1.0**	**100**	**1.0**
L44F	0.3 ± 0.03	1.0 ± 0.02	228 ± 13	1.1 ± 0.1
Q51H	0.3 ± 0.01	1.3 ± 0.1	85 ± 3	1.2 ± 0.2
F55S	No detectable binding			1.3 ± 0.2
Y101C	0.3 ± 0.03	0.7 ± 0.1	155 ± 11	1.5 ± 0.2
Y128H	0.4 ± 0.1	3.5 ± 1.5	9 ± 2	1.7 ± 0.3
F204I	0.3 ± 0.04	1.5 ± 0.03	41 ± 4	0.8 ± 0.1
F204L	0.3 ± 0.06	1.0 ± 0.4	51 ± 7	0.9 ± 0.1
Y266C	0.3 ± 0.04	1.1 ± 0.2	55 ± 5	0.9 ± 0.2
D275E	0.3 ± 0.03	0.8 ± 0.1	102 ± 6	1.5 ± 0.5

[a] the reported ratio is normalized to that of agonist [Lys^7(NBD),Nle^{12}]α-factor binding to normal receptors. (Note that all other results in this table are normalized to the values of normal receptor interacting with antagonist [DTyr^3,Lys^7(NBD),Nle^{12}]α-factor.)

[b] computed as the ratio K_d(mutant)/K_d(wild type) (for [DTyr^3,Lys^7(NBD),Nle^{12}]α-factor)

[c] computed as 100× B_{max}(mutant)/B_{max}(wild type) (for [DTyr^3,Lys^7(NBD),Nle^{12}]α-factor)

Supplementary Table 4. Binding of the agonist [Lys^{7}(NBD),Nle^{12}]α-factor to α-factor receptor mutants exhibiting altered spectra of bound antagonist.

Mutation	Rel. B_{max} Ratio (FL1/FL2)	FL1	
		Rel. K_d[a]	Rel. B_{max}[b]
WT	**1.0**	**1.0**	**100**
M54I	0.9 ± 0.1	0.7 ± 0.2	94 ± 7
F55L	0.9 ± 0.1	0.7 ± 0.2	47 ± 4
M218K	0.9 ± 0.04	0.3 ± 0.03	43 ± 1
L222R	1.0 ± 0.1	0.5 ± 0.1	47 ± 3
Q253L	1.0 ± 0.1	0.8 ± 0.2	82 ± 7
P258L	1.0 ± 0.1	1.5 ± 0.3	66 ± 4
P258S	1.1 ± 0.1	0.3 ± 0.1	20 ± 1
P258T	1.0 ± 0.1	0.7 ± 0.2	56 ± 2

[a] computed as the ratio K_d(mutant)/K_d(wild type) (for [Lys^{7}(NBD),Nle^{12}]α-factor)

[b] computed as 100× B_{max}(mutant)/B_{max}(wild type) (for [Lys^{7}(NBD),Nle^{12}]α-factor)

Supplementary Table 5. Yeast strains.

Strain	Plasmid	STE2 allele	Mutagenic Oligonucleotide[a]	Original plasmid vector
A1239	pMD228	*ste2*-Δ	-	pMD228
A3694	pMD1230	Truncated *STE2*$^{+}$	-	pMD1230
A3785	pMD1623	F12L	ON1183	pMD1230
A3822	pMD1644	T27S	ON1191	pMD1230
A3786	pMD1624	S34F	ON1184	pMD1230
A3823	pMD1645	S34T	ON1192	pMD1230
A3787	pMD1625	T35S	ON1185	pMD1230
A3824	pMD1646	L44F	ON1193	pMD1230
A3716	pMD1609	N46I	-	pMD1349
A3655	pMD1594	S47R	-	pMD1349
A3401	pMD1446	V49A	ON1056	pMD1230
A3825	pMD1647	V49S	ON1194	pMD1230
A3826	pMD1648	T50F	ON1195	pMD1230
A3405	pMD1441	Q51H	ON1051	pMD1230
A3827	pMD1649	A52S	ON1196	pMD1230
A3654	pMD1593	I53F	-	pMD1349
A3828	pMD1650	M54S	ON1197	pMD1230
A3788	pMD1626	F55S	ON1182	pMD1230
A3402	pMD1447	T65A	ON1057	pMD1230
A3722	pMD1615	Y98C	ON1112	pMD1230
A3452	pMD1462	F99I	-	pMD1450
A3434	pMD1460	F99L	-	pMD1450
A3453	pMD1463	F99S	-	pMD1450
A3435	pMD1461	Y101C	-	pMD1450
A3789	pMD1627	L102S	ON1154	pMD1230
A3432	pMD1464	L113P	-	pMD1450
A3724	pMD1617	D124G	ON1150	pMD1230
A3433	pMD1465	Y128H	-	pMD1450
A3725	pMD1618	V136A	ON1163	pMD1230
A3790	pMD1628	L137P	ON1155	pMD1230
A3791	pMD1629	V178A	ON1091	pMD1230
A3438	pMD1467	V178D	-	pMD1230
A3792	pMD1630	V178G	ON1092	pMD1230
A3793	pMD1631	F204I	ON1186	pMD1230
A3436	pMD1473	F204L	-	pMD1230
A3673	pMD1605	N205D	ON1114	pMD1230
A3447	pMD1471	N205H	-	pMD1230
A3448	pMD1472	N205Y	-	pMD1230
A3674	pMD1606	S207G	ON1115	pMD1230
A3449	pMD1470	S207I	-	pMD1230
A3675	pMD1607	T208A	ON1116	pMD1230
A3450	pMD1468	S214T	-	pMD1230
A3794	pMD1632	I215M	ON1187	pMD1230
A3795	pMD1633	I215T	ON1153	pMD1230

A3796	pMD1634	M218T	ON1158	pMD1230
A3437	pMD1466	F220I	-	pMD1230
A3451	pMD1469	I227V	-	pMD1230
A3798	pMD1636	I263S	ON1189	pMD1230
A3805	pMD1643	Y266C	ON51	pMD1230
A3829	pMD1654	L268S	ON1200	pMD1230
A3832	pMD1655	K269A	ON846	pMD1230
A3830	pMD1652	P270S	ON1199	pMD1230
A3800	pMD1638	Q272P	ON1159	pMD1230
A3659	pMD1596	G273S	-	pMD1230
A3801	pMD1639	T274I	ON1161	pMD1230
A3833	pMD1656	D275A	ON467	pMD1230
A3660	pMD1597	D275E	-	pMD1230
A3802	pMD1640	D275G	ON1151	pMD1230
A3803	pMD1641	L277S	ON1156	pMD1230
A3804	pMD1642	T278A	ON1162	pMD1230

[a]Sequences of oligonucleotides are available upon request.

Supplementary Table 6. Binding of the antagonist [desTrp^1,Ala^3,Lys^7(NBD),Nle^{12}]α-factor to selected α-factor receptor mutants that alter the emission spectrum of receptor-bound [DTyr^3,Lys^7(NBD),Nle^{12}]α-factor.

Mutation	Rel. B_{max} Ratio[a] (FL1/FL2)	FL1	
		Rel. K_d[b]	Rel. B_{max}[c]
WT	**0.5**[a]	**1.0**	**100**
M218K	1.1 ± 0.1	0.2 ± 0.02	170 ± 8
Q253L	1.2 ± 0.1	0.3 ± 0.1	234 ± 13
P258S	1.3 ± 0.1	0.2 ± 0.03	112 ± 5

[a] The reported ratio is normalized to that of agonist [Lys^7(NBD),Nle^{12}]α-factor binding to normal receptors. Note that all other results in this table are normalized to the values of normal receptor interacting with desTrp^1,Ala^3,Lys^7(NBD),Nle^{12}]α-factor

[b] computed as the ratio K_d(mutant)/K_d(wild type) (for [desTrp^1,Ala^3,Lys^7(NBD),Nle^{12}]α-factor). The absolute value of the K_d for [desTrp^1,Ala^3,Lys^7(NBD),Nle^{12}]α-factor was 37 ± 5 nM (n = 1 triplicate assay)

[c] computed as 100× B_{max}(mutant)/B_{max}(wild type) (for [desTrp^1,Ala^3,Lys^7(NBD),Nle^{12}]α-factor)

Supplementary Text

Analytical treatment of receptor activation according to the two-state model.

We follow the formalism for describing the equilibrium between activated and un-activated states of ligand-bound and ligand-free receptor under conditions where the ligand-free and ligand-bound receptors activate downstream responses with equal efficiency [1]:

$$\text{Signaling Response} = a[R_T]\frac{\frac{\alpha K_L + [L]}{1+\alpha K_R}}{\frac{\alpha K_L (1+K_R)}{1+\alpha K_R}+[L]}$$

where a is a proportionality constant, $[R_T]$ is the total concentration of receptors, [L] is the ligand concentration, K_L is the dissociation constant for ligand binding to un-activated receptors, K_R is the equilibrium constant for the de-activation of ligand-free receptor, and α is the ratio of dissociation constants for ligand binding to activated vs. un-activated receptors.

For a neutral antagonist, α = 1; the ligand binds with equal affinity to the activated and un-activated states and the expression reduces to:

$$Signaling\ \text{Re}sponse = a[R_T]\frac{1}{1+K_R}$$

In this situaiton, the response is independent of ligand concentration under all conditions, so that modifications of K_R leading to enhanced constitutive activation of the receptor can not be responsible for inducing ligand-dependent signaling.

At saturating ligand concentrations ($[L] >> K_L$) and, considering weakly activating ligands, such as the α-factor antagonists/partial agonists, such that $\alpha \approx 1$

$$\text{Signaling Response} = a[R_T]\frac{1}{(1+\alpha K_R)}$$

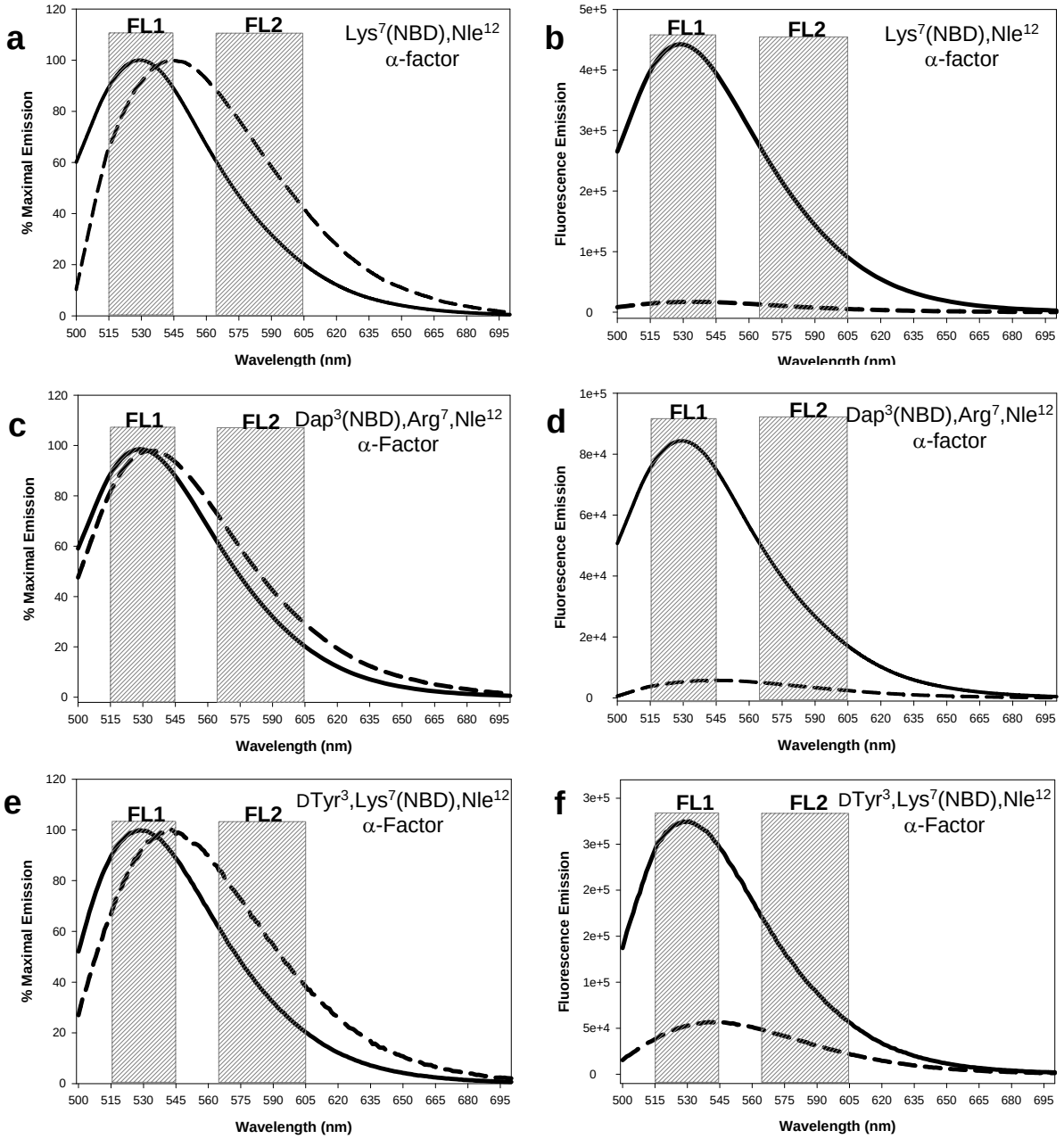
In the absence of ligand ([L] = 0) the expression again reduces to:

$$\text{Signaling Response} = a[R_T]\frac{1}{1+K_R}$$

Thus, both ligand-independent and maximal ligand-dependent signaling will increase as the equilibrium shifts toward the activated state of the receptor, decreasing the equilibrium constant K_R . This formalism predicts that for weakly activating ligands, such as the α-factor antagonists/partial agonists (for which $\alpha \approx 1$), the percentage increase in maximal ligand-dependent signaling resulting from a change in K_R should be the approximately equal to the percentage increase in the level of constitutive signaling. This is observed for certain of the stronger mutations recovered from the antagonist screen, such as Q253L and P258S, however, it is not observed for other mutations, such as M218K and L222R (Supplementary Fig. 6), suggesting either that the latter substitutions may do more than simply alter the equilibrium constant K_R for activation or that these effects occur via mechanisms outside the context of the two-state model.

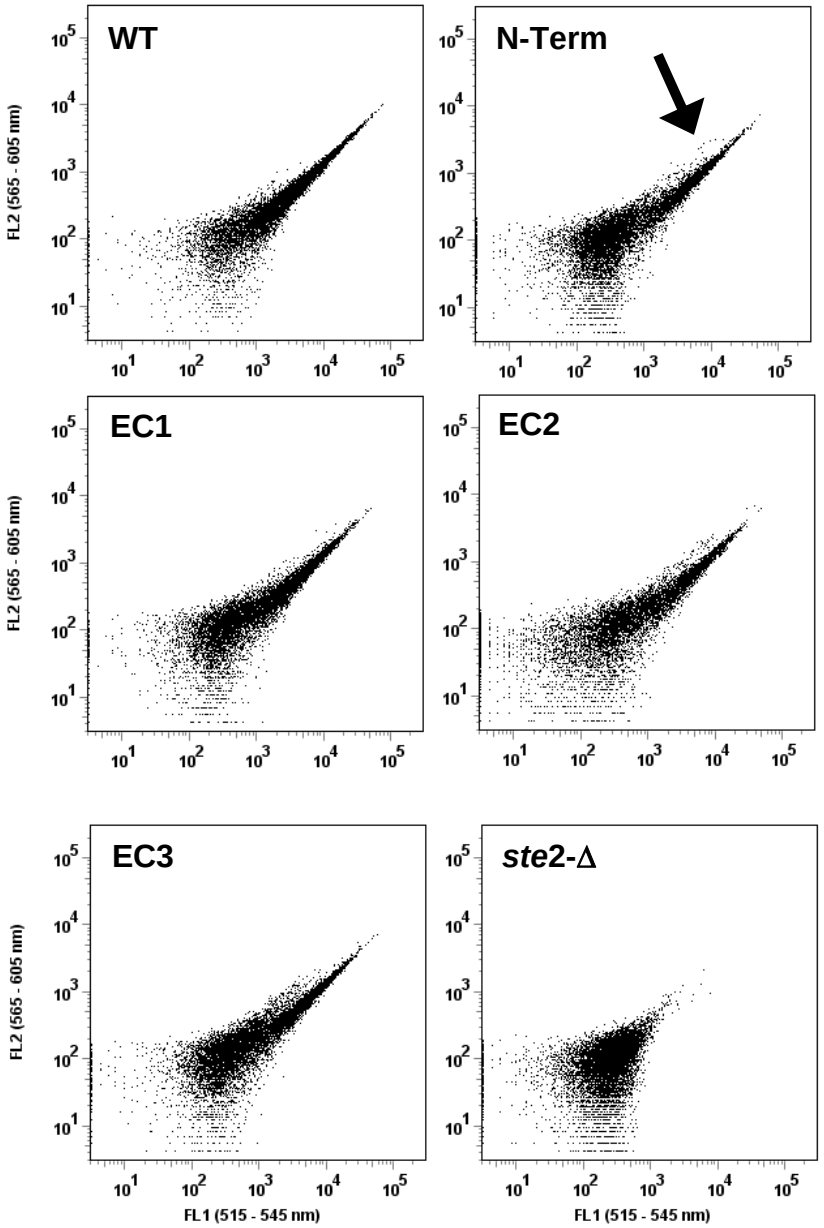
References

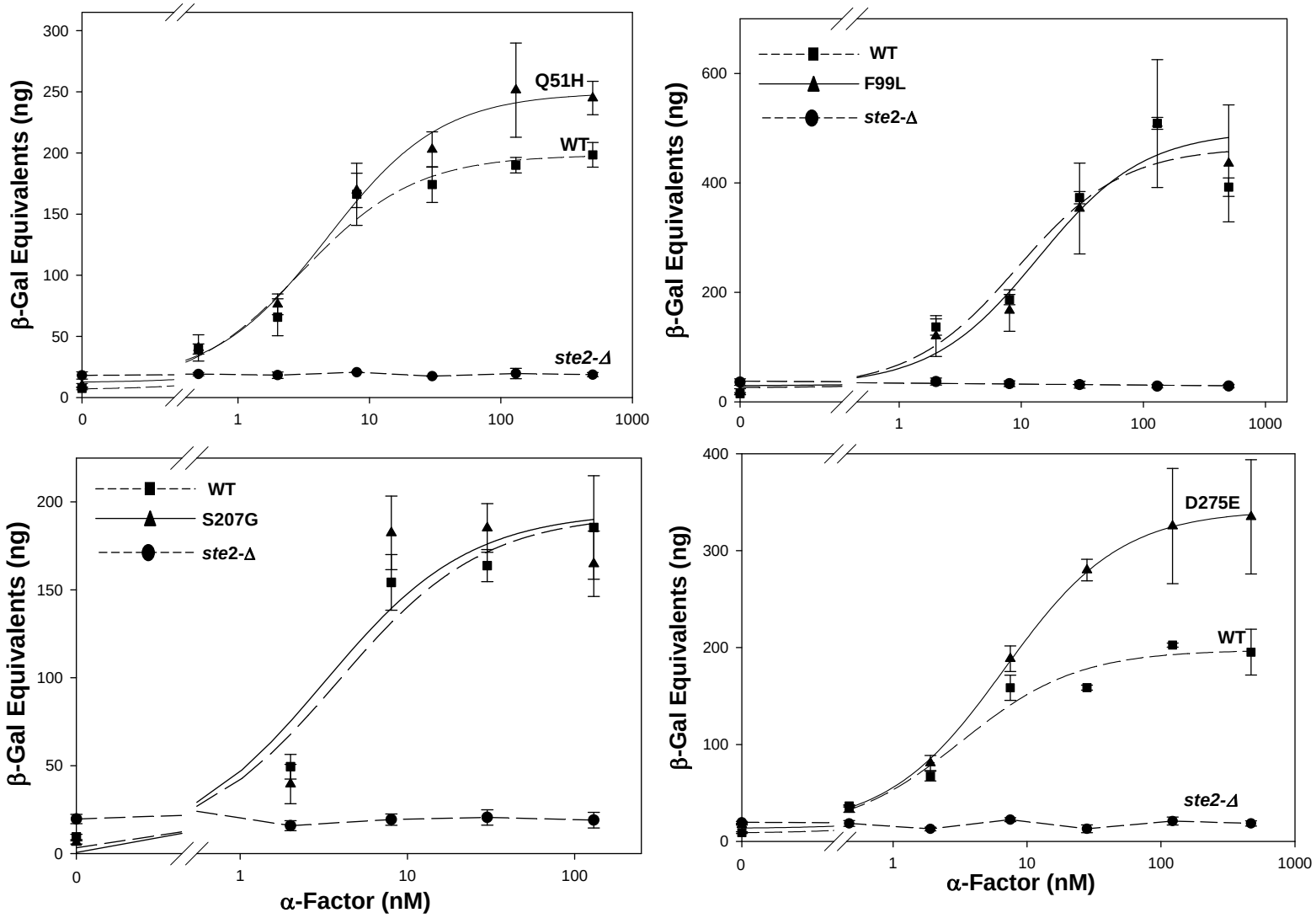
1. Taylor, P., Insel P.A. (1990). The Molecular Basis of Pharmacological Selectivity. In *Principles of Drug Action: The Basis of Pharmacology* (Pratt, W. B. a. T., P. , ed.). Churchill Livingstone Inc., New York, NY.



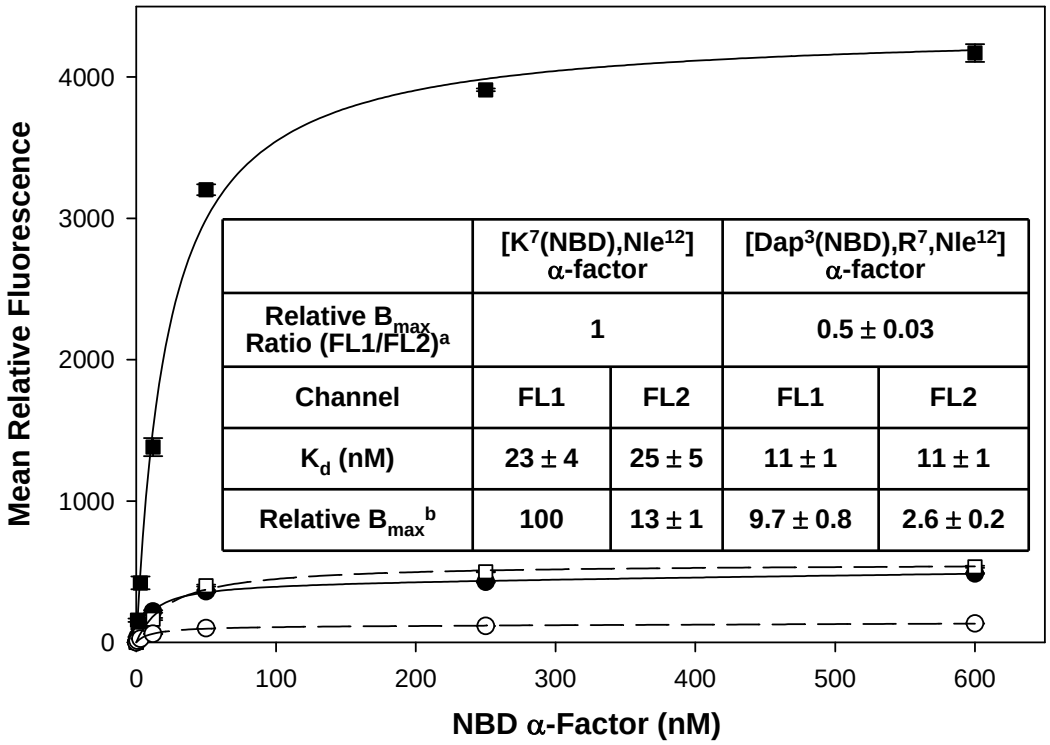
Supplementary Fig. 1: Emission spectra of three different NBD-a-factor analogs in environments of different polarity. Panels a and b - 50 nM [Lys^7(NBD),Nle^{12}] a-factor. Panels c and d - 50 nM [Dap^3(NBD),Arg^7,Nle^{12}] a-factor. Panels e and f - 50 nM [DTyr^3,Lys^7(NBD),Nle^{12}] a-factor. The Unbroken (___) lines represent spectra in 98% 2-propanol and the broken (- - -) ones in 20 mM sodium acetate buffer (pH 4.6). In panels a), c), and e) the spectra have been normalized to the same peak emission intensity. Panels b), d), and f) show the same spectra without normalization. The ranges of the flow cytometer channels FL1 (515 - 545 nm) and FL2 (565 - 605 nm) are represented by the shaded rectangles. The fluorimeter used was a Horiba Jobin Yvon FluoroMax4. The fluorescent a-factor was excited at 488 nm and emission was recorded between 500 and 700 nm. A slit width of 5 nm and an integration time of 1 second/nm were used.

Supplementary Fig. 2. Dot plots of fluorescence emission intensity in the FL1 (x-axis) and FL2 (y-axis) channels for the four libraries bound to a sub-saturating (50nM) concentration of [Lys^{7}(NBD),Nle^{12}] a-factor. Each dot plot is based on cytometry of 10,000 cells. Plots are shown for cell expressing un-mutagenized receptors (WT), for cells expressing libraries mutagenized in the four indicated regions of the receptor, and for *ste2*-Δ cells transformed with an empty vector. The arrow in the N-terminal region panel points out cells exhibiting the "mutant phenotype", of an increase FL2/FL1 ratio compared to unmatuagenized receptors.



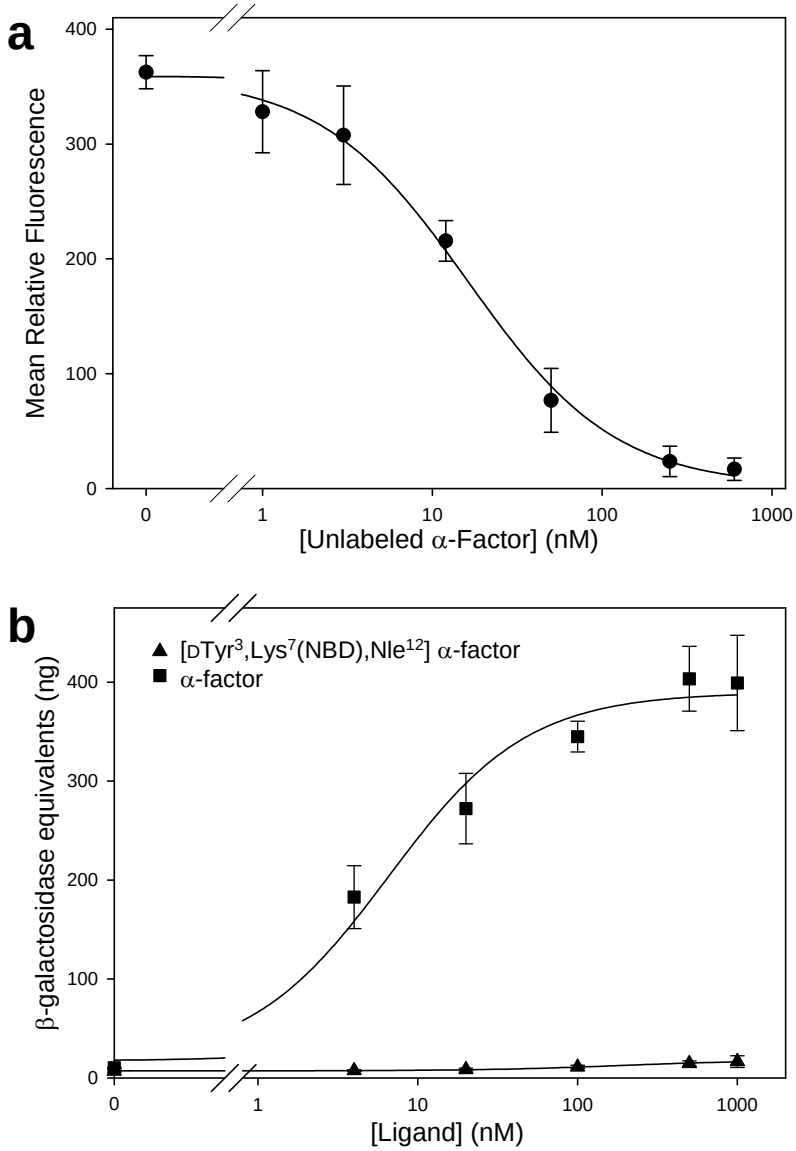


Supplementary Fig. 3. Induction of *FUS1*-lacZ reporter in response to α-factor by representative mutant and normal receptor alleles.

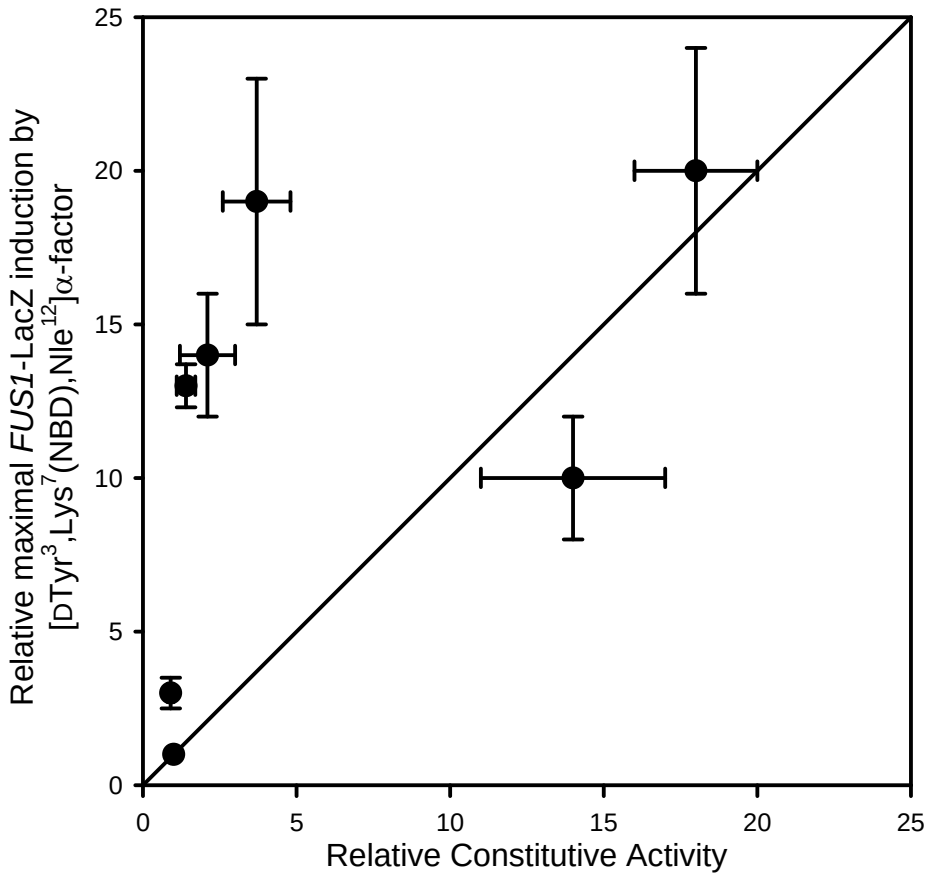


	[K⁷(NBD),Nle¹²] α-factor		[Dap³(NBD),R⁷,Nle¹²] α-factor	
Relative B_{max} Ratio (FL1/FL2)[a]	1		0.5 ± 0.03	
Channel	FL1	FL2	FL1	FL2
K_d (nM)	23 ± 4	25 ± 5	11 ± 1	11 ± 1
Relative B_{max}[b]	100	13 ± 1	9.7 ± 0.8	2.6 ± 0.2

Supplementary Fig. 4: Comparison of the binding curves of [Dap³(NBD),Arg⁷,Nle¹²] a-factor and [Lys⁷(NBD),Nle¹²] a-factor bound to Ste2p. The squares (■ □) represent the WT strain bound to [Lys⁷(NBD),Nle¹²] a-factor and the circles (● ○) represent binding of the same strain to [Dap³(NBD),Arg⁷,Nle¹²] a-factor. The closed symbols/unbroken lines and open symbols/broken lines indicate information obtained from the FL1 and FL2 channels, respectively. [a]Normalized to the ratio for [Lys⁷(NBD),Nle¹²] α-factor bound to wild-type Ste2p. [b]Normalized to the B_{max} in FL1 for [Lys⁷(NBD),Nle¹²] α-factor bound to wild-type Ste2p.



Supplementary Fig. 5. a. Inhibition of the binding of the fluorescent antagonist [DTyr^3,Lys^7(NBD),Nle^{12}] α-factor to Ste2p by α-factor. All experiments used yeast strain A3694, expressing Ste2p Δ305-431 from a multicopy plasmid. The concentration of the labeled antagonist was held constant at 6 nM. The curve shows a fit to 1-site competitive binding with an IC_{50} of 16 nM. **b.** Comparison of activation of the *FUS1*-LacZ reporter of pheromone response by normal α-factor and the antagonist [DTyr^3,Lys^7(NBD),Nle^{12}] α-factor.



Supplementary Fig. 6. Correlation of maximal signaling (induction of *FUS1*-LacZ reporter) versus relative constitutive activity of mutant receptor alleles that shift the emission of bound [DTyr^{3},Lys^{7}(NBD),Nle^{12}]α-factor (from Table 4). A line with a slope of 1 is shown for reference.