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GPCR-Gα protein precoupling: Interaction between Ste2p, a yeast GPCR, and Gpa1p, its Gα protein, is formed before ligand binding *via* the Ste2p C-terminal domain and the Gpa1p N-terminal domain

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ABSTRACT

G protein coupled receptors bind ligands that initiate intracellular signaling cascades *via* heterotrimeric G proteins. In this study, involvement of the N-terminal residues of yeast G-alpha (Gpa1p) with the C-terminal residues of a full-length or C-terminally truncated Ste2p were investigated using bioluminescence resonance energy transfer (BRET), a non-radiative energy transfer phenomenon where protein-protein interactions can be quantified between a donor bioluminescent molecule and a suitable acceptor fluorophore. Constitutive and position-dependent BRET signal was observed in the absence of agonist (α-factor). Upon the activation of the receptors with α-factor, no significant change in BRET signal was observed. The location of Ste2p–Gpa1p heterodimer was investigated using confocal fluorescence microscopy and bimolecular fluorescence complementation (BiFC) assay, a technique where two non-fluorescent fragments of a fluorescent protein reassemble *in vivo* to restore fluorescence property thereby directly reporting a protein-protein interaction. BiFC experiments resulted in a dimerization signal intracellularly during biosynthesis on the endoplasmic reticulum (ER) and on the plasma membrane (PM). The constitutive BRET and BiFC signals observed on ER between Ste2p and Gpa1p in their quiescent and activated states are indicative of pre-coupling between these two proteins.

This study is the first to show that the extreme N-terminus of yeast G protein alpha subunit is in close proximity to its receptor. The data suggests a pre-coupled heterodimer prior to receptor activation. The images presented in this study are the first direct *in vivo* evidence showing the localization of receptor - G protein heterodimers during biosynthesis and before reaching the plasma membrane.

1. Introduction

G protein-coupled receptors (GPCRs) belong to the largest cell surface receptor family in eukaryotic cells. Considering their role in sensing extracellular signals, the GPCR family plays very important roles in sensory systems and detect a large assortment of chemicals (hormones, neurotransmitters, chemo attractants and ions) and sensory signals (light, odorants and taste molecules) [1]. Activated GPCRs often transmit these extracellular signals to intracellular signaling cascades by heterotrimeric G proteins [2], which consist of three subunits, Gα, Gβ and Gγ. Mutations and functional problems of these receptors are linked to many human diseases such as diabetes, cardiovascular diseases, hypertension, cancer, hypothyroidism, retinitis pigmentosa and many psychotic disorders [3]. This is why GPCRs are the targets of ~25% of pharmaceuticals on the market thus being a major focus of molecular pharmacology research [4, 5]. However, the action mechanism of the GPCRs and the G proteins still has lots of unresolved discussions. Understanding the interactions between the GPCRs and their G proteins is pivotal to explain the plasticity of the signal transduction pathways.

GPCR structure and function has been studied in many model systems to avoid the complications arise from complex mammalian systems. In contrast to the human genome, which encodes nearly 1000 GPCRs [6], the budding yeast *Saccharomyces cerevisiae* encodes only

Abbreviations: BiFC, bimolecular fluorescence complementation; BRET, bioluminescence resonance energy transfer; C-EGFP, C-terminal domain of enhanced green protein; CFP, cyan fluorescent protein; EGFP, enhanced green fluorescent protein; ER, endoplasmic reticulum; FRET, Förster resonance energy transfer; GFP, green fluorescent protein; GPCR, G-protein coupled receptors; Gα, G protein alpha subunit; Gβ, G protein beta subunit; Gγ, G protein gamma subunit; MAPK, mitogen activated protein kinase; MLT, medium lacking tryptophan; MLTU, medium lacking tryptophan and uracil; MLU, medium lacking uracil; N-EGFP, N-terminal domain of enhanced green protein; PM, plasma membrane; RET, resonance energy transfer; RGS, regulators of G-protein; RLuc, *Renilla luciferase*; SDS, sodium dodecyl sulfate; TM, transmembrane; Tα, transducin; YFP, yellow fluorescent protein

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three known G protein coupled receptors and has one of the best-studied heterotrimeric G protein signaling pathways [7]. Basic principles of G protein signaling and regulation were first elucidated by genetic and biochemical assays analyzing the response of yeast to its mating pheromone peptide. Many of the important mechanisms of G protein and mitogen-activated protein (MAP) kinase signaling, the three-tiered structure of MAP kinase module [8] and the existence of kinase scaffold proteins [9] were first shown in *S. cerevisiae*. The first demonstration of mono-ubiquitination as a signal for receptor endocytosis [10, 11], the positive signaling role of Gβγ subunits [12] were discovered in yeast. The desensitization of signaling by regulators of G protein signaling (RGS) proteins were shown for the first time using the genetic disruption of *SST2* in yeast [13]. Homologs of Sst2 also exist in higher eukaryotes, named RGS proteins, were shown to accelerate G protein GTPase activity, thus leading to subunit re-association [7]. Furthermore, most of the GPCR mediated signaling pathway and its elements in mammalian cells are structurally and functionally similar to yeast pheromone signaling pathway [14]; the G protein and kinase components share extensive sequence similarity with their mammalian counterparts [15]. Thus, in this study we used Ste2p and Gpa1p to investigate GPCR Gα interactions.

The most studied contact site between Ste2p, the yeast mating pheromone α-factor GPCR and its cognate Gα is the C-terminal domain of Gα with the third intracellular loop of Ste2p. It was also shown that the N-terminus, the αN–β1 loop, the α4–β6 region and the α5 helix of Gα are involved in the interactions between Gα and GPCRs [16–23]. In rhodopsin transduction (Tα) complex, it was shown that both the N and the C termini are in close vicinity to the third cytoplasmic loop of rhodopsin Tα [24] but in most x-ray studies the extreme N- and C-termini of Gα cannot be depicted since they are disordered [25].

The mechanism of the dynamics of GPCR–Gα interaction is still under debate. Two different models were proposed: the “collision model” assuming that random collisions occur between receptors and G proteins in their inactive state without activating the signaling pathway that upon the stimulation of the receptors with their ligand, conformational changes on the receptor activates the G protein [26, 27], and a second model that presumes receptors and G proteins reside as a “pre-coupled” complex and stimulation of the receptors leads to immediate activation of the pre-coupled G protein [28–31]. One way to investigate these two models is to use resonance energy transfer (RET) assays in live cells.

Ability of tagging proteins with a fluorescent tag allows researchers to use these tagged proteins in order to study interactions *in-vivo*, in real-time [32]. Bioluminescence resonance energy transfer (BRET), a quantitative assay preferred in this study, is an electrodynamic phenomenon that occurs when an excited state “donor” molecule such as *Renilla luciferase*, transfers its energy to a ground state “acceptor” molecule, a fluorescent protein, in a non-radiative process through long range dipole-dipole interactions. Förster distance, which is defined as the distance at which RET efficiency is 50% [33] ranges from 2 to 9 nm. Such distances are far below the spatial resolution of a optical microscopy and are comparable to the size of biomolecules and/or the distance between sites on multi-subunit proteins. Therefore, in physiological conditions, RET can only be observed when two proteins are interacting since the average molecular distance is in the order of the Förster distance. The second technique used in this study is bimolecular fluorescence complementation (BiFC) assay, which allows to visualize protein interactions in live cells, and based on the facilitated association of complementary fluorescent protein fragments fused to interaction partners. Complex formation by the interaction partners brings the fluorescent protein fragments into close proximity thereby facilitating their complementation [34, 35]. The BiFC assay enables sensitive visualization of protein complexes with high spatial resolution, however the temporal resolution may be limited by the time required for fluorophore formation, as well as the stabilization of complexes by association of the fluorescent protein fragments [32, 36].

The heterotrimeric G protein cycle was quantitatively characterized recently in yeast measuring the FRET between CFP tagged Gpa1p (Gα) and YFP tagged Ste18p (Gγ) [37]. In another study, Gillen et al. reported that the first 67–36- or 9-amino acids of Gpa1p are sufficient to direct GST to the plasma membrane in yeast [38]. Gpa1p was shown to be myristoylated at the 2nd amino acid residue [39] and palmitoylated at the 3rd amino acid residue [40], and these post-translational modifications are required for plasma membrane targeting and membrane anchorage. Harashima et al. tagged Gpa2p (Gα of yeast glucose receptor) with GFP after position 10 without altering the protein function and showed that Gpa2p has lipid modifications, a myristoylation site on the Gly2 and possible palmitoylation sites at cysteine residue near this myristoylation site of its N-terminus, which is required for membrane localization similar to Gpa1p [41].

In this study, the interactions of the N-terminal residues of Gpa1p (Gα) with the C-terminal residues of its cognate receptor, Ste2p was investigated. For this purpose, Gpa1p was tagged with EGFP at various positions on the N-terminus, while a full-length Ste2p and a C-terminally truncated receptor (Ste2p-Δ305) were tagged with RLuc at position 304. GPCR–Gα interaction was quantified *in vivo* with BRET. Whereas the location of Ste2p–Gpa1p heterodimer, was assayed by BiFC and colocalization of reconstructed EGFP signal with organelle markers. This study is the first to show that the extreme N-terminus of yeast G protein alpha subunit is in close proximity to its receptor and both proteins exist in a pre-coupled heterodimer prior to receptor activation. The *in vivo* visualization data presented in this study also suggest that the heterodimerization occurs at the endoplasmic reticulum during the biosynthesis, before reaching the plasma membrane.

2. Methods

2.1. Yeast strains, plasmids and media

DK102 (MAT***a***, ura3-52 lys2–801^{am} ade2-101^{oc} trp1-Δ63 his3-Δ200 leu2-Δ1 ste2::HIS3 sst1-Δ5) [42], BJS21 (MAT***a***, prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::Kan^{R}) [43] and TM5117 (MAT***a***, trp1-Δ63, ade2-101^{oc}, bar1, his3-Δ200 leu2-Δ1 ste2::HIS3, FUS1-lacZ::URA3, gpa1Δ, far1Δ) [44] *Saccharomyces cerevisiae* strains were used. TM5117 was used as a Gpa1p knock-out strain to investigate the Ste2p–Gpa1p interaction. DK102 was used for alpha-factor induced growth arrest assay and the protease deficient BJS21 strain was used in Western blot analysis due to the decreased protein degradation.

For constitutive expression of Ste2p and Gpa1p, pSP-G1 and pST-G1 bidirectional double promoter plasmids [45], with 2-μm based shuttle vector, a *TEF1* promoter-*ADH1* terminator region and *PGK1* promoter–*CYC1* terminator region were used. pSP-G1 carrying a *URA* marker and pST-G1 carrying a *TRP* marker for selection in yeast.

The pBS-35S-RLUC plasmid, *Renilla Luciferase* cDNA vector, was a generous gift from Dr. Albrecht von Arnim (University of Tennessee Knoxville, USA). pEGFP-N2, enhanced green fluorescence cDNA vector was obtained from Prof. Dr. Henry Lester's laboratory (California Institute of Technology, USA) [46]. ER marker plasmid, YIplac204/TKC-DsRed-HDEL was a generous gift from Dr. Benjamin S. Glick (University of Chicago, USA).

Yeast strains were grown in YEPD (yeast extract-peptone-dextrose) broth at 30 °C and were maintained on agar plates at 4°C for short-term storage. For selection of successful yeast transformants with constructed plasmids, media lacking tryptophan (MLT, Supplementary materials), media lacking uracil (MLU, Supplementary materials) and media lacking both Tryptophan and Uracil (MLTU, Supplementary materials), were used respectively.

2.2. Chemical reagents and other materials

Phusion Hot Start II High-Fidelity DNA Polymerase was purchased from Thermo-Fisher Scientific (MA, USA). All restriction enzymes were

purchased from Thermo-Fisher Scientific (MA, USA). Coelenterazine 400a (DeepBlueC) used for BRET experiments was purchased from Biotium Inc. (CA USA), Yeast β-Galactosidase Assay Kit was purchased from Thermo-Fisher Scientific (MA, USA). The peptide pheromone α-factor used in biological activity assays was synthesized and purified by Dr. Naider's laboratory [47]. Primers used in this study were purchased from Invitrogen (MA, USA). Paper filter disks were from BD (Franklin Lakes, NJ, USA) and glass bottom dishes used in imaging experiments were ordered from Fisher Scientific (MA, USA). All other chemicals used in buffers and mediums were obtained from Sigma-Aldrich Inc. (NY, USA) and AppliChem (Darmstadt, Germany). Leica Laser Scanning Confocal Microscope equipped with a Leica 63 ×/1.32 HCX PL APO Oil DIC objective, VT-Hawk 2D array laser scanning confocal microscopy system (Visitech International, UK) with an Olympus IX-83 inverted microscope equipped with 100 ×/1.49 Oil UAPO lens (Olympus) and EM-CCD digital camera (Hamamatsu) (Department of Microbiology and Department of Biochemistry, Cell, and Molecular Biology, University of Tennessee, Knoxville, Tennessee) and Leica DMI4000 with Andor DSD2 Spinning Disc Confocal Microscope equipped with a Leica 100 ×/1.49 Oil DIC objective (Department of Biological Sciences, Middle East Technical University, Ankara, Turkey) was used for imaging experiments.

2.3. Constructs

For the BRET experiments, *Renilla Luciferase* (RLuc) was inserted between amino acids 304–305 on Ste2p. Two plasmids were constructed; one to express a C-terminally truncated Ste2p tagged with RLuc, other to express a Ste2p with an intact C-tail tagged with RLuc between positions 304–305. For Gpa1p, EGFP was inserted between amino acids 10–11, 36–37 and 67–68 to generate three separate constructs.

For the BiFC experiments, EGFP was split between 158th and 159th amino acids. N- EGFP (1–158) was inserted between amino acids 10–11 on Gpa1p and C-EGFP (159–238) was inserted between amino acids 304–305 on Ste2p (see Supplementary materials for primers and PCR conditions).

2.4. FUS1-lacZ assay using yeast β-galactosidase protocol

Liquid assays of *FUS1-lacZ* were performed as described by Martin et al. [48] with some modifications. In this assay, the biological function of Gpa1p and Ste2p constructs were quantified as a decrease of β-galactosidase activity due to the sequestration of Gβ/Gγ subunits with the expression of a functional Gα, and upon treatment of cells with α-factor, as an increase in the β-galactosidase activity due to the induction of the *FUS1-LacZ* reporter gene. For this, yeast cultures expressing the constructs were grown to stationary phase and then diluted and cultured overnight to an OD_{600} of approximately 0.4–0.6 in minimal media. 100 μL of cell culture was then dispensed into a 96-well plate containing 10 μL samples of 1 μM final concentration of α-factor. The cells were incubated with the pheromone for 105 min at 30 °C and the OD_{660} were measured using an absorbance plate reader (SpectraMax Plus, Molecular Devices Corp.). *Ste2Δ* and *Gpa1Δ* yeast cells transformed with an empty vector and cells transformed with a plasmid coexpressing Ste2p WT and Gpa1p WT were used as negative and positive controls, respectively. Activity was determined by measuring the absorbance at 420 nm using a fluorescence plate reader (SpectraMax Plus, Molecular Devices Corp.). The β-galactosidase activity of the cells was quantified using the calculations in the manufacturer protocol (Thermo-Fisher Scientific, MA, USA). Assays were conducted in triplicate using three independent yeast transformants of each strain.

2.5. Membrane preparation and Western blot analysis

Protein expression of all the constructs was determined by Western blotting using the protease-deficient yeast strain BJS21. Cells transformed with empty plasmid and cells expressing Ste2p WT or Gpa1 WT were used as controls. Yeast cells were grown in 50 mL of the selective proper medium (MLT/MLU) at 30°C overnight and membranes prepared as previously described [44]. Total protein concentration was determined by BioRad (BioRad, Hercules, CA) protein assay [44] and membrane protein extract was solubilized in SDS sample buffer (BioRad, Hercules, CA) and 5 μg was loaded in each lane of 10% SDS-PAGE. Following resolution according to molecular weight, proteins were transferred to Immobilon-P membrane (Millipore Corporation, Bedford, MA) for immunoblot analysis. Immunoblotting was carried out using affinity-purified antireceptor antiserum directed against the N-terminal domain of the α-factor receptor [49], which were kindly provided by James Konopka, State University of New York, Stony Brook, NY, anti-Gpa1 antibody (Santa Cruz Biotechnology, TX) and anti-GFP Polyclonal antibody (ThermoFisher Scientific, MA). Bands were observed with Odyssey CLx Imaging System (LI-COR Biosciences, NE).

2.6. BRET analysis

BRET assay were performed using the Turner designs TD-20/20 instrument equipped with the dual color accessory equipped with blue (333 nm–463 nm with > 90% transmission from 370 to 410 nm) and yellow (520 nm long pass with > 80% transmission from 550 nm up) filters. BRET technique, sometimes referred to as BRET1, with *Renilla luciferase* as a donor molecule and EGFP as an acceptor was used in this study. Coelenterazine was used as a substrate of *Renilla luciferase*, whose oxidation results in a ~475 nm emission peak and EGFP was used as a BRET acceptor with an excitation peak at ~490 nm and an emission peak at ~510 nm.

For the BRET analysis, yeast cells were grown overnight in a shaker incubator at 30 °C in 5 mL fresh media. Following day, the cells were subcultured and grown to OD_{600} of 1. From this culture, 3 mL was centrifuged at 4000 rpm for 5 min and the supernatant was removed. The cell pellet was resuspended in 200 μL of BRET buffer containing 0.01% (w/v) magnesium ($MgSO_4.7H_2O$) and 0.1% (w/v) glucose in phosphate-buffered saline (PBS) (0.14 M NaCl 10 mM NaH_2PO_4, pH 7.4) [50]. Cells were spun down at 4000 rpm for 3 min, the supernatants were removed, and the cell pellets were placed on ice.

Before the BRET measurements freeze-thaw treatment was applied placing the cell pellets in a dry ice/isopropanol bath for 3 min followed by an immediate thawing at room temperature bath for another 3 min as explained previously [50]. The cell pellets were then resuspended in 200 μL of BRET buffer and inserted into luminometer. 5 μM final concentration of coelenterazine was added to the cells and the signal was immediately measured with sequential readings switching between blue/yellow filter set. The BRET ratios were calculated by dividing the number of photons counted through the yellow filter with the number of photons counted through the blue filter and represent as the average for three independent experiments, the errors reported as standard error of the mean. Data presented as percentage since RLuc emission decays rapidly with time [51, 52]. The net BRET ratio was calculated by subtracting background obtained from cells only expressing RLuc tagged Ste2p as explained above. The BRET ratios calculated by this method remained constant throughout the experiment (see Supplementary materials for BRET calculations).

2.7. Imaging with laser scanning confocal microscope

For image acquisition, yeast cells were grown overnight at 30 °C in 5 mL fresh media. Following day, the cells were subcultured and grown to OD_{600} of 1. From this culture 1 mL was taken, spun down and the cells were resuspended again in 200 μL fresh media. For detection of fluorescent signal in live cells, yeast cells were observed using a Leica SP2 Laser Scanning Confocal Microscope equipped with an Leica

100 ×/1.49 Oil DIC objective and VT-Hawk 2D array laser scanning confocal microscopy system (Visitech International, UK), the preliminary imaging of the EGFP tagged Gpa1p was done with an Olympus IX-83 inverted microscope equipped with 100 ×/1.49 Oil UAPO lens (Olympus) and EM-CCD digital camera (Hamamatsu) (Department of Microbiology and Department of Biochemistry, Cell, and Molecular Biology, University of Tennessee, Knoxville, Tennessee) and colocalization of Ste2p-Gpa1p signal with ER marker was imaged using Leica DMI4000 with Andor DSD2 Spinning Disc Confocal Microscope equipped with a Leica 100 ×/1.49 Oil DIC objective (Department of Biological Sciences, Middle East Technical University, Ankara, Turkey).

3. Results

In this study, constructs of Ste2p were prepared considering previous studies reporting successful insertion of various fluorescent proteins while maintaining the biological function of the receptor [50, 53]. The importance of N-terminus of Gpa1p for membrane localization and function has been previously shown in several studies [38–40], thus taking the previous literature into consideration and avoiding disruption of the known post-translational modification sites on the Gpa1p N-terminus domain, four constructs of Gpa1p were prepared by inserting EGFP between residues 10–11; 36–37; 67–68 and inserting N-EGFP between residues 10–11 for BiFC experiments (Fig. 1, Table 1). Considering the test pairs, all these constructs were cloned into double promoter yeast expression vectors; functional assays, Western blots and image acquisitions were done using cells transformed with these plasmids.

Table 1
The abbreviated names of the Ste2p and Gpa1p constructs (1st column) explanation of constructs (2nd column).

Abbreviation	Explanation
Ste2p WT	*Wild-type* Ste2p receptor
Gpa1p WT	*Wild-type* Gpa1p
Ste2p[RLuc]	Ste2p tagged with RLuc inserted between 304–305th amino acids
Ste2p-Δ305[RLuc]	C-terminally truncated Ste2p tagged with RLuc at 304th a.a.
Ste2p[C-EGFP]	Ste2p tagged with C-EGFP (159–238) inserted between 304–305th a.a.
Ste2p-Δ305[C-EGFP]	C-terminally truncated Ste2p tagged with C-EGFP (159–238) at 304th a.a
Gpa1p[10-EGFP]	Gpa1p tagged with EGFP inserted between 10–11th a.a.
Gpa1p[36-EGFP]	Gpa1p tagged with EGFP inserted between 36–37th a.a.
Gpa1p[67-EGFP]	Gpa1p tagged with EGFP inserted between 67–68th a.a.
Gpa1p[10-N-EGFP]	Gpa1p tagged with N-EGFP (1–158) inserted between 10–11th a.a.

3.1. Functional assay for Gpa1p and Ste2p constructs

The signaling capacity of the Gpa1p and Ste2p constructs were evaluated using a reporter assay. The *Ste2Δ* and *Gpa1Δ* cells transformed with empty plasmid gave high and constitutive β-Galactosidase activity both in the absence and presence of receptor agonist due to the continuous induction of the *FUS1-LacZ* reporter gene by the free Ste4p (Gβ) and Ste18p (Gγ) [7]. Cells coexpressing Ste2p WT and Gpa1p WT were used as a positive control and showed decreased β-Galactosidase

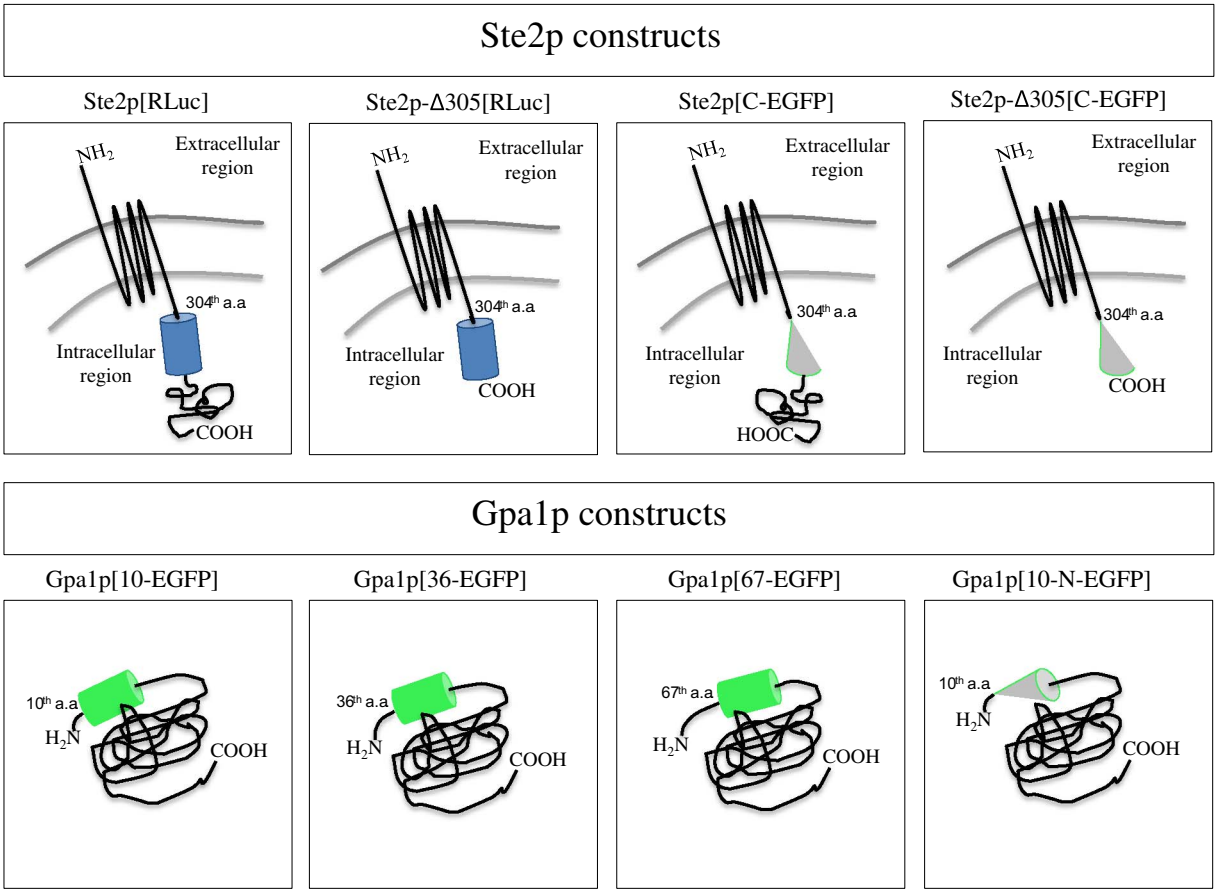

Fig. 1. The schematic representation of all the Ste2p and Gpa1p constructs used in this study. The first row of images shows full-length Ste2p receptor tagged with *Renilla Luciferase*, C-terminally truncated Ste2p-Δ305 receptor tagged with *Renilla Luciferase*; full-length Ste2p receptor tagged with C-EGFP (159–238) inserted between positions 304–305 and C-terminally truncated Ste2p-Δ305 receptor tagged with C-EGFP (159–238) appended at position 304 respectively. The second row of images show, Gpa1p G-protein tagged with EGFP (1–238) inserted between positions 10–11, 36–37, 67–68 and finally Gpa1p G-protein tagged with N-EGFP (1–158) inserted between positions 10–11.

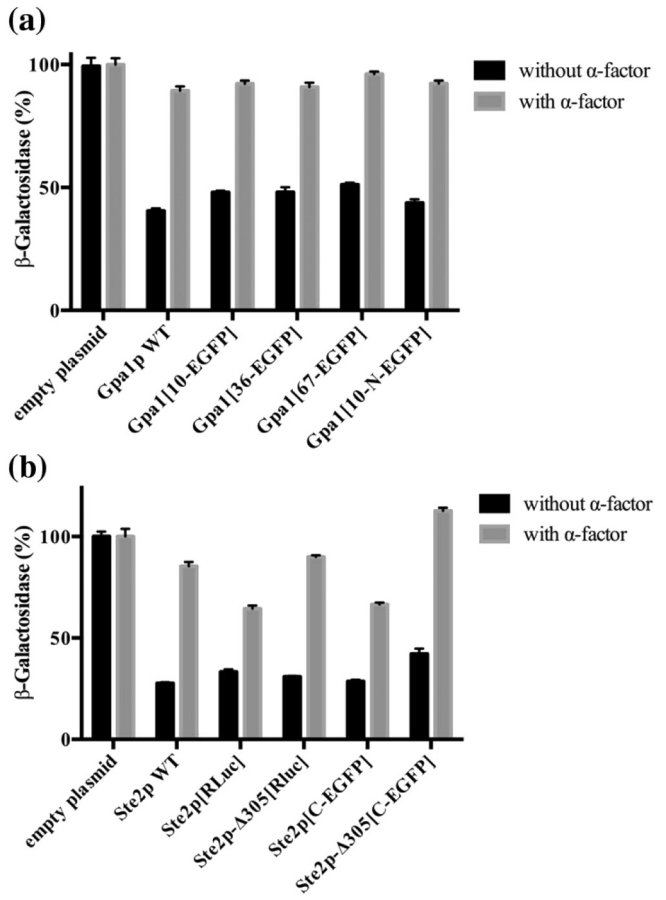

Fig. 2. (a) Functional Assay for Gpa1p constructs, β-Galactosidase activity (%) of the constructs was evaluated in the absence and presence of α-factor. As a negative control TM5117 cells transformed with an empty plasmid was used and as positive control TM5117 cells were transformed with a plasmid expressing Ste2p WT and Gpa1p WT. Grouped columns from 3 to 6 corresponds to Gpa1p tagged with EGFP at position 10, 36, 67 and Gpa1p tagged with N-EGFP at position 10 respectively. (b) Functional Assay for Ste2p constructs, β-Galactosidase activity (%) of the constructs was evaluated in the absence and presence of α-factor. As a negative control TM5117 cells transformed with an empty plasmid was used and as positive control TM5117 cells were transformed with a plasmid expressing Ste2p WT and Gpa1p WT. Grouped columns from 3 to 6 corresponds to Ste2p constructs tagged with RLuc and Ste2p constructs tagged with C-EGFP at receptor C-tail respectively.

activity as a result of the sequestering of Gβ/Gγ by Gpa1. For evaluating the signaling capacity of Gpa1p constructs, cells coexpressing Ste2p WT and Gpa1p constructs tagged with EGFP or N-EGFP were used and all the Gpa1p constructs showed decreased β-Galactosidase activity similar to the *wild type*. For evaluating the signaling capacity of Ste2p constructs, cells coexpressing Gpa1p WT and Ste2p constructs tagged with RLuc or C-EGFP were used. All Ste2p constructs showed decreased β-Galactosidase activity as expected. Treatment of cells expressing either Gpa1p constructs or Ste2p constructs with α-factor resulted in an increased β-galactosidase activity, indicating the induction of *FUS1-LacZ* reporter gene by the stimulated Gβ/Gγ (Fig. 2a, b). The results showed that Gpa1p constructs fused with EGFP at position 10, 36 or 67 and N-EGFP at position 10 behaved similar to Gpa1p WT and Ste2p constructs fused with RLuc or C-EGFP at receptor C-tail behaved similar to Ste2p WT in the absence and presence of pheromone, thereby suggesting that all the Gpa1p and Ste2p constructs made in this study are biologically functional.

3.2. Western blot experiments

Extract of cells expressing full-length Ste2p receptor tagged with RLuc (Ste2p[RLuc]: calc. M.W. 87 kDa); extract of cells expressing C-terminally truncated Ste2p receptor tagged with RLuc (Ste2p-Δ305[RLuc]: calc. M.W. 73 kDa); extracts of cells expressing BiFC constructs, full-length receptor tagged with C-EGFP (Ste2p[C-EGFP]: calc. M.W. 57 kDa) and the C-terminally truncated construct (Ste2p-Δ305[C-EGFP]: calc. M.W. 42 kDa) were resolved on SDS-PAGE in accordance with their calculated molecular weights (Supplementary Fig. S2a and b). The concentration of membrane protein extract was determined with Bradford dye binding method and equal amounts of protein was loaded on SDS-PAGE. All Ste2p constructs were resolved as multiple bands showing the glycosylated forms of the receptor on SDS-PAGE as previously reported [54, 55]. Gpa1p constructs were also resolved on SDS-PAGE to confirm the expression of these fusion proteins with the correct size. Western blot of cells expressing Gpa1p constructs tagged with EGFP at position 10, 36 or at position 67 and (Gpa1p[10-EGFP], Gpa1p[36-EGFP or Gpa1p[67-EGFP]: calc. M.W. 82 kDa) were probed with anti-Gpa1p and anti-GFP antibodies and resolved on SDS-PAGE in accordance with their calculated molecular weights (Supplementary Fig. S3a, b). The BiFC construct (Gpa1p[10-N-EGFP]: calc. M.W. 72 kDa) was also resolved on SDS-PAGE yielding a band with correct molecular weight (Supplementary materials, Supplementary Fig. S3c).

3.3. Imaging of Gpa1p constructs Gpa1[10-EGFP], Gpa1[36-EGFP] and Gpa1[67-EGFP]

For the investigation of Ste2p–Gpa1p heterodimerization using BRET, Gpa1p, was tagged with EGFP at its N-terminus at positions 10, 36 and 67 and imaged *in vivo*. Prior to BRET experiments the Gpa1p constructs were imaged, two sets of representative images are given in Fig. 3a–c (for additional images see Supplementary Fig. S4a–c). All Gpa1-EGFP fusion proteins were observed both intracellularly and on the plasma membrane (PM).

3.4. BRET experiments of Ste2p–Gpa1p heterodimer

In first experiment set, the BRET ratio for the heterodimerization of C-terminally truncated receptor, Ste2p-Δ305[RLuc] was measured with three Gpa1p constructs tagged with EGFP at positions 10, 36 and 67 (represented in Fig. 4, top row, and data in Fig. 5a, bars 2, 3 and 4). In the second experiment set, to investigate the effect of receptor C-tail, BRET ratios between full-length receptor, Ste2p[RLuc] were quantified with Gpa1p[10-EGFP], Gpa1p[36-EGFP] and Gpa1p[67-EGFP] (represented in Fig. 3, bottom row, and data in Fig. 5a, bars 5, 6 and 7). Gpa1p construct tagged with EGFP at position 10 gave the highest BRET ratio both with the C-terminally truncated and full-length receptors (Fig. 5a). Gpa1p constructs tagged with EGFP at distant residues on N-terminus (Gpa1p[36-EGFP] and Gpa1p[67-EGFP]) resulted in a significantly lower but detectable BRET ratio (Fig. 5a, one-way ANOVA test was performed, $P < 0.0001$).

The BRET measurements for Ste2p constructs and Gpa1p[10-EGFP] were carried out both in the absence and presence of receptor agonist, α-factor (30 min of incubation) (Fig. 5b). The treatment of cells with α-factor at various time intervals (0, 30, 60 min) did not result in a significant BRET decrease or increase (data not shown). The constitutive BRET ratio measured between the inactive receptor and the G protein (Fig. 5b) suggests a pre-coupled Ste2p–Gpa1p complex rather than a random molecular collisions model. The unspecific BRET due to the random molecular collisions is highly unlikely within the limits of cellular concentrations, still to overrule the possibility of BRET which might occur through random molecular collisions in cell, a negative control was used. Cells coexpressing Ste2p[RLuc] and Gpr1p[EGFP], the glucose sensing GPCR in yeast, were used in BRET experiments and no detectable BRET ratio was measured between these two membrane proteins (Supplementary Fig. S5).

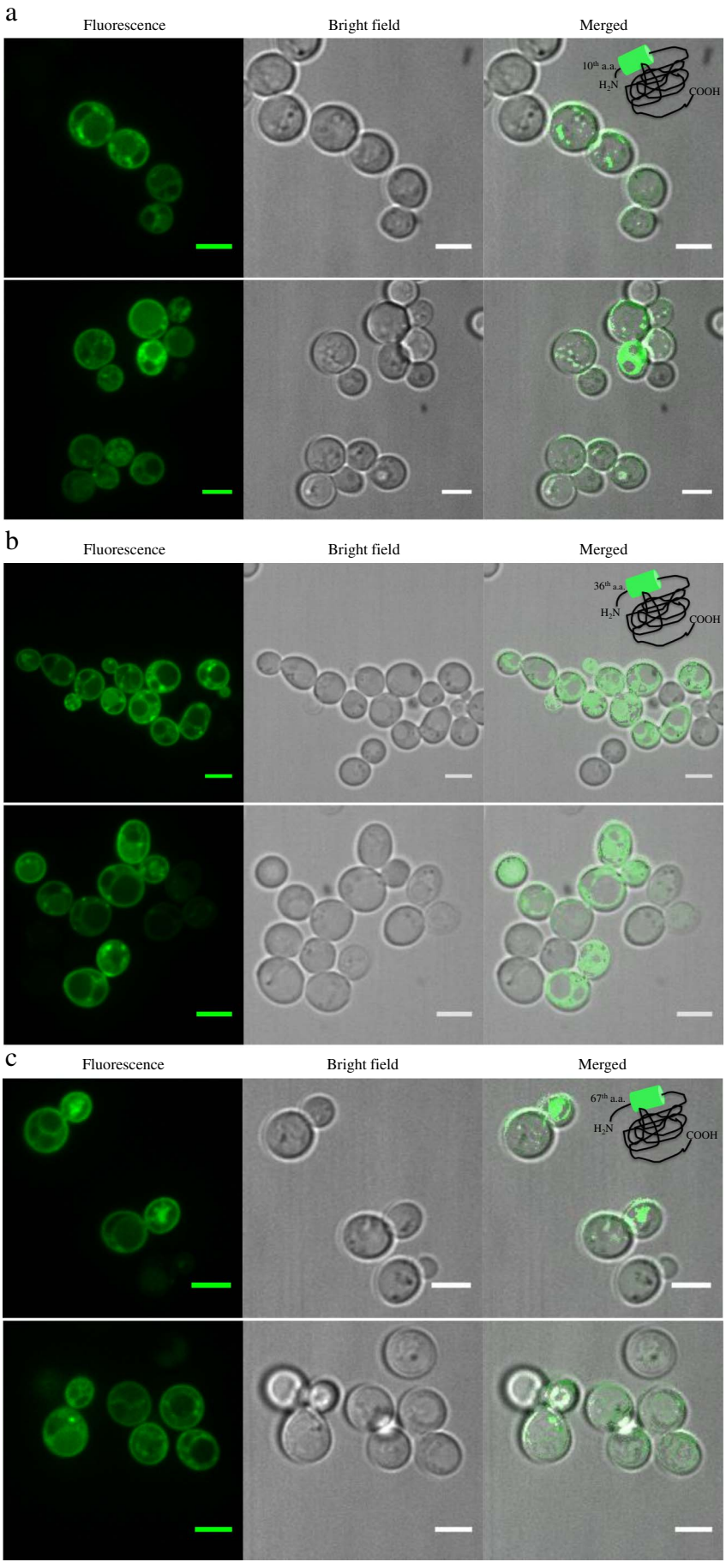

Fig. 3. Cells expressing EGFP tagged Gpa1p. Two sets of representative images are given for each construct; scale bars correspond 5 μm length. (a) Shows cells expressing Gpa1p tagged with EGFP at position 10; Gpa1p[10-EGFP]. (b) Shows cells expressing Gpa1p tagged with EGFP at position 36; Gpa1p[36-EGFP]. (c) Shows cells expressing Gpa1p tagged with EGFP at position 67; Gpa1p[67-EGFP].

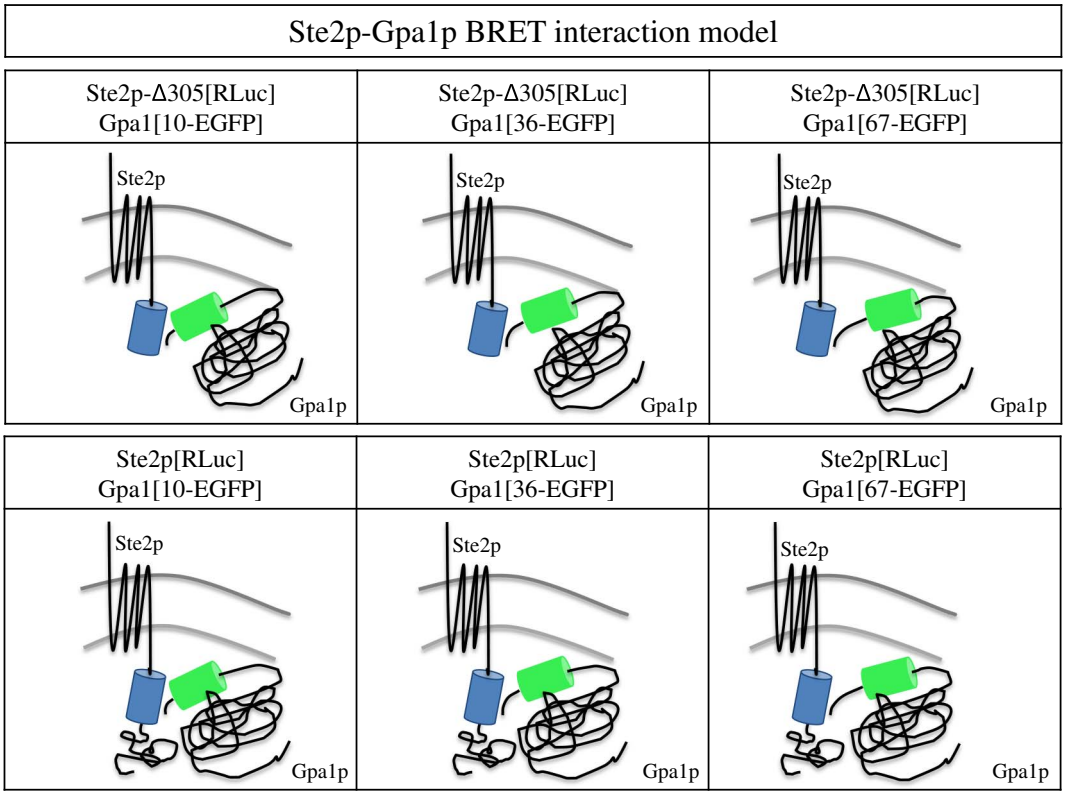

Fig. 4. The schematic representation of *Renilla luciferase* tagged Ste2p receptor–EGFP tagged Gpa1p G-Protein interaction model assayed with BRET. The first row of images shows the BRET model for C-terminally truncated Ste2p-Δ305[RLuc] and Gpa1p G-Protein tagged with EGFP at positions 10, 36 and 67. The second row of images shows the BRET model for full-length receptor, Ste2p [RLuc] and Gpa1p G-Protein tagged with EGFP at positions 10, 36 and 67.

3.5. Imaging the BiFC heterodimerization signal between Ste2p[C-EGFP] and Gpa1p[10-N-EGFP]

The BRET experiments indicated that the first residues on N-terminal domain of Gpa1p are in close proximity of the C-terminus of Ste2p. To investigate the *in vivo* location of heterodimerization, yeast transformants coexpressing the C-EGFP tagged Ste2p and N-EGFP tagged Gpa1p were imaged as described in the Methods and two sets of representative images for each condition are presented. The BiFC heterodimerization signal was observed in the absence and presence of α-factor, both for C-terminally truncated Ste2p receptors (represented in Fig. 6, Fig. 7a and b, for additional images see Supplementary Fig. S6a and b) and full-length receptors (represented in Fig. 6, Fig. 7c and d, for additional images see Supplementary Fig. S6c and d). The BiFC signal observed from the C-terminally truncated receptor; Ste2p-Δ305[C-EGFP]–Gpa1p[10-N-EGFP] heterodimer was mainly on the plasma membrane but EGFP signal was also detectable intracellularly (Fig. 7a). When these cells were treated with α-factor, the BiFC signal was detected both on the PM and intracellularly (Fig. 7b). The BiFC signal from full-length receptors; Ste2p[C-EGFP] and Gpa1p[10-N-EGFP] was observed on the PM and as brighter fluorescent regions intracellularly (Fig. 7c). When these cells were treated with α-factor, an increase in the intracellular fluorescent puncta presumed to be the endocytic vesicles was observed (Fig. 7d).

To assess the location of intracellular BiFC signal, cells expressing DsRed-HDEL ER marker was used. The colocalization of the Ste2p-Gpa1p heterodimer (EGFP signal) and ER marker (DsRed signal) was observed both from cells expressing C-terminally truncated (Fig. 8a, for additional images see Supplementary Fig. S7a) or full-length receptor (Fig. 8b, for additional images see Supplementary Fig. S7b) and Gpa1p.

4. Discussion

Previous work investigating GPCR Gα subunit interactions including x-ray studies could not resolve extreme N-terminal domain of Gα subunit. The work presented in this study highlights this region, and investigates the spatial proximity of first residues of the N-terminal domain of Gpa1p a G protein alpha subunit to the C-terminal cytoplasmic domain of Ste2p which is a model GPCR. This study also reports the location of the heterodimerization between Ste2p and Gpa1p using BiFC which is a novel biophysical method developed to study protein-protein interactions.

In agreement with the previous literature [56] [37, 38, 50] and the functional assays reported herein, tagging Ste2p with RLuc or C-EGFP and tagging Gpa1p with EGFP or N-EGFP fragment at reported positions on the N-terminus did not alter the function of these proteins (for the constructs Table 1 and Fig. 1, for data see Fig. 2a and b). The Gpa1p-EGFP constructs were imaged *in vivo* and the location of fluorescence signal was observed intracellularly and on the PM as expected (Fig. 3a–c). Altogether, these results suggest that Ste2p and Gpa1p constructs represent wild type protein function and dynamics.

It was previously reported that C-terminal cytoplasmic domain of Ste2p promoted the formation of receptor–G protein complexes [57]. To assess the effect of C-tail on the Ste2p–Gpa1p heterodimerization, Gpa1p EGFP constructs were coexpressed with C-terminally truncated receptor (Ste2p-Δ305[RLuc]) and with receptors having an intact C-tail (Ste2p[RLuc]) (Fig. 4 for schematic presentation of constructs). A constitutive BRET was observed between C-terminally truncated receptor and Gpa1p (Ste2p-Δ305[RLuc]–Gpa1p[10-EGFP]), and full-length receptor and Gpa1p (Ste2p[RLuc]305–431–Gpa1p[10-EGFP]). The BRET ratio measured from full-length receptor and Gpa1p (Ste2p [RLuc]–Gpa1p[10-EGFP]) was > 30% higher than the BRET ratio measured from truncated receptor and Gpa1p (Ste2p-Δ305[RLuc]–Gpa1p[10-EGFP]) (Fig. 5b). This increase in BRET ratio

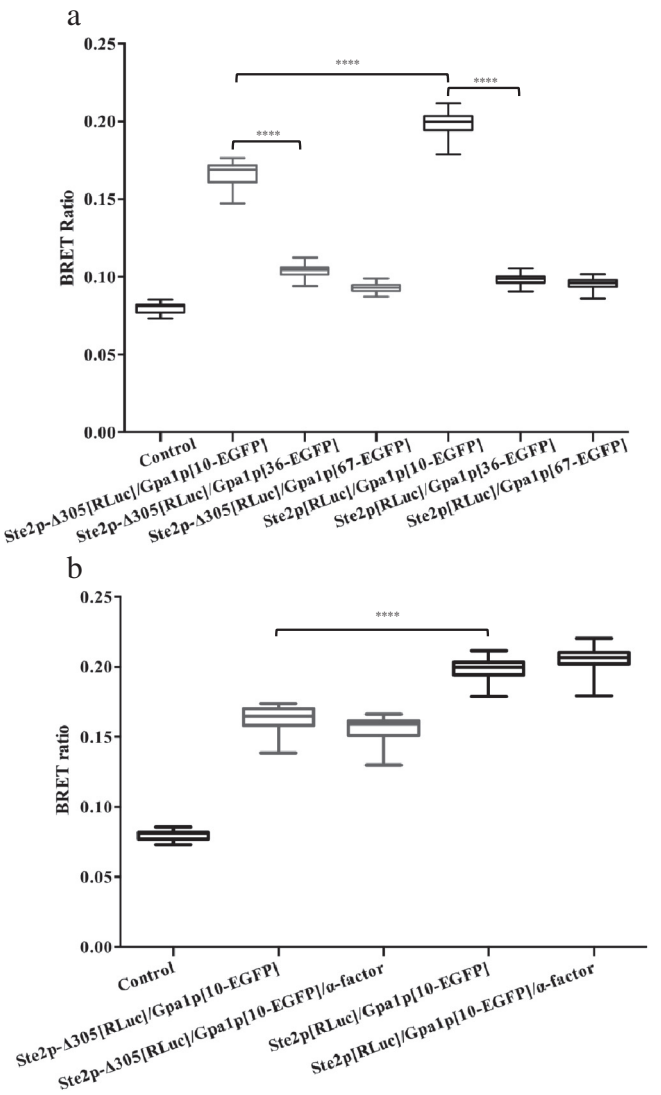

Fig. 5. BRET ratios for cells expression Ste2p[RLuc]–Gpa1p[EGFP] constructs, one-way ANOVA test was applied to data. (a) BRET ratios for cells expressing Ste2p[RLuc] and various Gpa1p[EGFP] constructs carrying EGFP at positions 10, 36 and 67. First column shows the background BRET ratio from the cells only expressing RLuc tagged Ste2p. Columns 2, 3 and 4 represent the BRET ratio between C-terminally truncated Ste2p-Δ305[RLuc] and Gpa1p tagged with EGFP at positions 10, 36 and 67 respectively. Columns 5, 6 and 7 show the BRET ratio between Ste2p[RLuc] and Gpa1p tagged with EGFP at positions 10, 36 and 67 respectively. (b) First column shows the background BRET ratio measured using cells only expressing RLuc tagged Ste2p, in the absence of a BRET acceptor. Second and third columns show the heterodimerization of C-terminally truncated receptor; Ste2p-Δ305 with Gpa1p tagged with EGFP between positions 10–11; second column shows untreated cells, whereas the third column shows cells treated with α-factor. Fourth and fifth columns show the heterodimerization of Ste2p receptor with Gpa1p tagged with EGFP between positions 10–11, the fourth column represents the BRET ratio of untreated cells and fifth column represents the BRET ratio for α-factor treated cells.

suggests that the C-terminal cytosolic domain on Ste2p significantly contributes to the receptor–Gα interaction, either by directly stabilizing the heterodimer or creating domains for other proteins which might interact with the receptor C-tail and improve the heterodimerization. Sst2p, the regulator protein for G protein signaling, was previously reported to carry a N-terminal DEP domain, which binds to the cytosolic tail of Ste2p [58] for PM delivery and places the C-terminal RGS domain of Sst2p in a position to stimulate the catalysis of GTP to GDP on Gpa1p [59]. Therefore, binding of Sst2p or such effector proteins on the Ste2p C-tail might be a reason for the measured Ste2p–Gpa1p heterodimer stabilization. Further experiments are necessary to resolve whether the stabilizing effect of the C-tail is direct or through other proteins.

The spatial proximity of Gpa1p N-terminus in the receptor–G protein complex, was assessed using BRET with Gpa1p tagged with EGFP at three positions on the N-terminus. The BRET ratio significantly decreased as the position of EGFP moved from 10th amino acid to further positions, such as 36th and 67th residues on the Gpa1p N-terminus. BRET ratio was still detectable from the Gpa1p constructs tagged with EGFP between residues 36–37 and 67–68. BRET as a resonance energy transfer technique, is very much sensitive to the distance between the donor molecule and the acceptor molecule, therefore the low but detectable BRET can be interpreted as EGFP on Gpa1p[10-EGFP] construct is the closest to the Rluc on receptor C-tail, whereas the distance between the EGFP and the receptor increases as EGFP moves to 36th and to 67th positions. These results suggest that, the extreme N-terminus of Gpa1p is in close proximity with Ste2p receptor. Although such energy transfer methods are indicative of specific molecular interaction and does not occur randomly within the limits of cellular concentration of proteins, a negative control was also devised using Ste2p Rluc constructs and Gpr1[EGFP], yeast glucose sensing receptor. There was no BRET detected between these two membrane proteins (Supplementary Fig. S5).

Although, the signal transduction through GPCR activation was previously believed to occur with subunit dissociation of the heterotrimeric G proteins [60, 61], numerous recent studies shed light on a different mechanism involving molecular rearrangements rather than a distinctive molecular dissociation. The activation of G Proteins was even shown to occur at different intracellular organelles, such as Golgi [62] and such activations were also shown to play important roles in cells [63, 64]. Using CFP tagged Gpa1p (Gα) at an identical position reported herein and YFP tagged Ste18p (Gγ) in yeast, Yi *et al.* reported not a loss but a decrease in FRET efficiency between Gpa1p-CFP and Ste18p-YFP upon the activation of the receptors [37]. In a similar study, measuring the FRET between YFP tagged rat $G\alpha_{i1}$ and Gβγ tagged with CFP at various positions, Bunemann et al. showed that the G protein activation involves a subunit rearrangement rather than a molecular dissociation [65]. It was also reported that Ste4p (Gβ)–Gpa1p fusion protein transmitted the pheromone signal and activated the mating pathway as effectively as when Ste4p and Gpa1p were coexpressed as separate proteins [66]. In this study, the effect of agonist on the GPCR–Gα interaction was evaluated by treating the yeast cells with α-factor. The results suggest that, stimulation of Ste2p receptors with their ligand, resulted no significant BRET decrease or increase between the Ste2p C-terminal and Gpa1p N-terminal domains (Fig. 5b).

Based on recent literature and the results of this study, within the limits of BRET dynamics, we propose that the transduction of receptor activation to its cognate Gα subunit does not occur through molecular dissociation, the Gpa1p N-terminal domain continues to interact with the Ste2p C-terminal domain during pheromone-induced receptor signaling. The constitutive BRET measured suggests that the Ste2p and Gpa1p heterodimer exists as a pre-coupled protein complex even in their quiescent (non-ligand activated) state and also after receptor stimulation. A molecular rearrangement might occur elsewhere on Gpa1p, probably close to the Gα C-terminus, as Gpa1p(Q323L) mutant was reported to fail GTP hydrolysis and permanently activates the pheromone response pathway [67]. Also, various studies showed that C-terminal domain of Gα is another contact site with the receptor [18–21].

To visualize the pre-coupled receptor–Gα interaction and the location of this heterodimer, yeast cells coexpressing Ste2p tagged with C-EGFP on C-tail and Gpa1p tagged with N-EGFP between residues 10–11 were imaged *in vivo* using a confocal microscope. The fluorescence signal observed from these cells was generated through the reassembly of EGFP fragments due to the heterodimerization of receptor with its Gα subunit. In the BiFC experiments, yeast cells coexpressing a C-terminally truncated receptor construct Ste2p-Δ305[C-EGFP] and

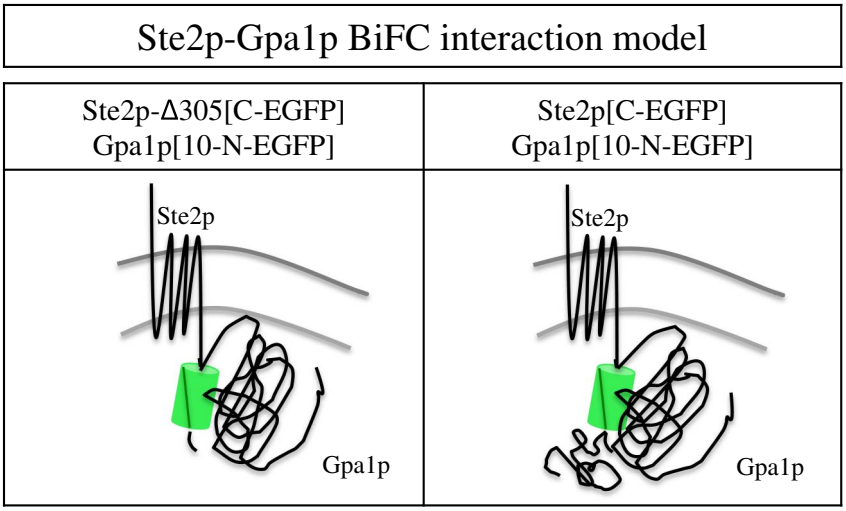

Fig. 6. The schematic representation of interaction model for C-terminally truncated and full-length Ste2p receptor tagged with C-EGFP (159–238) at 304th and Gpa1p tagged with N-EGFP (1–158) at 10th position in BiFC technique.

Gpa1p[10-N-EGFP] were imaged *in vivo*, the fluorescence signal was observed mainly on the PM and as distinct fluorescent regions intracellularly [68–70] (Fig. 7a, for additional images see Supplementary Fig. S6a). Colocalization of BiFC signal with ER marked with DsRed-HDEL suggests that the interaction between Ste2p and Gpa1p occurs on the ER during biosynthesis before reaching the PM (Fig. 8a, for

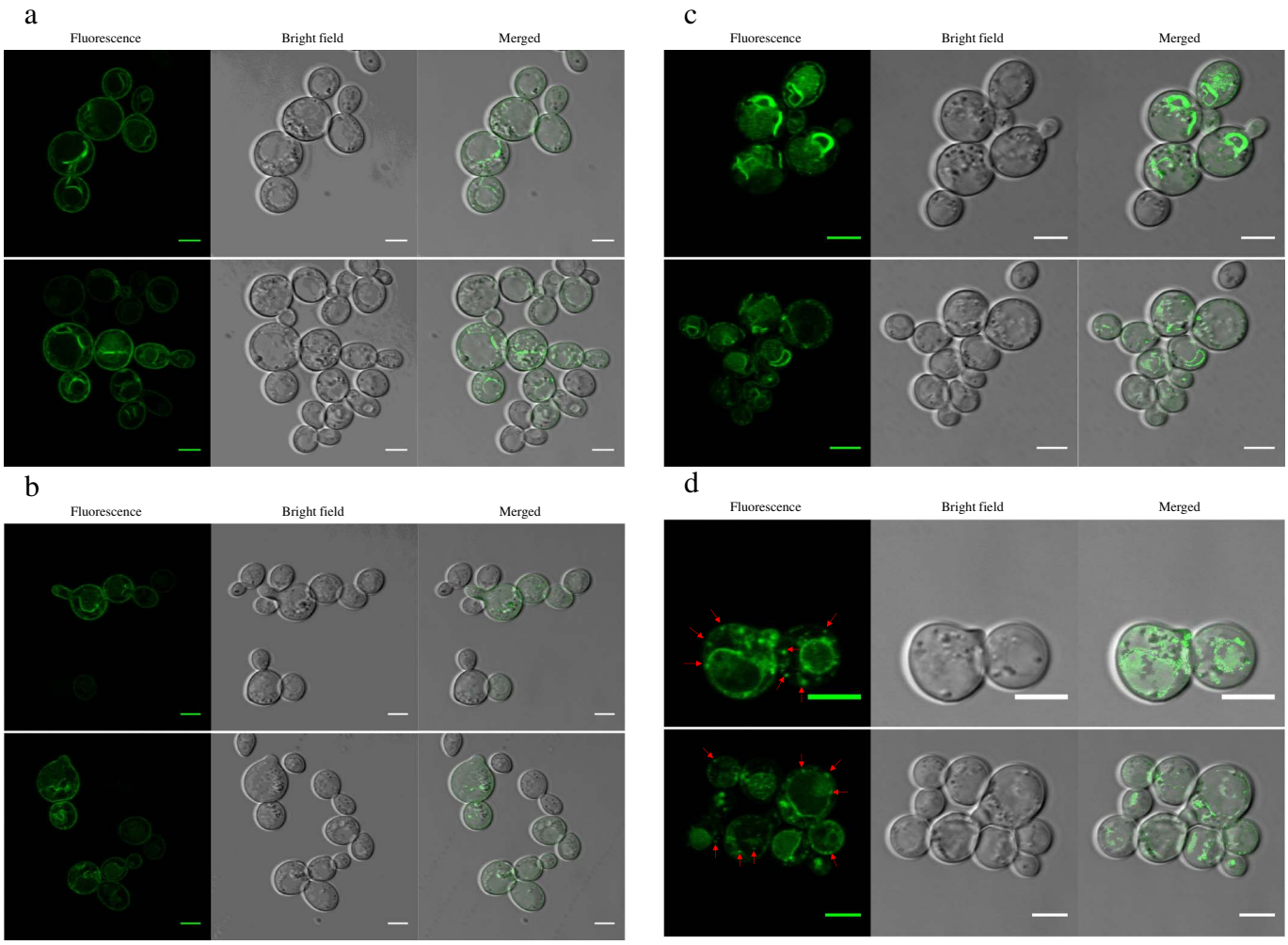

Fig 7. Cells coexpressing Ste2p tagged with C-EGFP and Gpa1p tagged with N-EGFP. Two sets of representative images are given for each construct, scale bars correspond 5 μm. (a) Shows cells coexpressing Ste2p-Δ305[C-EGFP]/Gpa1p[N-EGFP], a C-terminally truncated Ste2p is tagged with C-EGFP at position 304; on Gpa1p, N-EGFP is inserted between positions 10–11. (b) Shows cells coexpressing Ste2p-Δ305[C-EGFP]/Gpa1p[N-EGFP] treated with α-factor. (c) Shows cells coexpressing Ste2p[C-EGFP]/Gpa1p[N-EGFP], full-length Ste2p is tagged with C-EGFP between positions 304–305; on Gpa1p N-EGFP is inserted between positions 10–11. (d) Shows cells coexpressing Ste2p[C-EGFP]/Gpa1p[N-EGFP] treated with α-factor, arrows show the endocytotic vesicles.

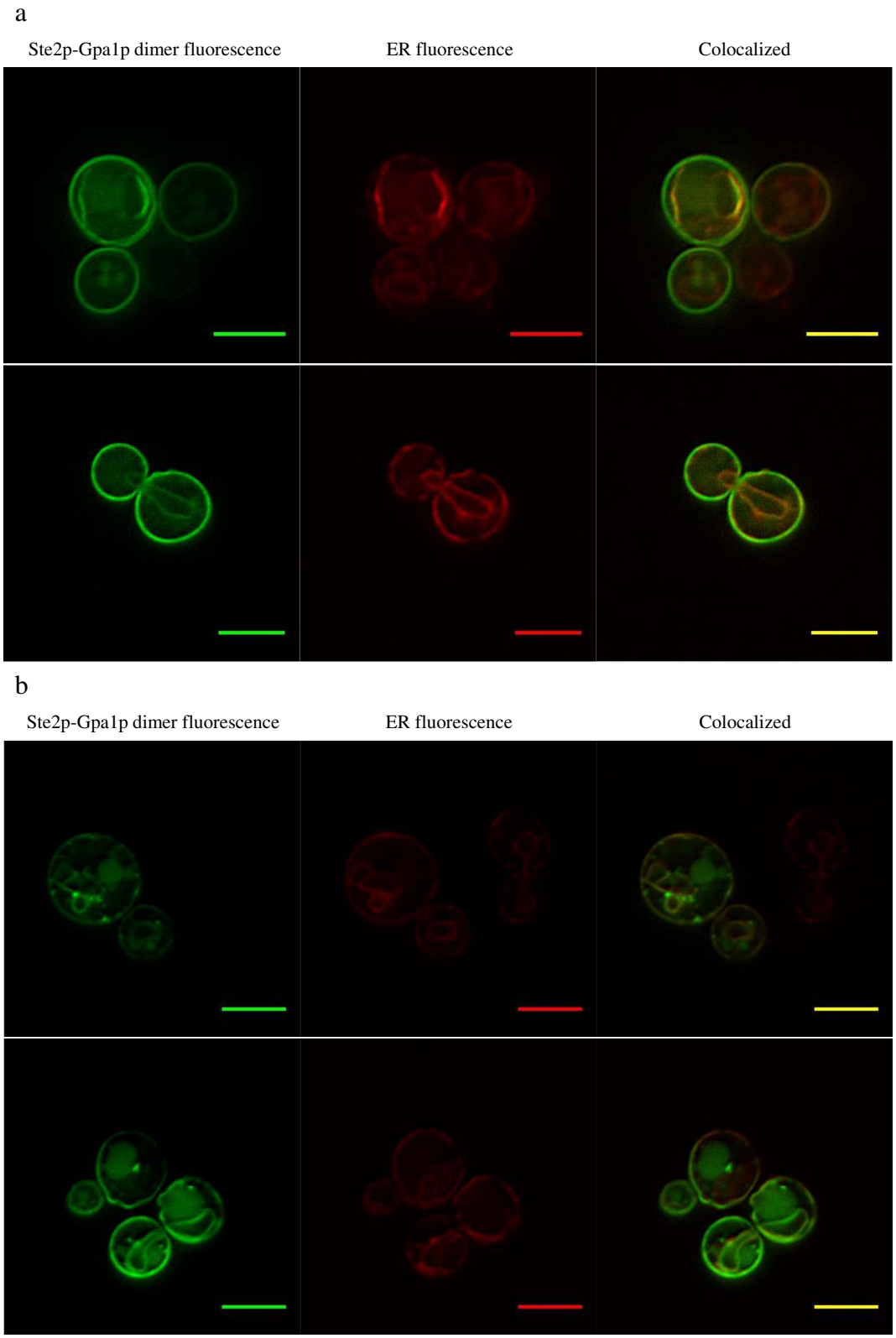

Fig. 8. Cells coexpressing Ste2p tagged with C-EGFP and Gpa1p tagged with N-EGFP and ER marker DsRed-HDEL. Two sets of representative images are given for each construct; scale bars correspond 5 μm. (a) From left to right, first image shows cells coexpressing Ste2p-Δ305[C-EGFP]/Gpa1p[N-EGFP], the second image shows cells expressing DsRed-HDEL and the last image shows the colocalized Ste2p-Gpa1p heterodimer signal and ER marker signal. (b) Shows cells coexpressing Ste2p[C-EGFP]/Gpa1p[N-EGFP], the second image shows cells expressing DsRed-HDEL and the last image shows the colocalized Ste2p-Gpa1p heterodimer signal and ER marker signal.

additional images see Supplementary Fig. S7a). When these cells were treated with α-factor the heterodimerization was still detectable both on the PM and intracellularly suggesting that the receptor and the Gα remains together (Fig. 7b, for additional images see Supplementary Fig. S6b).

In contrast to the C-terminally truncated receptor–Gα heterodimer (Fig. 7a), cells coexpressing full-length receptor construct Ste2p[C-EGFP] and Gpa1p[10-N-EGFP] had brighter cytosolic fluorescence on ER and intracellular fluorescent puncta presumed to be the endocytotic vesicles (Fig. 7c, for additional images see Supplementary Fig. S6c). Furthermore, when the cells coexpressing GPCR–G protein heterodimer were treated with α-factor, the intracellular puncta resembling the endocytotic vesicles increased, which agrees with the down regulation of the receptor–Gα heterodimer after the receptor stimulation through endocytosis (Fig. 7d, red arrows, for additional images see Supplementary Fig. S6d).

In agreement with the BRET results and as a further evidence for the proposed pre-coupled receptor–G protein model, BiFC experiments also resulted a constitutive heterodimerization signal between Ste2p–Gpa1p heterodimer in their quiescent (non-ligand activated) state (Fig. 7a, c). The BiFC images both from C-terminally truncated Ste2p–Gpa1p and full length Ste2p–Gpa1p suggests that Ste2p couples with Gpa1p before reaching the PM, during biosynthesis on the ER (Fig. 8a, b). Previous studies reported that endocytosis of Ste2p is independent of G protein mediated signal transduction [71] but significantly increases upon treatment with α-factor [72]. Furthermore, the internalization of Ste2p tracked by reporter genes [73–75] showed its localization on PM and transport to the vacuole for degradation through endocytotic vesicles and Gpa1p was also shown to be present in endosomes [76]. These previous studies and the results reported herein, suggests that the intracellular fluorescent puncta most likely originate from endocytotic vesicles containing Ste2p[C-EGFP] and Gpa1p[10-N-EGFP] heterodimer. Increase of these puncta upon the treatment of cells with α-factor (Fig. 7d, red arrows) suggests the endocytosis of receptors as a heterodimeric complex with their cognate Gα.

5. Concluding remarks

Bioluminescence resonance energy transfer (BRET) was used to detect the interacting regions between yeast pheromone receptor Ste2p and its cognate Gα subunit Gpa1p. The constitutive and position-dependent BRET reported in this study suggests that Gpa1p extreme N-terminus is in close proximity (2–10 nm) with Ste2p C-terminus (around 304th residue) and the two proteins exist as a pre-coupled heterodimer in receptor quiescent (non-ligand activated) state. In addition, the stabilizing effect of C-tail of the Ste2p on the GPCR–G Protein complex was assessed using BRET. Furthermore, Bimolecular Fluorescence Complementation (BiFC) assay was used to detect the cellular location of Gpa1p - Ste2p heterodimerization by monitoring the generation of EGFP signal generated upon G Protein - GPCR heterodimerization and reassembly of the EGFP. The results suggest that Gpa1p–Ste2p heterodimerization occurs during biosynthesis on endoplasmic reticulum before trafficking to the plasma membrane. Upon the activation of the receptors with α-factor, no significant change in BRET or loss of fluorescence in BiFC was observed. Altogether, these findings strongly indicate that a molecular rearrangement occurs between G Protein and GPCR rather than a dissociation.

Further experiments are being conducted to resolve whether the contribution of the C-tail to heterodimer structure is through directly stabilization of the interaction between receptor and G proteins or by creating domains for other proteins such as Sst2p to interact with the receptor C-tail and/or G protein for stabilization. In addition, the quantitative BRET assay optimized in this study is being used to determine importance of specific C-terminal residues of Ste2p on Ste2p-Gpa1p interaction.

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Appendix A. Supplementary data

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