DETECTING G-PROTEIN COUPLED RECEPTOR INTERACTIONS USING ENHANCED GREEN FLUORESCENT PROTEIN REASSEMBLY

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ABSTRACT

DETECTING G-PROTEIN COUPLED RECEPTOR INTERACTIONS USING ENHANCED GREEN FLUORESCENT PROTEIN REASSEMBLY

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The largest class of cell surface receptors in mammalian genomes is the superfamily of G protein-coupled receptors (GPCRs) which are activated by a wide range of extracellular responses such as hormones, pheromones, odorants, and neurotransmitters. Drugs which have therapeutic effects on a wide range of diseases are act on GPCRs. In contrast to traditional idea, it is recently getting accepted that G-protein coupled receptors can form homo- and hetero-dimers and this interaction could have important role on maturation, internalization, function or/and pharmacology.

Bimolecular fluorescence complementation technique (BiFC); is an innovative approach based on the reassembly of protein fragments which directly report interactions. In our study we implemented this technique for detecting and visualizing the GPCR interactions in yeast cells. The enhanced green fluorescent protein (EGFP) fractionated into two fragments at genetic level which does not possess fluorescent function. The target proteins which are going to be tested in terms of interaction are modified with the non-functional fragments, to produce the fusion proteins. The interaction between two target proteins, in this study Ste2p receptors which are alpha pheromone receptors from *Saccharomyces cerevisiae*, enable the fragments to come in a close proximity and reassemble. After reassembly, EGFP regains its fluorescent function which provides a direct read-out for the detection of interaction.

Further studies are required to determine subcellular localization of the interaction. Moreover, by using the fusion protein partners constructed in this study, effects of agonist/antagonist binding and post-translational modifications such as glycosylation and phosphorylation can be examined. Apart from all, optimized conditions for BiFC technique will guide for revealing new protein-protein interactions.

Keywords: Split EGFP, Bimolecular fluorescence complementation technique (BiFC), GPCR dimerization, Ste2p

ÖZ

GELİŞTİRMİŞ YEŞİL FLORESAN PROTEİNİN YENİDEN BİRLEŞMESİ KULLANILARAK G PROTEİNE KENETLİ RESEPTÖRLERİN EŞLEŞMESİNİN TESPİTİ

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Memeli genomunda en geniş sınıfı kapsayan hücre yüzeyi reseptörleri olan G proteinine kenetli reseptör (GPKR) süperfamilyası hormon,feromon, koku ve nörotransmiter gibi çok sayıda hücre dışı faktör tarafından aktive edilmektedir. Birçok hastalık üzerinde tedavi edici etkisi olan ilaçlar GPKRL'lere etki etmektedir. Geleneksel düşüncenin aksine, son zamanlarda G proteinine kenetli reseptörlerin homo- ve hetero-dimerler oluşturduğu ve bu etkileşimin, reseptörün olgunlaşması, internalizasyonu, fonksiyonu ve/veya farmakolojisi üzerinde etkili olduğu kabul edilmektedir.

Biyomoleküler floresan tamamlama tekniği (BIFC); protein parçalarının tekrar bir araya gelmesine dayanarak moleküller arası etkileşimleri doğrudan gösteren yenilikçi bir yaklaşımdır. Çalışmamızda bu teknik, maya hücrelerinde GPKR etkileşimlerini belirlemek ve görüntülemek için kullanılmıştır. Geliştirilmiş yeşil floresan proteini floresan özelliği olmayan iki parçaya bölünerek test edilecek olan proteinlere eklenmiştir. İki hedef reseptör arasındaki etkileşim, bu çalışma için *Saccharomyces cerevisiae'da* alfa feromon reseptörü olan Ste2p, floresan protein parçalarının birbirine yaklaşarak yeniden bir araya gelmelerini sağlar. Geliştirilmiş yeşil floresan proteininin birleşmesiyle kazanılan floresan özellik, etkileşimin doğrudan belirlenmesini mümkün kılar.

Etkileşimin hücre içi lokalizasyonunun belirlenmesi için ileride yapılacak çalışmalara gerek duyulmaktadır. Ayrıca bu çalışmada oluşturulmuş olan birleştirilmiş protein partnerleri kullanılarak agonist/antagonist bağlanması ve fosforilasyon, glikozilasyon gibi translasyon sonrası modifikasyonlar belirlenebilir. Bunların dışında, BIFC tekniği için optimize edilen koşullar yeni protein-protein etkileşimlerini ortaya çıkarmak için kolaylık sağlayacaktır.

Anahtar sözcükler: Bölünmüş EGFP, Biyomoleküler floresan tamamlama tekniği(BiFC), GPKR dimerizasyonu, Ste2p

To my family,

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6'-GNTI	6'-guanidinonaltrindole
AFM	atomic force microscopy
Bar	Barrier (to α-factor diffusion)
Bem	Bud emergence
BiFC	Bimolecular Fluorescence Complementation
Bp	base pair
BRET	Bioluminescence Resonance Energy Transfer
BSA	Bovine Serum Albumin
cAMP	cyclic AMP
Cdc	Cell division control
DHFR	Dihydrofolate reductase
DHFR	dihydrofolate reductase
Dig	Down-regulator of invasive growth
DLS	dynamic light scattering
DNA	Deoxyribonucleic Acid
EDTA	Ethylenediamine tetraacetic acid
EGFP	Enhanced Green Florescent Protein
EL	Extracellular lopp
ER	Endoplasmic Reticulum
Far	Factor arrest
FRET	Fluorescence/Förster Resonance Energy Transfer
GDP	Guanosine Diphosphate
GFP	Green Fluorescence Protein
G_i	Inhibitory G_α subunit
G_{olf}	Olfactory G_α subunit
Gpa1	G-protein alpha subunit
G_s	Stimulatory G_α subunit

GTP	Guanosine Triphosphate
IL	Intracellular loop
kb	Kilobase pair
Kss1	Kinase-supressor of Sst2
LB	Luria Bertani
MAP	Mitogen Activated Protein
MLT	Media lack of Tryptophane
MLTU	Media lack of Tryptophane and Uracil
MLU	Media lack of Uracil
Msg5	Multicopy suppressor of *GPA1* deletion
PAK	p21-activated protein kinase
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
Ptp	Protein tyrosine phosphatese
RE	Restriction Enzyme
Rpm	Revolution per Minute
SDS-PAGE	Sodium dodecyl sulfate-polyacrylamide gel electrophoresis
Sst	Supersensitive
Ste	Sterile
Taq	*Thermus aquaticus*
TBE	Tris Borate EDTA
TM	Transmembrane
UV	Ultraviolet
YEPD	yeast extract-peptone-dextrose

CHAPTER 1

INTRODUCTION

1.1 G Protein-coupled Receptors: Structure, Function and Importance

G protein-coupled receptors (GPCRs) are one of the largest cell surface receptor families. Considering the role of these receptors in sensing extracellular signals in eukaryotic organisms, GPCR receptor family is one of the most important sensory systems which are able to detect and transmit a large scale of extracellular chemical (hormones, neurotransmitters, chemoattractants, calcium ions and pain killers) and sensory (light, odorants and taste molecules) signals into cells (Gurevich & Gurevich, 2008). The importance of understanding the mechanisms of GPCR signal trunsduction pathways can be summarized in several main points. Firstly, GPCRs are the targets of ~50% of pharmaceuticals on the market and are the main center of interest of the pharmaceutical industry (Gonzalez-Maeso, 2011). Mutations and misfunction of these receptors are also linked to many human diseases (Vilardaga, Agnati, Fuxe, & Clruela, 2010) such as diabetes insipidus and mellitus, hypercalcemia, obesity, hypertension, cancer, hypothyroidism, retinitis pigmentosa and psychotic disorders. In addition, pathways involving GPCRs were used to control virulence mechanism of fungal pathogens both for human and plant (Hoffman, 2005).

Table 1.1 Some prescription drugs acting via G-protein coupled receptors (Veulens & Rodríguez, 2009).

Drug	API	Company	Disease	Receptor
Claritin	loratadine	Schering-Plough	allergies	H1 antagonist
Zyprexa	olanzapine	Eli LillyTatemoto	schizophrenia	mixed D2/D1/5-HT2
Cozaar	losartan	Merk & Co	hypertension	AT1 antagonist
Risperdal	risperidone	Johnson & Johnson	psychosis	mixed D2/5-HT2A
Leuplin/Lupron	leuprolide	Takeda	cancer	LH-RH agonist
Neurontin	gabapentin	Pfizer	neurogenic pain	GABA B agonist
Allegra/Telfast	fexofenadine	Aventis	allergies	H1 antagonist
Imigran/Imitex	sumatriptan	GlaxoSmithKline	migrane	5HT1 agonist
Serevent	salmaterol	GlaxoSmithKline	asthma	β2 agonist
Zantac	ranitidine	GlaxoSmithKline	ulcers	H2 antagonist
Pepcidine	famotidine	Merk & Co	ulcers	H2 antagonist
Zofran	ondansetron	GlaxoSmithKline	antiemetic	5-HT3 antagonist
Dovan	valsartan	Novartis	hypertension	AT1 antagonist
Duragesic	fentanyl	Johnson & Johnson	pain	opioid agonist

Despite revealing a wide diversity in primary sequence and function, all G-protein coupled receptors (GPCR) share some characteristic structural features such as alpha-helical seven-transmembrane domains, C-terminal domain is always in cytosol and N-terminal domain in extracellular region (Chezerov, Abola, & Stevens, 2010). An extracellular agonist 'ligand' binding starts the signal transduction by the activation of receptor with a conformational change. The switch of the receptor from an inactive state to an active state generates an intracellular response through activation of an associated G-protein. The inactive form of a G-protein consists of three heteromeric subunits which are Gα bound to GDP, Gβ and Gγ. Following the ligand binding to a GPCR, GDP is released and Gα subunit binds to GTP which leads to a conformational change of the subunit. As a consequence of this exchange, disassociation of Gβγ dimer occurs and both Gα and Gγβ subunits modulate the effector molecules (e.g.

adenylyl cyclases, phosphodiesterases, phospholipases) and ion channels either together or separately which in fact determined by signaling pathway. These ion channels regulate the generation and propogation of secondary messengers such as cAMP, cGMP, diacylglycerol or inositoltriphophate which coordinate cell function (Panetta & Greenwood, 2008). In addition G-protein independent signaling is also possible through binding to β-arrestins proteins (Lefkowitz & Shenoy, 2005). To complete the activation-inactivation cycle of G-protein, GTP bound Gα subunit hydrolyzes GTP to GDP by its intrinsic GTPase activity and re-associate with other subunits (Gβγ). For most Gα subunits GTPase activating proteins (GAPs) stimulate the intrinsic GTPase activity, and for some others RGS proteins (regulators of G-protein signaling) activate the GTPase activity (Hoffman, 2005; Panetta & Greenwood, 2008) . The schematic illustration of the cycle is shown below on Fig 1.1.

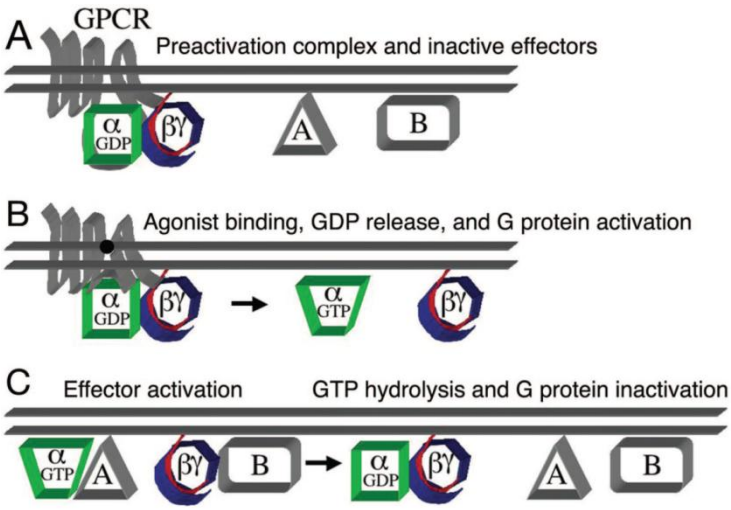

Figure 1.1 The G-protein activation-inactivation cycle (Hoffman, 2005). (A) Inactive G-protein prior to ligand binding. (B) Ligand binding to G-protein coupled receptor and activation of G-protein. (C) Transmission of signal to effector molecules and G-protein inactivation. (Green color indicates Gα, blue color indicates Gβ and red color indicates Gγ; A and B are downstream effectors)

1.2 Yeast GPCRs and Ste2p receptors as a model

In *Saccaromyces cerevisiae* (also known as budding yeast or baker's yeast), the number of the identified G-protein coupled receptors are only three as opposed to human genome which encodes nearly 1000 GPCRs (Fredriksson & Schioth, 2005). In yeast, pheromone and glucose signaling both occur through the action of two different GPCR systems.

S.cerevisiae prefers to utilize glucose as a carbon source and the presence of glucose activates a shift to the fermentative state by stimulating cAMP synthesis. Glucose/cAMP pathway in *S.cervisiae* has been shown to be Ras-dependent pathway in which Ras is coupled to a adenylate cyclase (Toda *et al.*, 1985). Recent studies have shown that a GPCR system is also responsible for the activation of this pathway which possesses a glucose receptor; Gpa1 and Gα protein; Gpa2 as main components. (Kraakman *et al.*, 1999; Yun, Tamaki, Nakayama, Yamamoto, & Kumagai, 1998). The glucose receptor Gpa1 activates the Gpa2 but the exact role of Gpa2 protein in activation of adenylate cyclase is not known, whereas Ras is a direct activator of adenylate cyclase. Overall, activation of Gpa2 and Ras proteins as a result of two different specific signaling pathways is stimulating adenylate cyclase synthesis and therefore activating PKA (cAMP-dependent protein kinase) which ends up with pseudohyphal differentiation, loss of stress resistance (high osmolarity, salinity, heat, freezing, etc), storage of carbohydrates (trehalose and glycogen), stimulation of growth and lower life-span (Fig 1.2). In G-protein coupled signal transduction, glucose induced cAMP signaling inhibited by an RGS protein (regulators of G-protein signaling),Rgs2, which stimulate the GTPase activity of Gα protein could be indicated as a negative regulator. (Versele, Lemaire, & Thevelein, 2001). There has not been any Gβγ dimer identified yet; however, a recent study has found that Gpb1/Krh2 and Gpb2/Krh1 proteins bind to Gpa2 by acting together with Gpg1 protein which leads to the idea that those proteins could be proposed to serve as an Gβγ mimic even if they do not function parallel to the role of Gβγ (Harashima & Heitman, 2002)

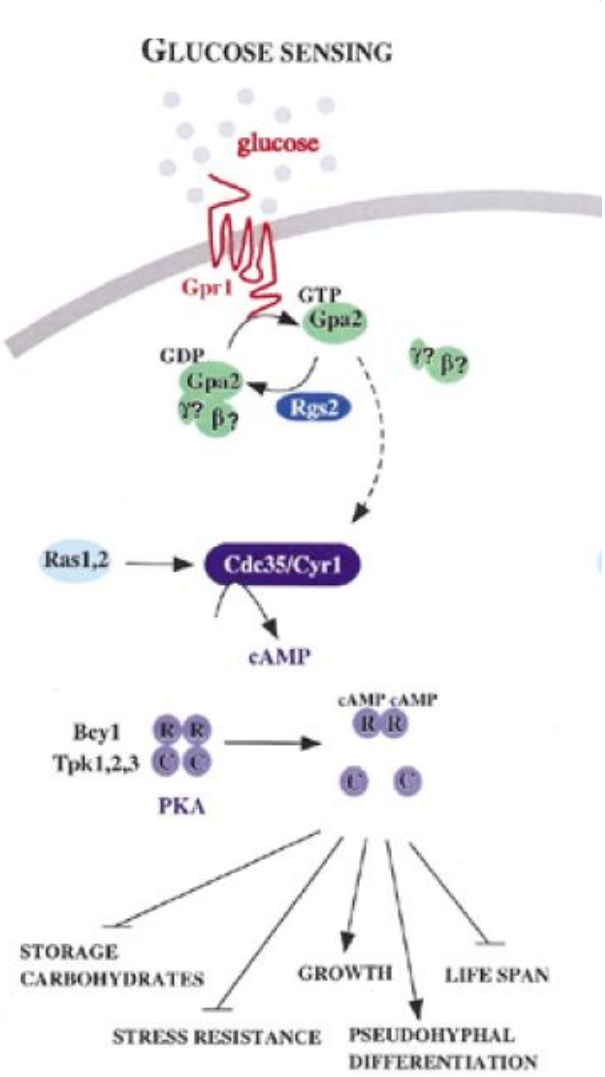

Figure 1.2 Glucose sensing and signaling in *Saccahormyces cerevisiae* (Versele *et al.*, 2001)

The pheromone response pathway of *S. cerevisia* is the other GPCR system which has been well identified and characterized with all major sensing and signaling components and targets. Moreover, many of the GPCR-mediated signaling elements in mammalian cells closely resemble the pathway (Pausch, 1997). Indeed, many of the important aspects and elements were firstly discovered in yeast such as RGS proteins, signaling

by Gβγ and mitogen activated protein kinase (MAPK) (Dohlman & Thorner, 2001). Therefore, the pheromone response pathway of *S. cerevisia* is used as a model system and plays an important role for studying GPCR systems *in vivo,* also understanding ligand-GPCR interactions, both in other fungi and animals and served as an exemplary tool which is utilized in the deorphanization of GPCRs.

Conserved cellular pathways in *S.cerevisiae* are a determining feature that makes this organism suitable to be used as recombinant expression system for mammalian proteins, beside low cost and simplicity. To characterize heterologous GPCRs, a bioassay has been developed based on pheromone response pathway. However, there have been several pre-requirements; firstly appropriate membrane expression of target GPCR should be obtained. Secondly, the target receptor should accurately couple with Gα protein (Gpa1) to activate downstream effectors and finally to monitor the expression of receptor and signal activation, a reporter gene is required. In addition to these requirements, optimization of the pathway might be needed to improve signal sensitivity (Panetta & Greenwood, 2008; Xue, Hsueh, & Heitman, 2008). In Table 1.2 examples of successfully expressed receptors are given.

Additionally, robust and fast growth, easy genetic manipulation, completely sequenced genome and simple requirements for nutrition are other benefits of the system that pheromone response pathway of *S.cerevisiae* could offer (Lee *et al.*, 2007).

Table 1.2 Examples of successful expression of heterologous GPCRs in yeast (adapted from (Xue, *et al.*, 2008))

Receptor	Species	G protein	Ligand
Ste2, Ste3	*S. cerevisiae*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. kluyveri*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. uvarum*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. bayanus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. castellii*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. kudriavzevii*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. mikatae*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. paradoxus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. pastorianus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. servazzii*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. unisporus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. zonatus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. exiguus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. dairenensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. rosinii*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. spencerorum*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. transvaalensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. telluris*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. turicensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. unibasalis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. zonatus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. exiguus*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. dairenensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. rosinii*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. spencerorum*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. transvaalensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. telluris*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. turicensis*	Gpa1	α-factor, **a**-factor
Ste2, Ste3	*S. unibasalis*	Gpa1	α-factor, **a**-factor

1.3 Pheromone response pathway

Saccaromyces cerevisiae could be found in different cell types which are the haploid mating types *MAT***a** or *MATα* and these haploid cells can mate to generate the diploid cell type *MAT***a**/*MATα*. Both haploid cells secrete peptide pheromones, namely, **a** and α factor, respectively (Dohlman, 2002). Pheromones are defined as “substances that mediate communication between individuals of the same species” and mating pheromones induce the subsequent mating response in haploid yeast cells by enabling communication between cells (Karlson & Luscher, 1959). These peptide pheromones are recognized by the G-protein coupled receptors which are located on the surface of opposite mating types. *S.cerevisiae* Ste2 pheromone receptor is expressed by *MAT***a** cells and recognizes α-factor pheromone, whereas Ste3 pheromone receptor is expressed by *MATα* cells and recognizes **a**-factor pheromone (Elion, 2000). The α-factor is originally translated as pre-protein which is composed of 165 amino acids. The

final state of the pheromone with 13 amino acids (WHWLGLKPGQPMY) is obtained by the maturation process that takes place in ER and golgi (Jones Jr. & Bennett, 2011). On the other hand, **a**-factor is composed of 12 amino acids (YIIKGVFWDPAC [Farnesyl]-OCH3) and for full activity, post-translational modification is required which occurs in cytoplasm (Naider & Becker, 2004). Peptide pheromone binding to Ste2p and Ste3p receptors triggers a conformational change in the extracellular regions and intermembrane domains that are transmitted to cytosolic domains. Although primary sequence of Ste2 and Ste3 pheromone receptors are not related, both activated receptors couple to the same G-protein that is composed of Gα (Gpa1p), Gβ (Ste4p) and Gγ (Ste18p) subunits (Lee, *et al.*, 2007). Mating starts with pheromone binding to Ste2/Ste3 receptor and upon ligand binding a conformational change occurs in receptor that promotes trimeric G-protein activation resulting in a change. GTP replaces GDP in subunit Gpa1 (Gα). It also results in disassociation of Ste4/Ste18 (Gβγ) from Gpa1, as explained earlier. Signal is transmitted with Ste4/Ste18 complex by binding three different effector molecules. These are Ste5 which is an adaptor and scaffold protein, Ste20 p21-activated protein kinase (PAK) and Far1 which is a MAPK substrate and also forms a complex with a guanine nucleotide exchange factor (GEF) namely Cdc24. With the phosphorylation of Ste20 signal is passed on Ste11. This autophosphorylation is facilitated by Cdc42 binding to Ste20 and also localizes the protein kinase to membrane. The function of second effector Ste5 is to bind both Gβ and Ste11 serving as an adaptor molecule with no catalytic activity. The binding site of Ste5 in Gβγ is in close proximity to Ste20 binding site. Hence, with the activation of Ste20 signal transition to Ste11 occurs. Ste11 is the first component of MAPK module termed as MAPKKK (MAPKK kinase) and starts a sequential activation through phosphorylation of the second module. Additionally, Ste50 binding occurs to aid Ste11 activation.

The second module is a MAPKK (MAPK kinase) called Ste7 which in turn activates two different MAPK namely Kss1 and Fus3 via phosphorylation again. During this transduction Ste5 protein utilized as a scaffold through binding to Ste7 and two different MAPKs for improving signal transmission among MAPK modules. Substrates

for the third and the last modules of the cascade are Ste12/Dig1/Dig2 complex and Far1 protein. The phosphorylation of Far1 protein is required for pheromone-induced cell cycle arrest. The cell division control is inactivated by binding of Far1 protein to Cdc28/Cln kinase. Ste12 is a DNA binding transcription factor which indeed regulates activation/repression of transcription of genes in response to pheromone whereas Dig1 and Dig2 proteins are the repressors of Ste12. The genes involving cell fusion (FIG1, FIG2, FUS1, FUS2, AGA1), regulation of pathway (SST2, GPA1, MSG5) and also genes which encodes some of the main components of pathway (FUS3, STE2, FAR1) could be given as examples. Kss1 is also a regulatory protein that is required for Ste12 repression. The un-phosphorylated Kss1 protein binds to Ste12 and represses the potential gene expression induction.

The third effector molecule is Far1/cdc24 complex where Far1 also functions as an adaptor molecule for cdc42 which brings cdc42 to plasma membrane. In plasma membrane cdc24 promotes GDP-GTP exchange in cdc42 which binds to a number of effectors involving the regulation of cell polarity and actin cytoskeleton besides the ste20. The formation of this Gγβ-Far1-Cdc24-Cdc42 complex is mainly necessary for polarized cell growth. Bem1 is another protein acting on actin cytoskeleton in polarized growth of cell. In the cell, this protein shows its function by interacting with several proteins including Ste5, Ste20, Cdc42, and Cdc24.

Phosphatases also take action throughout the pathway. For instance, Ptp2/Ptp3 tyrosine phosphatases and dual-specificity phosphatase Mgs5 control Ste12 phosphorylation by acting on Fus3 and Kss1. The whole process takes nearly 4 hours and consequences of pheromone-induced mating response can be summarized as follows: 1) expression of mating related genes 2) cell cycle arrest in G_1 phase 3) formation of elongated pear-shape extensions called shmoo and the structure that generated by asymmetrical growth in direction to mating partner is called mating projection and 4) cell fusion (see reviews (Bardwell, 2005; Elion, 2000; Xue, *et al.*, 2008)).

The illustration of the pathway that is explained above is given in Fig 1.3

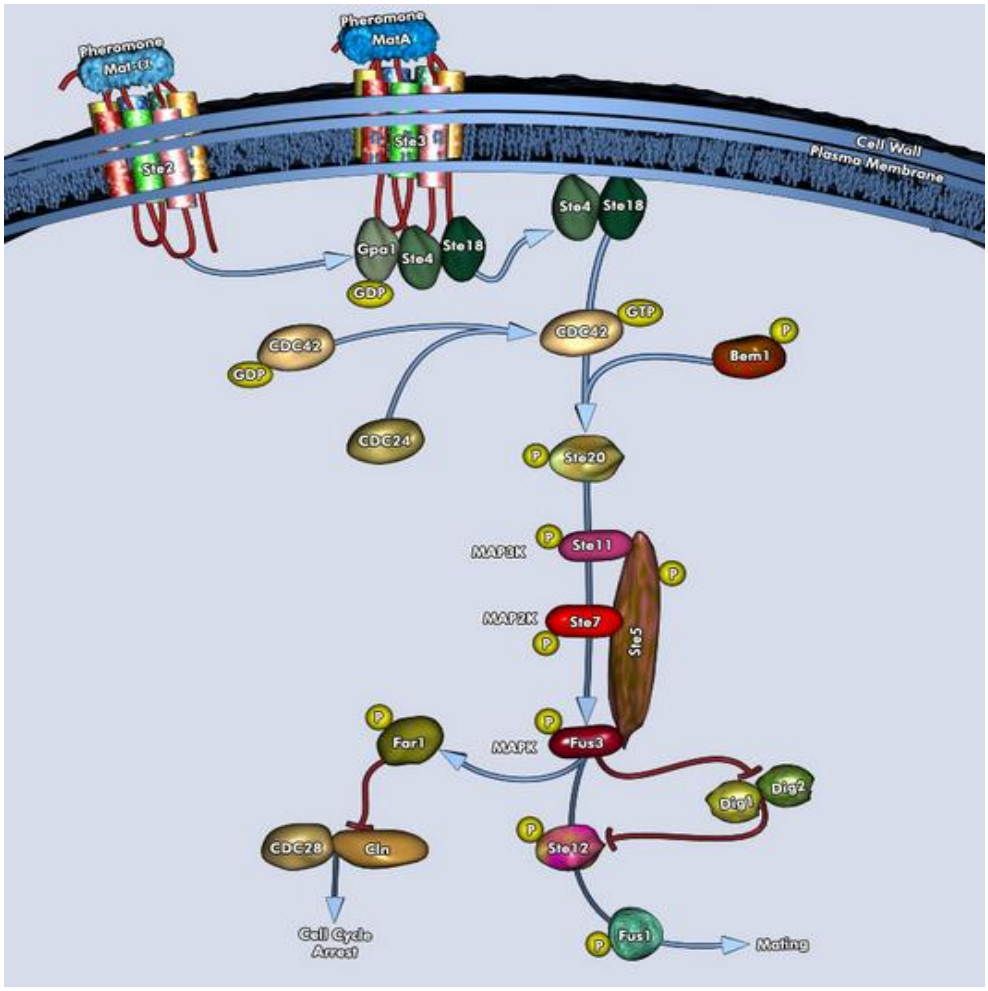

Figure 1.3 Mating-Pheromone Response Pathway in *S.cerevisiae* (© 2009 QIAGEN, all rights reserved)

Ste2p is one of the widely used model receptors which has similar structural properties with other GPCRs (Fig 1.4). C-terminal of the receptor contains phosphorylation and ubiquitination sites which are involved in desensitization and endocytosis of the receptor. It is also associated with heteromeric G-protein and stimulates downstream signaling with intracellular loop 3 (IC3). Extracellular loops (EC) 2 and 3 and multiple

sites in N-terminal of the receptor have a role in the recognition of α-factor with transmembrane domains (TM) 1,5 and 6. TM1, TM4 and TM7 are shown to be related with oligomerization of receptors (see review (Jones Jr. & Bennett, 2011)).

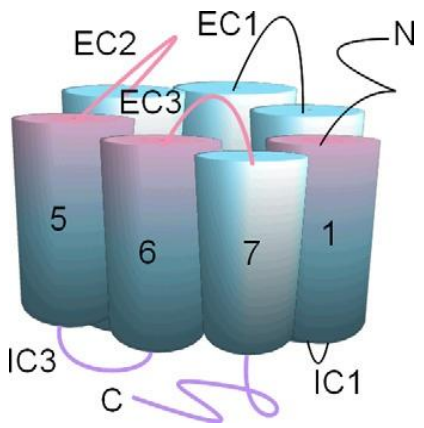

Figure 1.4 Model structure of Ste2 pheromone receptor in Saccharomyces cerevisia (Jones Jr. & Bennett, 2011)

1.4 Dimerization of GPCRs: Background, methods used for detecting dimerization and understanding the phenomenon

Dimerization is an accepted concept for most of the cell surface receptors and the dimers can be generated either between same types of proteins (homodimerization) or different types of proteins (heterodimerization). The formation of dimers offers many different properties that a monomer does not possess such as increasing the ligand specificity, versatility or affinity; trafficking; internalization of receptor and cell surface mobility (Lohse, 2010).

Traditionally G protein-coupled receptors which are one of the largest cell surface receptor family are thought to exist as monomers and one ligand-one receptor complex is functional unit for signal transduction. Indeed, there is some recent evidence for G

protein-coupled receptors do not need to dimerize to carry out its basic function. Whorton *et al.* (2007, 2008) demonstrated that the purified monomeric rhodopsin and β_2-adrenergic receptor (β_2AR) are capable to activate the G protein in reconstituted phospholipid bilayer systems which indicate that monomeric signaling of receptor is possible (M. R. Whorton *et al.*, 2007; M.R. Whorton *et al.*, 2008).

However, during the last decade, there has been plenty of evidence accumulated about the notion that GPCRs form homo- and/or hetero dimers and even larger oligomers (Palczewski, 2010). The first concept of GPCR dimer/oligomerization is introduced in 1980's by Aganti and Fuxe with the studies that propose GPCRs could interact in plasma membrane. The idea of this intermembrane receptor interaction based on the influences in binding characteristics of the neuropeptide to monoamine receptors in the plasma membrane preparations of brain.(as cited in (Vilardaga, *et al.*, 2010)). After that, by using saturation and competition binding experiments, evidence was accumulated which supports the existence of interaction between certain glutamate and adenosine receptors (for review see (Agnati, Ferre, LLuis, Franco, & Fuxe, 2003).

In the nineties, the hypothesis that GPCRs exist in hetero/homodimers and formation of heteromeric complexes through the interaction of these dimers was introduced, and also the first evidence reported that "G protein-coupled receptor can exist in dimer" by using immunoblot analysis for 5-HT_{1B} receptor in Sf9 insect cells (Ng *et al.*, 1993). In a separate experiment to overcome the denaturation effects of SDS, treatments with covalent cross-linkers were applied. And by using this strategy, existence of homomeric forms of D2 and A1 receptors was verified in brain tissue, *in situ* (Ciruela *et al.*, 1995; Ng *et al.*, 1996).

Co-immunoprecipitation which is an important technique to study protein-protein interactions was also used to study GPCR dimerization in 1996. Hebert *et al.* co-expressed c-myc- and HA-tagged β_2 adrenergic receptors in Sf9 cell line and

differently tagged receptors were identified in both anti-HA and anti-c-myc immunoprecipitates (Hebert *et al.*, 1996).

With the development of fluorescent procedures especially resonance energy transfer (RET) techniques detection of GPCR interactions in living cells has become possible. FRET (Fluorescence-RET) and BRET (Bioluminisence) became popular fluorescent strategies for dimerization studies. In 2000, Blumer *et al.* observed homodimers of Ste2p with FRET methodology (Overton & Blumer, 2000). And in another study combining co-immunoprecipitation with BRET, Angers *et al.* observed the occurrence of β2-adrenergic receptor homodimerization in mammalian HEK-293 cells (Angers, Salahpour, & Bouvier, 2001). In a more recent study oxytocin receptor dimers and oligomers have been shown in their native tissues (mammary gland) by using time-resolved FRET method (Albizu *et al.*, 2010). Another evidence for which homodimerization is visualized comes from rhodopsin. Fotiadis *et al.*, obtained topographic images of rhodopsin using atomic-force microscopy and showed organization of rhodopsin dimers in native retinal disks (Fotidais *et al.*, 2003).

A different approach has also been utilized which is based on mutant receptors that are complementary to each other. For instance, transgenic mice which can co-express binding deficient and signaling deficient forms of luteinizing hormone receptor (LHR) were generated and with complementation of deficient receptors, normal hormone function of LHR can be restored which support the evidence for dimerization of GPCR. This study is also important with respect to its demonstrating dimerization in native physiological environment (Rivero-Muller *et al.*, 2010).

The results obtained from different biochemical or biophysical experiments including SDS-PAGE (sodium dodecyl sulfate-polyacrylamide gel electrophoresis) with covalent cross-linking, co-immunoprecipitation, FRET, BRET and functional complementation assay all lead to the dimerization of GPCR, not only *in vitro* but also in native tissues. In spite of experimental evidence mentioned above, many questions arise regarding the mechanism of dimer/oligomerization. The life cycle stages of G protein-coupled receptors in living cells can be arranged in; biosynthesis and modification in ER and

golgi, transportation to membrane, glycosylation, activation by ligand and signal transduction and internalization subtitles (Shi, Paige, Maley, & Loewen, 2009). When it comes to the question concerning “What are the possible effects of dimerization to the stages mentioned above?” or “Why GPCRs need to be dimerized?”, there is no consensus on the answer. The early data in studies suggested that dimerization occurs during biosynthesis. A study done with co-immunoprecipitation and BRET techniques confirmed that Vasopressin V1a and V2 and oxytocin receptors’ homo-/hetero- dimer formation take place early during biosynthesis indicating the idea that dimerization of the GPCRs is a constitutive process which means dimerization is not dependent on ligand binding (Terillon *et al.*, 2003). Additionally, in another study, it has been reported that in the absence of an agonist, oligomerization of a chemokine receptor CC5 occurs which supports the constitutive oligomerization hypothesis (Issafras *et al.*, 2002). Similarly, direct evidence for serotonin 5-HT2C receptor homodimerization is found which takes place during biosynthesis in ER and also trafficking through the golgi in intact living cells (Herrick-Davis, Weaver, Grinde, & Mazurkiwicz, 2006). The requirement of dimerization for proper transportation of GPCRs to plasma membrane has been demonstrated with $GABA_B$ receptor subunits. There are two subunits of the receptor, $GABA_{B1}$ and $GABA_{B2}$. The first interaction between subunits of the receptor was shown to occur in the ER. Concerning their functions, $GABA_{B2}$ is essential for membrane trafficking and coupling with G protein; while $GABA_{B1}$ is required for agonist activation.(see review (Prézeau, Rondard, Goudet, Kniazeff, & Pin., 2011)). The modification of receptor selectivity for agonist with dimerization is an important subject which has possible effects on receptor pharmacology. Waldhoer *et al.* showed that 6’-guanidinonaltrindole (6’-GNTI) is an agonist with an analgesic characteristic recognized only by opioid receptor heterodimers (κ-opioid/δ-opioid) not homodimers (Waldhoer *et al.*, 2005). In other words, heterodimerization may alter the functional characteristic of the receptor that results in extended pharmalogical diversity by the development of a new binding site. The effect of dimerization to G protein coupling has also been examined. Dopamine receptors, namely D_1R and D_2R normally couples with $G\alpha_{s/olf}$ and $G\alpha_i$, respectively. However, heteromerization of these receptors results in $G\alpha_{q/11}$ mediated signal transduction which induces phospholipase C activity rather than

adenylate cyclase activity (Rashid *et al.*, 2007). Hence, as a consequence, receptor dimers may be demanded for exhibiting specific signaling cascade. An example to the effects of dimerization to internalization could be given for δ-opioid receptors. Homodimerization of these receptors are required for internalization controversially heterodimerization between β_2-adrenergic receptor (β_2AR) and κ –opioid receptor has an inhibitory effect on agonist induced endocytosis (Jordan, Trapaidze, Gomes, Nivarthi, & Devi, 2001).

Homodimerization of α-factor receptor (Ste2p) of S*. cerevisiae* has been surveyed with some of the methods that are mentioned earlier. One of the first examples supporting the hypothesis of homodimerization is 'regulatory carboxyl-terminal cytoplasmic domain deleted' Ste2 receptors. Normally receptors lacking carboxyl tail which includes DAKSS endocytosis signal fail endocytosis. But endocytosis function is regained when wild type receptors co-expressed with mutant receptors lacking C-tail. Besides the genetic experiments, biochemical evidence supporting the homodimerization of Ste2p receptor has been obtained. Co-immunopreciptation of the influenza HA epitope (Ste2-HA) tagged and GFP tagged (Ste2-GFP) receptors was found when both of the tagged receptors precipitants treated with anti-HA antibody (Yesilaltay & Jenness, 2000). In another study, Overton and Blumer firstly demonstrated Ste2p oligomerization in intact cells using FRET method, and showed that it is a process independent of agonist or antagonist binding indicating a constitutive homodimer formation. The results of the same study also showed that internalization of Ste2p occurs as a dimeric complex (Overton & Blumer, 2000). In 2007 another study was conducted using BRET method and its results are consistent with the ones obtained by Overton and Blumer. This study also concludes signaling defective receptors are obtained with the heterodimerization of normal and dominant negative mutated receptors indicating two functional receptors are required for proper signaling process (Gehret, Bajaj, Naider, & Dumont, 2006). In a more recent study the ligand related dimerization of recombinant Ste2p receptor was investigated *in vitro* by using different methods including atomic force microscopy (AFM), dynamic light scattering (DLS) and chemical cross-linking. Dimer form of the receptor found both in the absence and more dominantly in the presence of

pheromone. So results could lead to the hypothesis that GPCR dimerization is constitutive, however ligand binding may induce dimer stability and also oligomeric assembly of the receptor (Shi, *et al.*, 2009). The particular contact sites for homodimer formation are also investigated and TM1 is found to be critical for dimerization with the assistance of TM2 and N-terminal domain. A GXXXG motif which is located in TM1 was offered to mediate direct interaction between receptors results from impairing effect of amino acids substitution in GXXXG motif to receptor homodimerization (Overton & Blumer, 2002). Further studies of this group revealed that mutant receptors which possess disrupted dimer formation sites can bind ligand but signaling is abolished which point out the effect of oligomerization in signal transduction (Overton, Chinault, & Blumer, 2003).

1.5 Split-EGFP: A protein complementation assay to study *in vivo* detection and characterization of protein interactions

There have been various methods to detect the interactions between proteins as mentioned above under the 'dimerization' title, and these methods have been utilized for identification of interactions and interacting partners. The methods investigating these mechanisms *in vitro*, for instance co-immunoprecipitation and constitutive complexes, provide benefits for further structural and biophysical characterization of the interaction with several techniques such as NMR spectroscopy, atomic force microscopy etc. However, they require extraction and purification of the target components from their native environment (Langelaan, Ngweniform, & Rainey, 2011).

The other genetic and cell biology approaches, for example functional complementation assays or yeast-two hybrid systems, enable studying interactions in the cell through the analysis of functional alterations caused by mutations, but it is not possible to decide whether it is a direct or indirect interaction between the gene products that cause these consequences (Magliery *et al.*, 2005). And they do not provide adequate information about localization of interaction at the subcellular level (Barnard, McFerran, Nelson, & Timson, 2007).

The resonance energy transfer (RET) methods defeat these problems and enable to distinguish direct interactions and also detect them in their native environment. Beside these benefits, they have some drawbacks. In FRET method over-expression of the potential interacting target protein and complex data analysis are required. In BRET method, signal detection is limited nearly in 10 µm resolution (Kerppola, 2009; Liu, Ahmed, & Wohland, 2008).

Protein complementation assays (or Protein-fragment complementation assays) are relatively new methods which allow detection of protein interactions directly. In this assay, reporter protein fragments which are not functional by themselves are fused to the potential interacting partners. Interaction between these partners brings the fragments of reporter protein in a close distance in which they can reassemble and make the reporter protein regain its function (Shekhawat & Ghosh, 2011). This method was first defined for ubiquitin in 1994 (Johnsson & Varshavsky, 1994). And since then, there has not been any procedure or algorithm reported to predict which proteins could be the possible candidates of protein reporters (Kerppola, 2009). In the meantime, fragments of dihydrofolate reductase (DHFR), β-lactamase, β-galactosidase, fluorescent proteins and several luciferases have been used to study protein-protein interactions in many different types of organisms i.e. bacteria, yeast, mammalian cells, plant (Barnard, McFerran, Trudgett, Nelson, & Timson, 2008). In the case of fluorescent proteins, the method is called bimolecular fluorescent complementation (BiFC) assay which enable to visualize interactions of proteins *in vivo* and also measure the effects of extracellular agents such as drugs or agonists on the protein complexes (for review see (Morell, Ventura, & Aviles, 2009)). As illustrated above in Fig1.5 the target proteins desired to be tested in terms of interaction that is indicated with pink color are tagged with non-functional fluorescent protein fragments (indicated with grey color). When the target proteins interact with each other, non-covalent reassembly of fluorescent protein and recovery of the function occurs which provides a direct read-out of interaction with fluorescence signal. The functional florescent protein is indicated as a green cylinder.

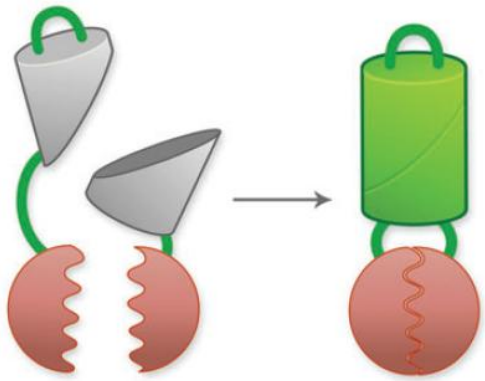

Figure 1.5 Fluorescent protein complementation (Frommer, Davidson, & Campbell, 2009)

In this study split-EGFP method is implemented to detect G-protein coupled receptor dimerization. Green florescent protein is a powerful tool used as a biological marker molecule. Early in 1960s, Osamu Shimomura first identified green fluorescence protein which is isolated from the jellyfish *Aequorea victorea,* and in 1974, he described the purification and crystallization of it. Afterwards in 1990s, Martin Chalfie's group firstly expressed this fluorescent protein not only in *E.coli* but also in the touch neurons of *Caenorhabditis elegans* which opens the way for GFP utilized as a florescent probe *in vivo*. Later on, Roger Tsien changed and improved its spectral properties that led to developing many other GFP variants. These tremendous studies and their contribution to biology and medicine were awarded in 2008 with the Nobel Prize in Chemistry ''for the discovery and development of the green fluorescent protein, GFP'' (as cited in Sanders 2009). With an eleven-stranded β-barrel, GFP has an α-helix running through the center of β-barrel. The chromophore which gives its color when excited with a certain wavelength and transmits to another is located in the center of the molecule.

Enhanced green florescence protein is a GFP variant that has only two amino acid substitutions; first in position 65 serine for threonine and second in position 64

phenylalanine for leucine (Day & Davidson, 2009). These amino acid substitutions improve properties of protein in terms of folding efficiency and brightness and make the EGFP molecule the brightest and the most photostable among the Aequorea based fluorescent proteins (Kerppola, 2009). In BiFC assay, EGFP molecule splitted into two fragments generally termed as N-EGFP and C-EGFP (Fig1.6.a). These EGFP fragments are then fused to the proteins whose interaction is under question and could led to the reassembly of the active protein. Co-expression of the differently tagged fusion proteins results in chromophore maturation and florescence signal in the cell. The structure of the chromophore and the excitation/emission spectra of EGFP molecule are shown below in Fig 1.6.b and 1.6.c.

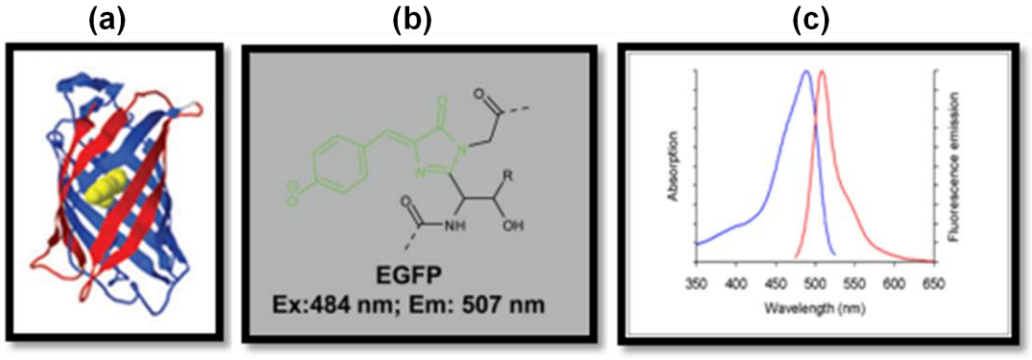

Figure 1.6. (a) X-ray crystal structure of GFP. N-EGFP is in blue and C-EGFP is grey. The images were generated using jmol (Kerppola, 2009) (b) Chromophore structure (Sample, Newman, & Zhang, 2009), (c) Absorption and fluorescence emission spectra of enhanced green fluorescent protein (EGFP) in pH 7 buffer (http:/www.invitrogen.com)

1.6 Aim of the study

GPCRs are cell surface receptors activated by a wide range of extracellular stimuli. They also are the targets for 50% of drugs on the market. It is recently getting accepted that they function as homo/heterodimers which have an effect on life cycle stages, function and/or pharmacology. Thus understanding the mechanism of dimerization is crucial in pharmaceutics industry for developing new and safer therapies.

In this study, split-EGFP (Enhanced Green Fluorescent Protein) method was implemented that is based on bimolecular fluorescence complementation technique to detect and visualize dimerization of receptors, directly and in intact membranes and cells *in vivo*. For this aim; yeast alpha pheromone receptor (Ste2p) was used as a model GPCR system. Firstly, EGFP molecule was splitted into two non-functional fragments, and fusion proteins with the Ste2p receptor was generated by using recombinant DNA techniques. Following the transformation into yeast cells, image acquisition with a laser scanning confocal microscope was carried out to detect fluorescent signal which can be seen only if the tagged receptors are dimerizing. Implementation of BiFC assay for demonstration of Ste2p dimerization in live cells will lead further analysis to specify localization of the dimerization. The method we are trying to implement also can be used to investigate effects of various factors on receptor dimerization such as: the effects of post translational modifications or agonist and/or antagonist binding. On the other side developing and optimizing the split-EGFP method will aid us use this method for revealing new protein-protein interactions.

CHAPTER 2

MATERIALS AND METHODS

2.1 Material

2.1.1 Yeast Strains, Media and Growth Conditions

DK102 and BJS21 *Saccaromyces cerevisia* strains were used in this study and they were kindly provided as a gift by Prof. Dr. Jeffrey M. Becker from University of Tennessee, Knoxville, USA.

The genotype of DK102 is *MAT***a** *ura3*-52 *lys*2–801^{am} *ade2*-101^{oc} *trp1*-Δ63 *his3*-Δ200 *leu2*-Δ1 *ste2*::*HIS3 sst1*-Δ5 (Dohlman, Goldsmith, Spiegel, & Thorner, 1993) and BJS21 is *MAT***a**, *prc1-407 prb1-1122 pep4-3 leu2 trp1 ura3-52 ste2::Kan*^{R} (Son, Sargsyan, Naider, & Becker, 2004). To measure alpha-factor induced growth arrest, DK102 strain was used and protease deficient BJS21 strain was used for western blot analysis to decrease the receptor degradation.

Yeast strains were grown in YEPD (yeast extract-peptone-dextrose) broth at 30 °C and were maintained in agar plates at 4 C for short term storage. To prepare long term stocks at -80 °C, 50% glycerol was added as cryoprotectant agent until liquid yeast culture aliquots reach up to 25% final glycerol concentration. YEPD media ingredients were dissolved in distilled water and sterilized by autoclaving.

MLT, MLU and MLTU (Media lack of Tryptophane, Uracil and Tryptophane and Uracil, respectively) were used for selection of yeast cells after transformation of plasmids. Ingredients of the media are listed in Appendix B.

2.1.2 Bacterial Strains, Media and Growth Conditions

Top10 and NEB 5-alpha competent *E.coli* (USA) strains were used in this study. Bacterial strains were grown in LB (Luria Bertani) media, both solid agar plates and liquid broth. All ingredients of the media which are given in Appendix C were dissolved in distilled water, and after adjusting the pH to 7.4, media were sterilized by autoclaving at 121°C for 20 minutes. 100 mg/mL of Ampicillin or 50mg/mL Kanamycin was added to sterile media for bacterial selection.

E.coli cultures were incubated at 37 °C incubators (Nuve) and for liquid growing cultures shaker incubator (Zheiheng) was used.

2.1.3 Vectors

pEGFP-N2, enhanced green fluorescence cDNA vector was generously donated by Prof. Dr. Henry Lester, California Institute of Technology, USA.
The parental plasmids pBEC1 and pCL01 expressing the Ste2 receptor which was used as template for our constructs were kindly gifted by Prof. Dr. Jeffrey M. Becker, University of Tennessee Knoxville, USA.

2.1.4 Other Chemicals and materials

LA taq polymerase enzyme and Pfu high fidelity polymerase enzyme was purchased from Takara Bio Inc. (Japan) and Stratagene (CA,USA), respectively. DpnI, BamHI and EcoRI restriction enzymes were from New England Biolabs (Hertfordshire, UK). Alpha pheromone peptide used in biological activity assays was provided by our collaborator Prof. Dr. Jeffrey M. Becker, University of Tennessee, Knoxville. Paper filter disks were

from BD, Franklin Lakes, NJ and glass bottom dishes used in imaging experiments were ordered from *In vitro* Scientific (CA, USA).
All other chemicals used in buffers and mediums were obtained from Sigma-Aldrich Inc (NY, USA) and Applichem (Darmstadt, Germany).
LSM 510 laser scanning microscope (UNAM, Bilkent University) was used for live cell imaging studies.

2.2 Methods

2.2.1 Competent E.coli Preperation by $CaCl_2$ method

Top10 *E.coli* cells were inoculated in 50 ml of LB media and incubated at 37 °C and 200 rpm overnight. Next day, 4 mL from growth culture was transferred into 50 mL LB and they were incubated at 37 °C and 200 rpm for 3 hours. After that 50 mL culture was incubated in ice for 15 minutes and transferred to 50 mL falcon tube and centrifuged at 4000 rpm for 10 minutes. Supernatant was removed and later on the pellet was resuspended in 4 mL 0.1 M $CaCl_2$. After centrifuging at 4000 rpm for 10 minutes, supernatant was discarded and pellet was resuspended with 4 mL cold 0.1M CaCl2 / 15% glycerol. 100 µL aliquots were prepared and stored at -80 °C.

All glass and other autoclavable materials were sterilized at 121 °C for 20 minutes prior to protocol. And aliquots were handled in aseptic conditions.

2.2.2 High Efficiency Transformation of chemically competent E.coli Cells

Chemically competent *E.coli* cells were thawed on ice for 5 minutes until the ice crystals disappear. 1-5 µL of plasmid DNA was added to the cell mixture and tube was carefully flicked to mix cells and DNA. Cells were placed on ice for 30 minutes following 42 °C heat shock for 60 seconds. They were placed on ice for 5 minutes and 950 µL of room temperature SOC (Appendix C) was added into the mixture. After incubation at 37 °C for 60 minutes, cells were centrifuged at 15,000 rpm for 30 seconds. 800 µL of

supernatant was discarded and cells were resuspended in the left 150 µL SOC. Whole lot were spreaded onto the selective LB plates containing Amp/Kan and plates were incubated overnight at 37 °C.

2.2.3 Cloning strategy for labeling of Ste2p receptors with EGFP fragments

N-terminal and C-terminal of EGFP molecule were amplified by PCR using primers which have 30 bp-long overhanging regions with receptor sequence in the insertion position on Ste2p. A second PCR is used to integrate the PCR products with STE2p which amplifies the whole plasmid. The complementary regions present on the product of the first PCR reaction form the initial hybridization and upon the second round of PCR the label sequence is inserted in the target site. The verified constructs (by sequencing) are transformed to DK102yeast cells to test biological activity (Fig 2.1).

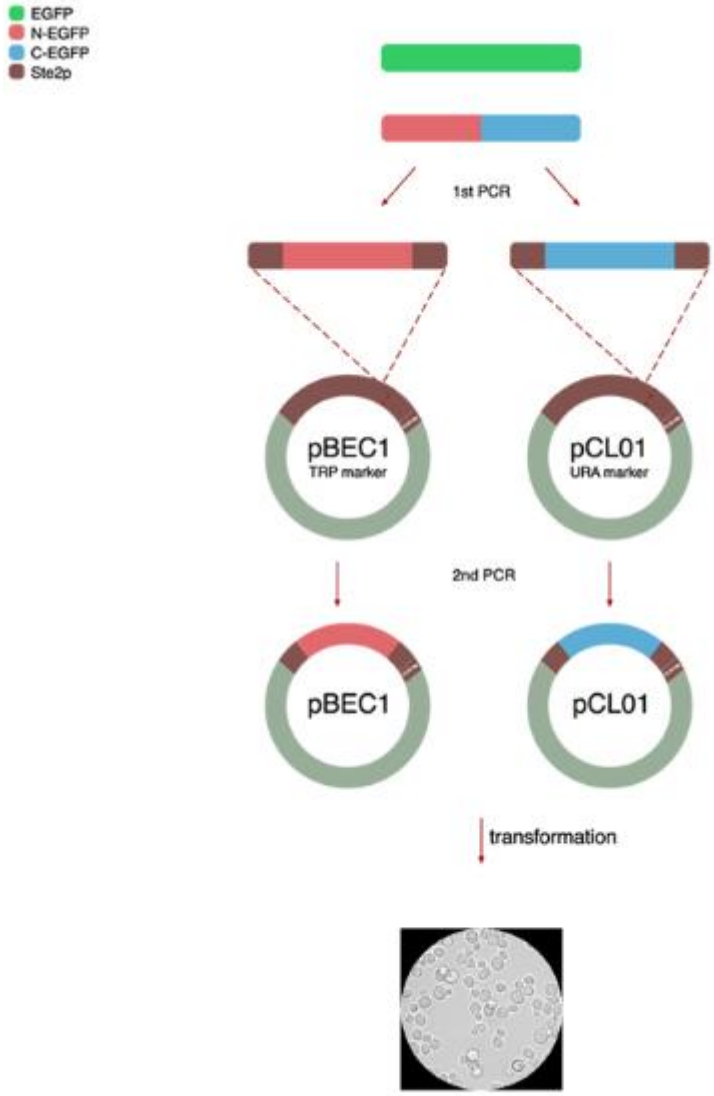

Figure 2.1 Cloning strategy for labeling of Ste2p receptors with EGFP fragments

Two different dissection sites in EGFP molecule were tested for the use of split-EGFP method. Firstly EGFP dissected at 128-129 and secondly at 158-159. Resultant EGFP fragments split at 128-129 were sandwiched with a full length Ste2p receptor between 304-305 residues and also between 441-442 residues. Similarly fragments of EGFP split at 158-159 were integrated between 304-305th residues of a full length receptor however these fragments were fused to Ste2p receptor whose C tail was truncated

after 304th residue. For positive controls labeling of Ste2p receptor at 304-305, 441-442 and 304 truncated with full length EGFP were carried out.

2.2.4 Primer Design

All primers composed of 2 parts; first part located in 3' end of the primers, was complementary to pEGFP-N2 sequence to amplify enhanced green fluorescence protein fragment at first PCR reaction and the second at 5' end of primers was complementary to Ste2p receptor sequence which enables the annealing to template in second PCR.

The total length of PCR primers were 50-51 base pairs and the primer melting temperature (T_m) and GC content of sequence varied. Primers which were used in this study were purchased from Invitrogen (USA) and Alpha DNA (Montreal, Quebec). Lists of the primers are given in Table 2.1.

In Table 2.1 (a) list of the primers that are used for splitting EGFP molecule from 128th residue is given. Resultant EGFP fragments are then integrated into full length Ste2p receptor both after 304th and 441th residues.

Table 2.1.a List of primers for EGFP dissection site 128-129

Position	Primer	Sequence
304	N-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAATGGTGAGCAAGGG C GAGGAGCTGT
	N-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTGCCCTTCAGCTCGA TGCGGTTCACCA
	C-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAATCGACTTCAAGGAG GACGGCAACA
	C-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTCTTGTACAGCTCGT CCATGCCGAGA
441	N-EGFP Forward	CAAGGACGACGATGACAAGACCGGTATGGTGAGCAAGG GCGAGGAGCTGT
	N-EGFP Reverse	GGCTGCTGCCGCTGCCGCGCGGCACGCCCTTCAGCTCG ATGCGGTTCACCA
	C-EGFP Forward	CAAGGACGACGATGACAAGACCGGTATCGACTTCAAGGA GGACGGCAACA
	C-EGFP Reverse	GGCTGCTGCCGCTGCCGCGCGGCACCTTGTACAGCTCG TCCATGCCGAGA

In Table 2.2(b) list of the primers that are used for splitting EGFP molecule from 158th residue is given. Resultant EGFP fragments are integrated into full length Ste2p receptor after 304th residue and C-terminal tail deleted receptor.

Table 2.2.b List of primers for EGFP dissection site 158-159

Position	Primer	Sequence
304 Full	N-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAATGGTGA GCAAGGGCGAGGAGCTGT
	N-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTCTGCTTG TCGGCCATGATATAGAC
	C-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAAAGAACG GCATCAAGGTGAACTTC
	C-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTCTTGTAC AGCTCGTCCATGCCGAGA
304 Truncated	N-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAATGGTGA GCAAGGGCGAGGAGCTGT
	N-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTTTACTGC TTGTCGGCCATGATATAGAC
	C-EGFP Forward	CACGGCTGCTAATAATGCATCCAAAAAGAACG GCATCAAGGTGAACTTC
	C-EGFP Reverse	GTAAAGTCTGAAGTAATTGTGTTTGTTTACTTGT ACAGCTCGTCCATGCCGAGA

To obtain Ste2p receptors that are labeled with a full length EGFP molecule, N-EGFP Forward and C-EGFP Reverse primers are combined according to desired position.

2.2.5 PCR for Amplification of enhanced green fluorescence protein

To amplify EGFP coding sequence (Accession Number: AAB02574) with 25 bp overhangs designed according to target sequence (Ste2p, Accession Number: X03010.1), two different optimized PCR protocol was applied for different polymerases as described below.

Table 2.3 Optimized PCR conditions for *Pfu* Turbo

PCR mix	Volume (µL)
dH2O	40
Pfu Buffer 10X	5
dNTP (25mM)	1
Forward primer (20 pmol)	1
Reverse primer (20 pmol)	1
Template (≈100ng)	1
Pfu polymerase	1

Cycling parameters			
Pre-denaturation	94 °C	5 min.	
Denaturation	94 °C	30 sec.	c*
Annealing	53 °C	45 sec.	c*
Extension	72 °C	1 min.	c*
Final extension	72 °C	5 min.	
***cycle x35**			

Table 2.4 Optimized PCR conditions for LA *taq*

PCR mix	Volume (µL)
dH2O	26
LA Taq Buffer 10X	5
dNTP (25mM)	5
Forward primer (10pmol)	2.5
Reverse primer (10pmol)	2.5
Enhancer	5
Template (≈100ng)	3.5
La Taq polymerase	1.5

Cycling parameters			
Pre-denaturation	98 °C	4 min.	
Denaturation	98 °C	45 sec.	c*
Annealing	58 °C	30 sec.	c*
Extension	68 °C	1 min.	c*
Final extension	68 °C	20 min.	

To check the size of PCR products, agarose gel electrophoresis was performed. For this purpose 1% agarose gel was prepared by dissolving 0.5 g of agarose in 50 mL of 1X TBE (Appendix D) in microwave. EtBr added after cooling down to nearly 55 °C and solution poured to gel casting tray carefully avoiding bubbles. When it completely solidified comb was removed and after that gel was placed in electrophoresis chamber and covered with TBE buffer. 6X sample loading buffer (Fermentas® see in Appendix D) mixed with DNA samples and mixture was loaded into wells carefully. Also for each gel, DNA ladder was loaded as a standard. Gel was run at 100V for 40 minutes and observed under UV light.

2.2.6 PCR Clean-up

Before the next step, PCR products cleaned up to eliminate excess amount of dNTP, primers, enzyme and salts. For this purpose Fermentas GeneJET™ PCR Purification Kit (#K0702) was applied to the products and instruction guide followed as required by manufacturer.

To analyze the quality of purification, post clean-up products run on an agarose gel as described before and to quantify the amount of the DNA, Nanodrop 2000 spectrophotometer (Thermo Scientific ®) was used.

2.2.7 PCR for Insertion

A second PCR was used for the insertion of fluorescent protein labels into target receptor (Ste2p). For this purpose, first PCR products were used as primers and template Ste2p vectors (pBEC1 and pCL01) with a ratio 5:1. High fidelity DNA polymerase enzymes were used for amplification to produce vector carrying labeled Ste2p gene.

Table 2.5 Optimized conditions for insertion of fluorescent label

PCR mix	Volume
dH2O	x µLto 50 µL
Buffer 10X	5 µL
dNTP (25mM)	1µL
PCR fragment	100 ng
Template	20 ng
***Pfu*LA *taq* polymerase**	1µL
Total	50 µL

Cycling parameters			
Pre-denaturation	95 °C	30 sec.	
Denaturation	94 °C	30 sec.	c*
Annealing	51 °C	1min.	c*
Extension	68 °C	2 min/kb	c*
***cycle x18**			

2.2.8 DpnI Digestion

In almost all bacteria (including *E. coli*), CH_3 groups are added to specific sequences of DNA as a bacterial immune system to make a distinction between native and foreign DNA by methylase enzyme. Thus we use DpnI enzyme which can recognize the sequence G^mA/TC and digest the methylated template used in the PCR reaction.

17 µL of second PCR product and 1 µL of DpnI enzyme incubated in Buffer-4 (Appendix D) that provided by manufacturer at 37 °C overnight. Next day 2 µL of digested product transformed into *E.coli* cells as explained earlier and transformants

were selected on ampicillin plates. 5-6 colonies were picked from each plate in order to miniprep and screening for positives.

2.2.9 Plasmid Isolation

Throughout this study, plasmid isolation from E.coli cells were done with Fermentas GeneJET™ Plasmid Miniprep Kit and protocol provided by the manufacturer was followed.
After isolation, concentration measurements were performed in Nanodrop 2000.

2.2.10 Restriction Enzyme Digestion for Size Control

BamHI and EcoRI restriction enzymes were used in this study which were purchased from New England Biolabs Inc. For double digestion of 1 µg of DNA, samples were incubated with 1 unit of restriction enzymes and 1X BSA in the provided NEB-Buffer at 37°C for 2 hours.
To check the sizes of products, agarose gel electrophoresis was performed as described before.

2.2.11 Sequencing

Sequences of all constructs were confirmed by DNA sequencing which was carried out in Refgen (Ankara,Turkey) and DNA sequencing facility located in the campus of UT Knoxville.

2.2.12 Yeast Transformation

For high efficiency transformation the LiAc/ss-DNA/PEG transformation method was modified and applied (Gietz & Schiestl, 1995). All solutions were prepared under aseptic conditions and sterilized either by filter or autoclave and the recipes of solutions were listed in the Appendix D.

DK102 or BJS21 cells were inoculated in 5 ml of appropriate medium and incubated with shaking at 30°C overnight. Next morning cells were counted by hemocytometer and inoculation of 50 ml medium in sterile flask to a cell concentration of 5 x 10^6 cells/ml was done. The culture was incubated at 30°C on a shaker at 200 rpm until cell density is 2 x 10^7 cells/ml. The culture harvested in a sterile 50 ml centrifuge tube at 4000 rpm for 5 minutes and supernatant was discarded. Cells were resuspended in 25 ml of sterile water and centrifuged again. The water was poured off and this time cells were resuspended in 1 ml 100 mM LiAc. The suspension was transferred to a 1.5 ml eppendorf tube and cells were pelleted at top speed for 15 seconds afterwards LiAc removed with micropipette. Cells were resuspended in 400 µl of 100 mM LiAc and 50 µl aliquots were prepared as needed. Cells were pelleted again following the removal of LiAc and those ingredients below were added in the following order;

240 µl PEG (50% w/v)

36 µl 1.0 M LiAc

25 µl salmon sperm DNA (boiled for 5 minutes before being used)

50 µl water and plasmid DNA (0.1 - 10µg)

Each tube was mixed by vortexing until the cell pellet disappears and incubated at 30°C for 30 minutes. Heat shock was applied at 42 °C for 25 minutes and they were centrifuged at 8000 rpm for 30 seconds. Transformation mix was removed with micropipette and cells were resuspended in 1 ml of sterile water. 200 µl of cell suspension was placed on selective media plates and incubated at 30 °C for 2 days.4 individual single colonies were picked from transformation plates and grown on another selective media plate for further analysis and long term storage at 4 °C.

2.2.13 Growth Arrest Assay (Halo assay)

DK102 cells expressing EGFP tagged Ste2p were inoculated in proper media (MLT/MLU) and grown at 30°C overnight. Next day, cells were harvested and washed for 3 times with sterile water and resuspended at a final concentration of 5x10^6 cells/ml. Later on cells were combined with 4 ml agar noble (1.1%) and poured as a top agar

lawn onto MLT/MLU medium agar plates. Filter disks (BD, Franklin Lakes, NJ) were placed on the top agar and impregnated with 10 µl of alpha-factor pheromone at various concentrations. Plates were incubated at 30°C for 20-30 hours and clear zones (halos) were observed around the disks. The diameter of halos including the diameter of disc were measured and plotted halo size versus the amount of alpha-factor and then analyze by linear regression using Prism software (GraphPad Software, San Diego, CA). Each assay was carried out at least three times with no more than a 2 mm variation in halo size at an individual amount of alpha-factor.

2.2.14 Membrane Preparation

Yeast cells were grown in 5 mL of proper media (MLT/MLU) at 30 ^{o}C for overnight. Yeast culture was washed two time with water and resuspended with HEPES solution (10mM HEPES and 4mM EDTA, pH 7.0). Cells were homogenized with glass beads and after that centrifuged for 5 minutes at 2000 g to get rid of cell debris and intact cells. Membranes were harvested by centrifuging supernatant at 15,000 g for 30 minutes. All centrifuging steps were carried out at 4 ^{o}C. Membrane pellet was resuspended with HEPES solution and for protein concentration Bradford Assay was applied. For this purpose, sterile water added to membrane samples with a dilution factor 1/10. Bradford reagent was added both to samples and protein standard which were prepared with BSA at various concentrations and their absorbance at 595 nm was measured. Protein concentrations were calculated using Prism software (GraphPad Software, San Diego, CA).

2.2.15 Western Blot Analysis

Labeled Ste2p receptor was seperated from membrane proteins with SDS-PAGE and for western blot anlysis proteins were transferred to Immobilon-P membrane (Milipore Corporation, Bedford, MA) . Immunoblotting was carried out using anti-FLAG antibody and bands were observed with West Pico chemiluminescent detection system (Pierce). Preparation of SDS-PAGE solutions is given in Appendix G.

For comassie staining another gel was loaded with same protein samples and then 25mL of coomassie stain was added and incubated at room temperature overnight. Following the staining step, gel was washed with distilled water thoroughly and kept in destaining solution for 30 min for 4 times in order to remove the unbound stain from the gel.

2.2.16 Imaging with Laser Scanning Confocal Microscope

For image acquisition, yeast cells were grown at 30 °C overnight. Next morning subculture in 5 ml fresh media were prepared and incubated for 3 hours. The cells were diluted with a dilution factor 1/10 prior to imaging. Sterile and fresh culture media was used for dilutions.

The schematic illustration of imaging pairs are given in Fig 2.1

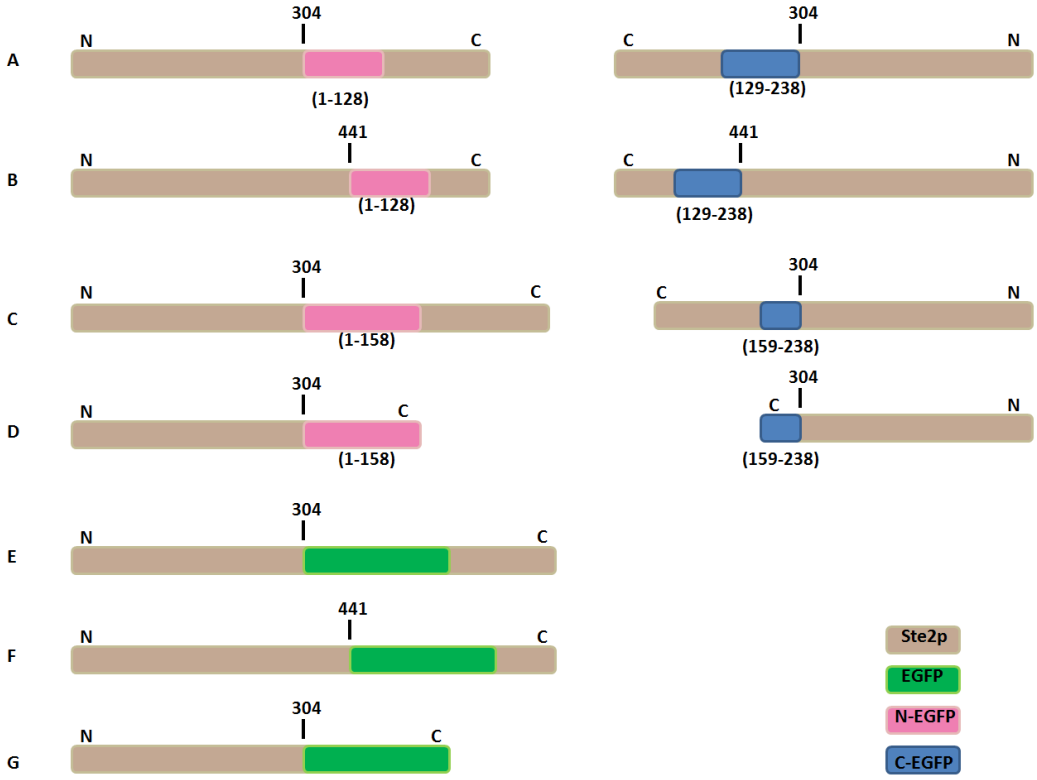

Figure 2.2 The schematic illustration of imaging pairs

For detection of fluorescent signal in live cells, yeast cells were observed using a Zeiss LSM510 confocal microscope with an objective; Plan-Apochromat 63x/1.40 Oil DIC M27. Cells were excited at 488nm and emission between 505-550 nm ranges was collected. All images have been taken with same parameters. And the modifications that were applied to the images are only sizing, cropping, alterations in brightness and contrast.

CHAPTER 3

RESULTS & DISCUSSION

2.3 Construction of Ste2p fusion proteins with fluorescent tags

The enhanced green fluorescent protein that was used in this study is composed of 239 amino acid residues. To investigate different points of dissection (Ghosh, Hamilton, & Regan, 2000; Ozawa, 2006), EGFP was divided into two fragments from two different positions; between 128-129th and 158-159th residues. Fragments of EGFP molecule were then integrated into Ste2p receptor of its C-terminal.

The strategy for cloning EGFP fragments to the receptor includes two consecutive PCR reactions was illustrated in Figure 2.1 in the previous chapter. In first PCR reaction, EGFP fragments had been amplified with 25-26 bp Ste2p overhangings and the product of first PCR was double stranded DNA with flanking regions which were homologous to Ste2p. pEGFP-N2 plasmid was used as a template for EGFP gene throughout the study. Following the first PCR, the product was then used as primers in a second PCR reaction in which plasmids carrying Ste2p gene was used as template. Through the amplification of the Ste2p gene with first PCR product, fusion of the receptor gene with EGFP fragments was generated.

2.3.1 Tagging Ste2p receptor with EGFP fragments splitted from 128th residue

EGFP coding sequence was amplified using the primers which were shown in Table 2.1.a. Two different sets of PCR reaction were performed, one for amplification of

N-EGFP (1-128) and C-EGFP (129-239) with Ste2p overhangs that enable insertion into Ste2p at position 304 and the other for amplification of the EGFP fragments that enable insertion into Ste2p at position 441. These two different insertion sites are shown below in Figure 3.1 with blue arrows.

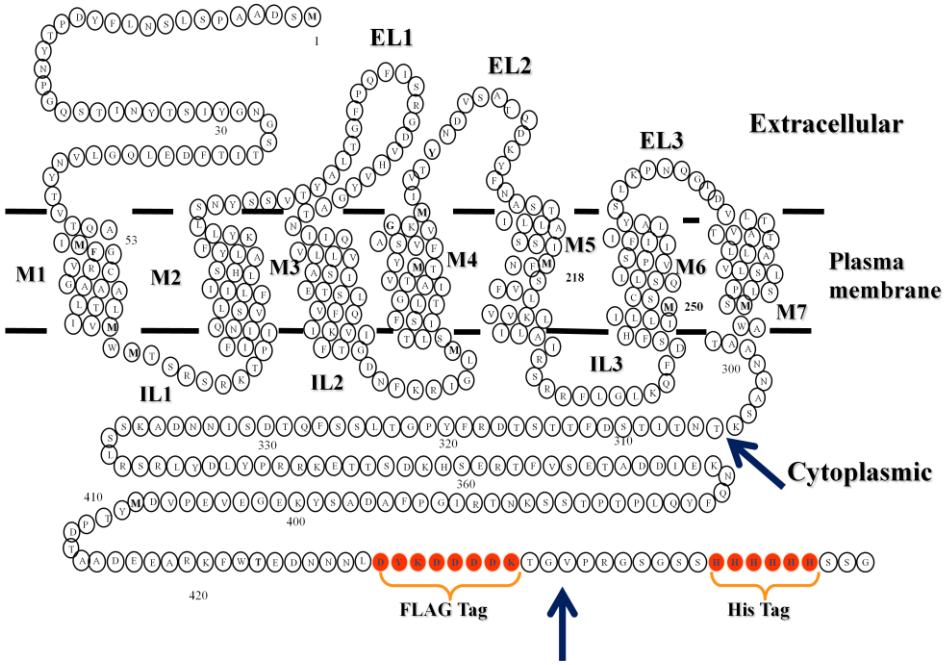

Figure 3.1 Snake diagram of yeast α-pheromone receptor (Ste2p)

The agarose gel electrophoresis photo of first PCR products are seen on Fig.3.2. Lane 1 indicates N-EGFP fragment whose dissection site was 128-129th residues. This fragment was amplified with flanking regions which enable insertion of the fragment into Ste2p receptor between 304-305th residues. Lane 2 indicates C-EGFP fragment also dissected from 128-129th residues and possess flanking regions for insertion into Ste2p receptor between 304-305th residues. The expected sizes for the two PCR products are 435 and 384 base pairs, respectively. Similarly, amplified N-EGFP (1-128) and C-EGFP (129-239) fragments are shown in lane 3 and lane 4, respectively and they have the flanking regions for integration into Ste2p between 441-442th residues.

As expected, all the products are below the 500 bp ladder band. Further verification of these fragments will be done after insertion to receptor by sequence analysis. Moreover, only one main product was obtained from all PCR experiments which is the reason of choosing PCR purification rather than gel extraction for the following procedures.

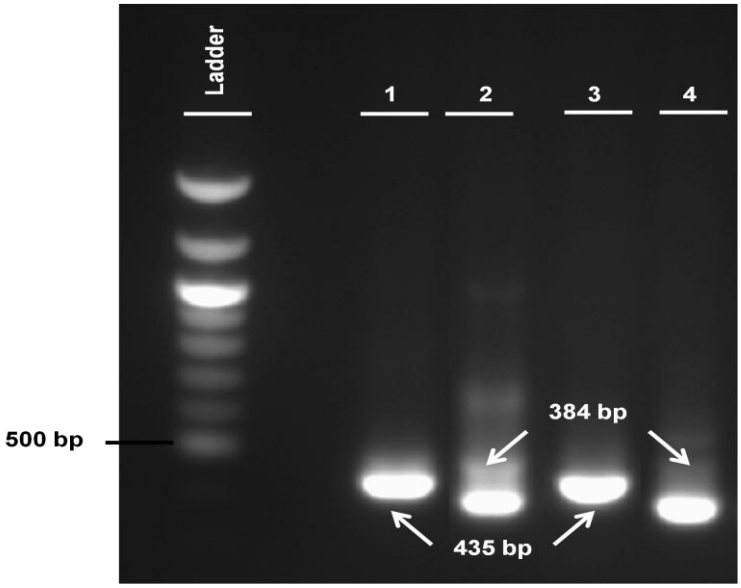

Figure 3.2 Agarose gel electrophoresis of PCR amplified EGFP fragments which were splitted from 128th residue with Ste2p flanking regions

Following the first PCR reaction, products were cleaned up with purification kits. Amplified and purified EGFP fragments were inserted into Ste2p receptor gene with a second PCR reaction. High fidelity DNA polymerase enzyme was used due to amplification of the whole vectors. pCL01 and pBEC1 vectors which possess different auxotrophic markers were used as a template for coding sequence of Ste2p receptor in this reaction. We inserted EGFP partners (N-EGFP and C-EGFP) into two different Ste2p plasmids separately because of the necessity for selection in yeast cells.

Prior to *E.Coli* transformation; to digest the parent vector and to get rid of any transformants that may occur from a plasmid other than our PCR amplification target; the PCR mixture is applied with DpnI enzyme and its buffer (NEB buffer-4) at 37 °C for overnight. After that, to increase the intended plasmid concentration, transformation to competent *E.coli* cells was made. Five colonies were picked from each transformation plate for plasmid isolation. To confirm the presence of inserts, all of the isolated plasmids were double digested with BamHI and ECoRI restriction enzymes. Digestion control with BamHI and EcoRI enzymes used to decide whether the fluorescent protein sequences are inserted into parent vectors or not, on gel agarose electrophoresis. (see Fig. 3.3, Lanes 1-5). Both PBEC1 and PCL01 vectors contain restriction sites for BamHI and ECoRI at the beginning and at the end of the Ste2p coding sequence, respectively. Therefore treatment with these two restriction enzymes cuts the Ste2p gene off from the plasmid. Considering the size difference, all of the plasmids were screened as seen in Fig. 3.3. The expected size for 'N-EGFP fragments inserted Ste2p receptors' was 435 base pairs and for 'C-EGFP fragments inserted Ste2p receptors' was 384 base pairs.

On Fig. 3.3, EGFP fragment inserted Ste2p fusion proteins are shown in red boxes. Plasmids 1-10 were constructed by using pCL01 vector whereas plasmids 10-20 were constructed with pBEC1 vector. For comparison with Ste2p, wild type pBEC1 and pCL01 vectors were also double digested and Ste2p gene was dropped between BamHI and EcoRI restriction sites. The upper bands which are seen in each gel photo indicate linearized empty vectors.

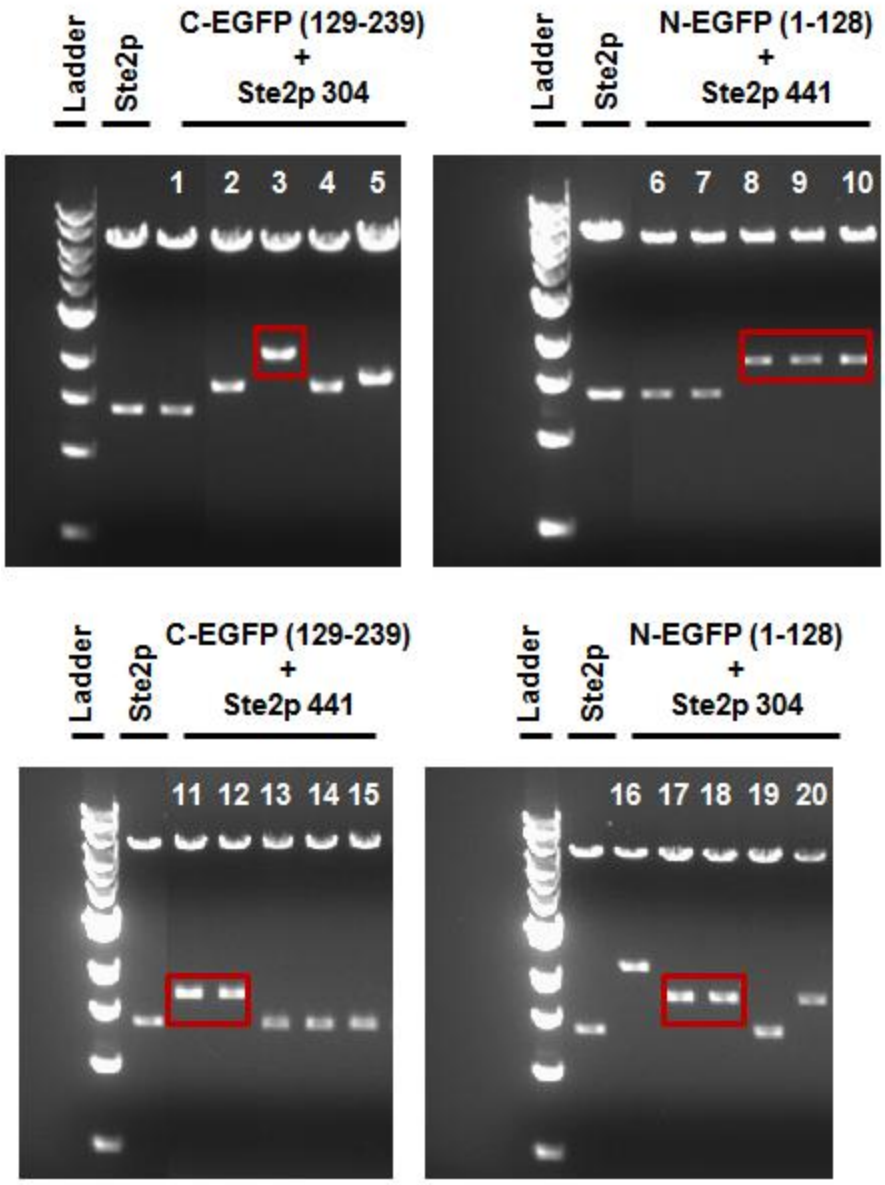

Figure 3.3 Insertion control of Ste2p fusion proteins tagged with N-EGFP (1-128) and C-EGFP (129-239)

In the next step, constructs were sent to sequence analysis to verify that the fusion proteins have the correct insert without any deletions, insertions or mutations. The results of sequencing showed that Ste2p receptor was successfully tagged with EGFP fragments in plasmids 3, 9, 12, and 18. The coding sequence of each EGFP fragment + Ste2p fusion receptor are given in Appendix F.

Newly constructed plasmids which contains tagged Ste2p receptors are named as seen in Table 3.1

Table 3.1 The list of constructed Ste2p plasmids with EGFP fragment tags which are splitted from 128th residue

Name	EGFP Fragment	Yeast Marker	Position in Ste2p
pGNU304	N-EGFP(1-128)	Uracil	304
pGCT304	C-EGFP(129-239)	Tryptophane	304
pGNT441	N-EGFP(1-128)	Tryptophane	441
pGCU441	C-EGFP(129-239)	Uracil	441

The verified plasmids which contain 'Ste2p receptors labeled with EGFP fragments' were then transformed to DK102 yeast cells for further analyses namely; biological activity assay and detection of fluorescence in laser scanning confocal microscope.

2.3.2 Tagging Ste2p receptor with EGFP fragments splitted from 158th residue

The other dissection site chosen for split-EGFP method was between 158-159th residues. However, regarding to insertion into Ste2p, only position 304 was studied. For this dissection point, in addition to the full length Ste2p, C-terminal truncation from 304th residue was carried out to study the effect of C-terminal truncation on the fluorescence signal.

The strategy to divide EGFP into two fragments from this new position and to insert into Ste2p receptor is similar with previous one. First PCR was performed for the amplification of EGFP coding sequence from corresponding vector with primers carrying matching sequences for 304th positions of Step2 gene. To generate C-terminal tail deleted constructs, “TAA” stop codon was added to primers which were shown in Table 2.2.b. The four gene constructs were size controlled with agarose gel electrophoresis by Low Molecular Weight DNA Ladder (Fermentas), the expected sizes of products are shown below in Fig. 3.4 and 3.5.

In Fig. 3.4, N-EGFP and C-EGFP fragments are shown to have approximately 525 and 294 bp, as expected.

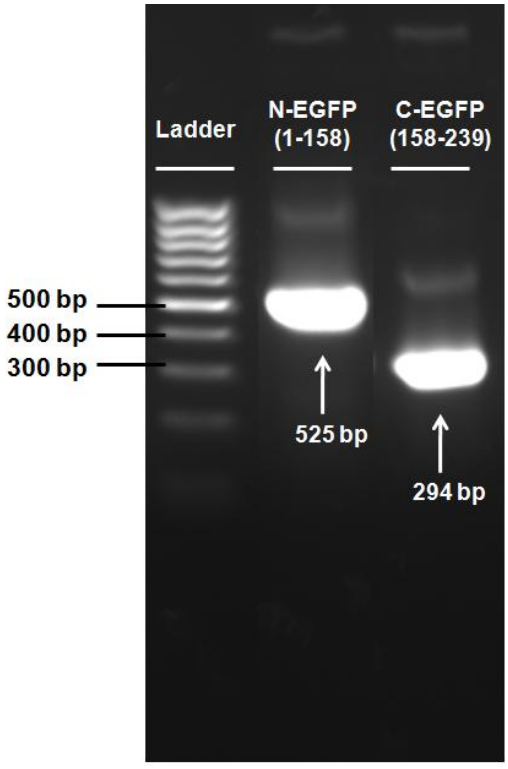

Figure 3.4 Agarose gel electrophoresis photo of PCR amplified EGFP fragments splitted from 158th residue.

Amplified EGFP fragments contain stop codons at the end of the EGFP coding sequence which enables C-terminal truncation after 304th residue. As a result, shown in Fig. 3.5 same patterns were observed with previous products because of the impossibility to detect 3 bp difference in gel. Further sequence analysis will be done for verification.

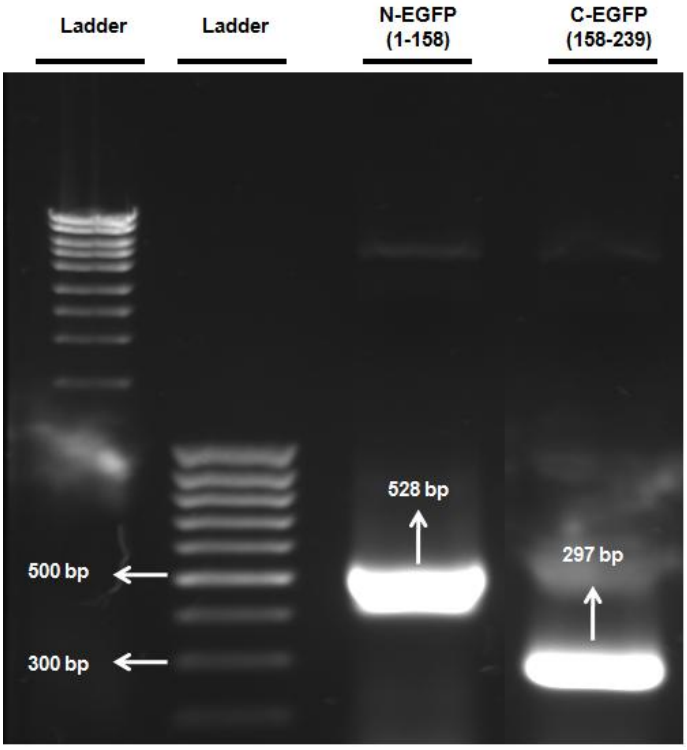

Figure 3.5 Agarose gel electrophoresis photo of PCR amplified EGFP fragments splitted from 158th residue with C-terminal truncation.

The products of first PCR reaction was cleaned up with purification kits before used as primers for PCR integration method. In second PCR, pBEC1 vector was used as a template for the insertion of N-EGFP fragments whereas pCL01 was used for C-ECGFP integration. DpnI digested 'second PCR products' transformed to *E.coli* cells and 5 colonies picked for plasmid isolation from each plate. For size control of tagged receptor, newly synthesized plasmids were double digested with BamHI and EcoRI

restriction enzymes which cut off Ste2p receptor from plasmid. Agarose gel electrophoresis results are shown in Fig. 3.6. and Fig 3.7.

The bands which are seen in red boxes imply the correct fusion of fluorescent protein sequences inside the Ste2p sequence. The expected size of wild-type Ste2p is 1374 bp, the expected size of Ste2p sequence carrying N-EGFP(1-158) tag is 1848 bp and the expected size for the C-EGFP(159-239) tagged Ste2p is 1617 bp. Inserts of the N-EGFP labeled plasmid #3 and C-EGFP labeled plasmid #1 was verified by sequencing. The coding sequences of fusion proteins are given in Appendix F.

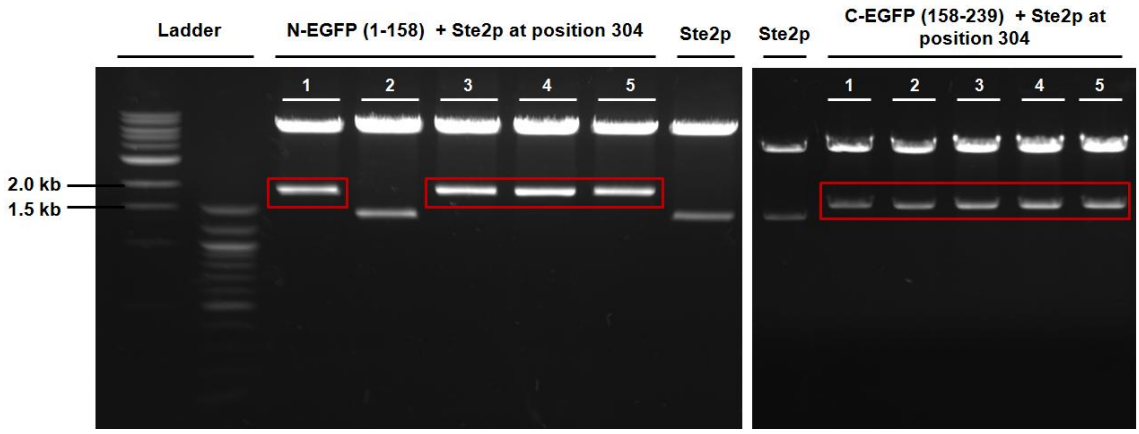

Figure 3.6 Insertion control of Ste2p fusion proteins tagged with N-EGFP (1-158) and C-EGFP (159-239). Insertion position in Ste2p gene is between 304-305th residues.

In Fig. 3.7, lane 1-5 represents pCL01 plasmids tagged with C-EGFP fragment while lane 6-10 represents pBEC1 plasmids tagged with N-EGFP fragment. The expected sizes for N-EGFP (1-158) tagged tailless Ste2p receptor is 1851 bp, and 1620 bp for C-EGFP (159-239) tagged one. All of the constructs had the inserts of the correct sizes which are shown in red boxes on Fig.3.7. The correct insertions into plasmid #3 and #4 were verified by sequencing. The coding sequences for EGFP fragments + Ste2p gene are given in Appendix F.

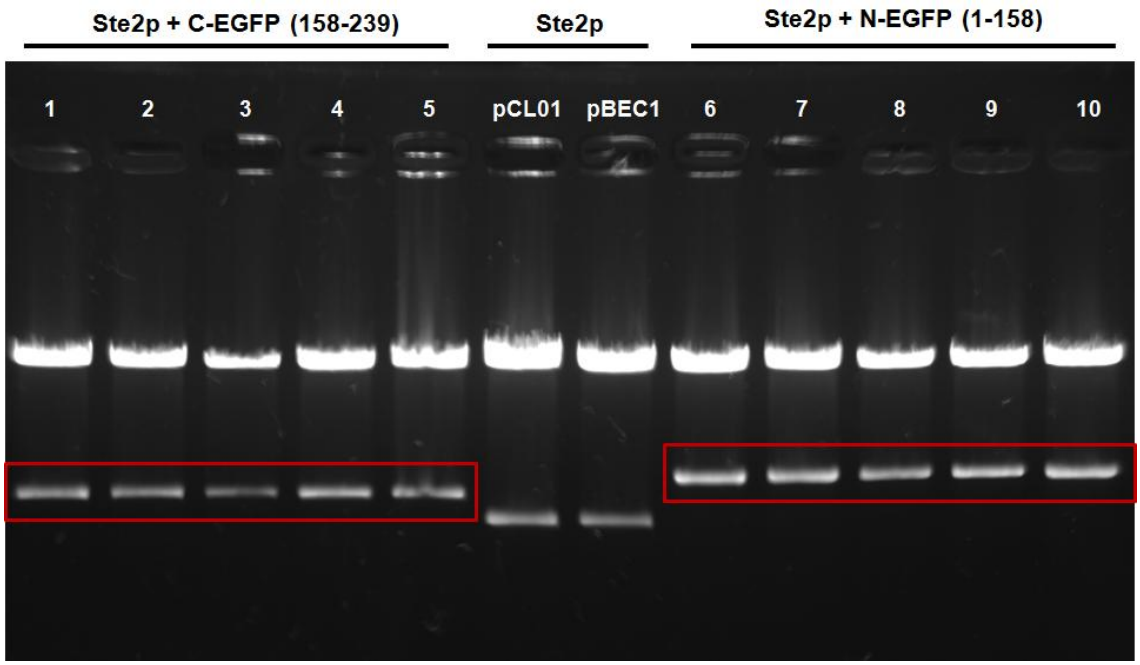

Figure 3.7 Agarose gel image of BamHI and EcoRI double digested, C-tail truncated Ste2p receptors tagged with EGFP fragments.

Newly constructed plasmids which contains EGFP fragments splitted from 158th residue are named as seen in Table 3.2

Table 3.2 The constructed Ste2p plasmids with EGFP fragment tags which are splitted from 158th residue

Name	EGFP Fragment	Yeast Marker	Position in Ste2p
pKNT304f	N-EGFP(1-158)	Tryptophane	304
pKCU304f	C-EGFP(159-239)	Uracil	304
pKNT304t	N-EGFP(1-158)	Tryptophane	304 Truncated
pKCU304t	C-EGFP(159-239)	Uracil	304 Truncated

Plasmids, which were verified with sequencing, were used for the transformation to yeast cells, to study biological activity and further imaging analyses.

2.3.3 Tagging Ste2p receptor with full length EGFP

Yeast alpha factor pheromone receptor (Ste2p) was also labeled with full length EGPF molecule for utilizing as positive controls during imaging analyses. Fluorescent protein tags were inserted into full length Ste2p receptor between the residues 304-305th and 441-442th and also inserted into tailless Ste2p receptor truncated after 304th residue. For this purpose, the same procedure which clarified earlier in this chapter was applied. The main steps of the procedure is as follows: amplification of fluorescence sequence with first PCR, purification of PCR products, insertion of fluorescence protein into Ste2p receptor with a second PCR reaction, DpnI digestion of newly synthesized plasmids to get rid of template vector, transformation to *E.coli*, plasmid isolation, double digestion with BamHI and EcoRI restriction enzymes to control insert size and transformation to DK102 yeast cell for further analyses.

The full length proteins; amplified in 1st PCR reaction; carrying the matching sequences from 304th and 441st positions of Ste2 are shown below on Fig. 3.8.

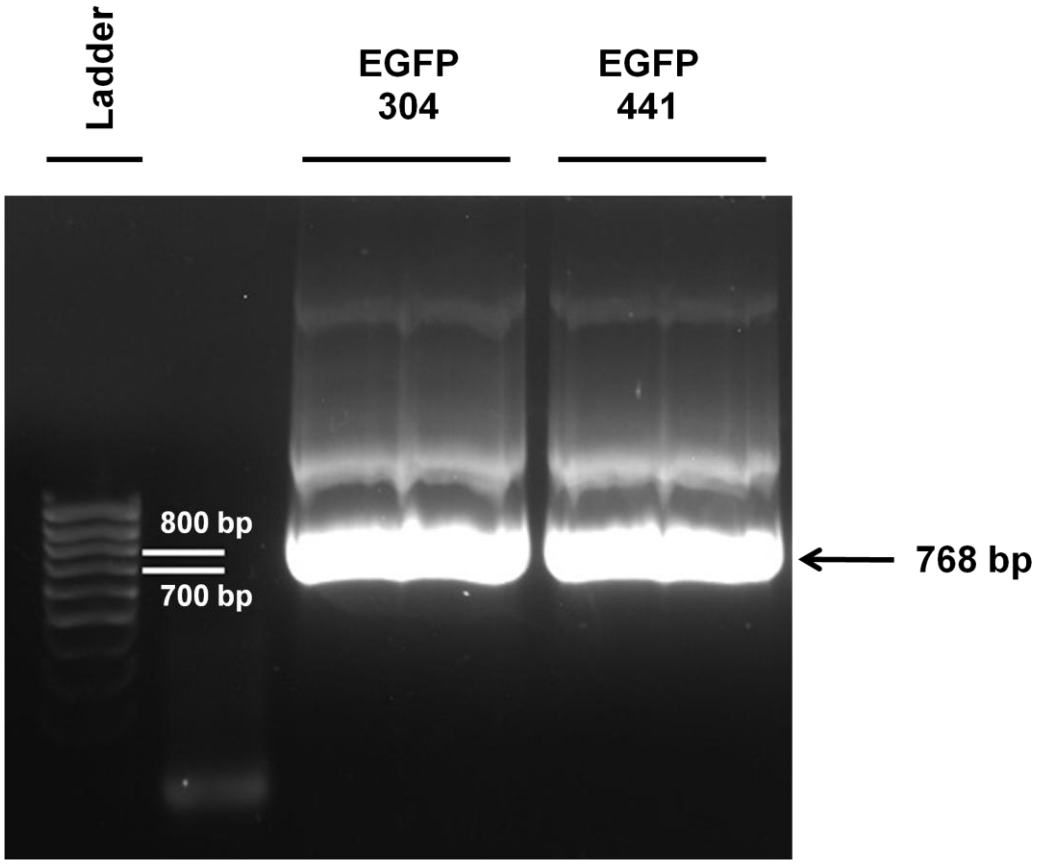

Figure 3.8 Agarose gel electrophoresis image of PCR amplified full length EGFP

The two amplified sequences were size controlled with agarose gel electrophoresis and found out to be between the interval 700 – 800 bp as expected, EGFP being 767 bp.

In the same way, EGFP coding sequence was amplified with the primers which contain stop codon to produce truncated Ste2p receptors at position 304. The products of this PCR reaction was also run in agarose gel and photo of the gel is shown on Fig. 3.9.

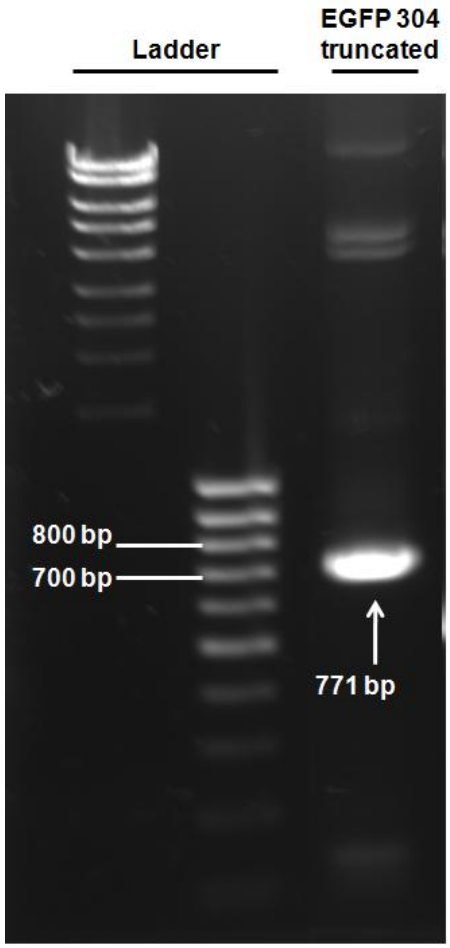

Figure 3.9 Agarose gel electrophoresis photo of PCR amplified EGFP with Ste2p flanking ends

In second PCR reaction pBEC1 vector was used as a template for labeling Ste2p at position 304 and PCL01 for labeling Ste2p at position 441.The plasmids which were produced in second PCR reaction transformed to competent *E.coli* cells. Isolated plasmids were screened with double digestion and size control (see Fig 3.10).

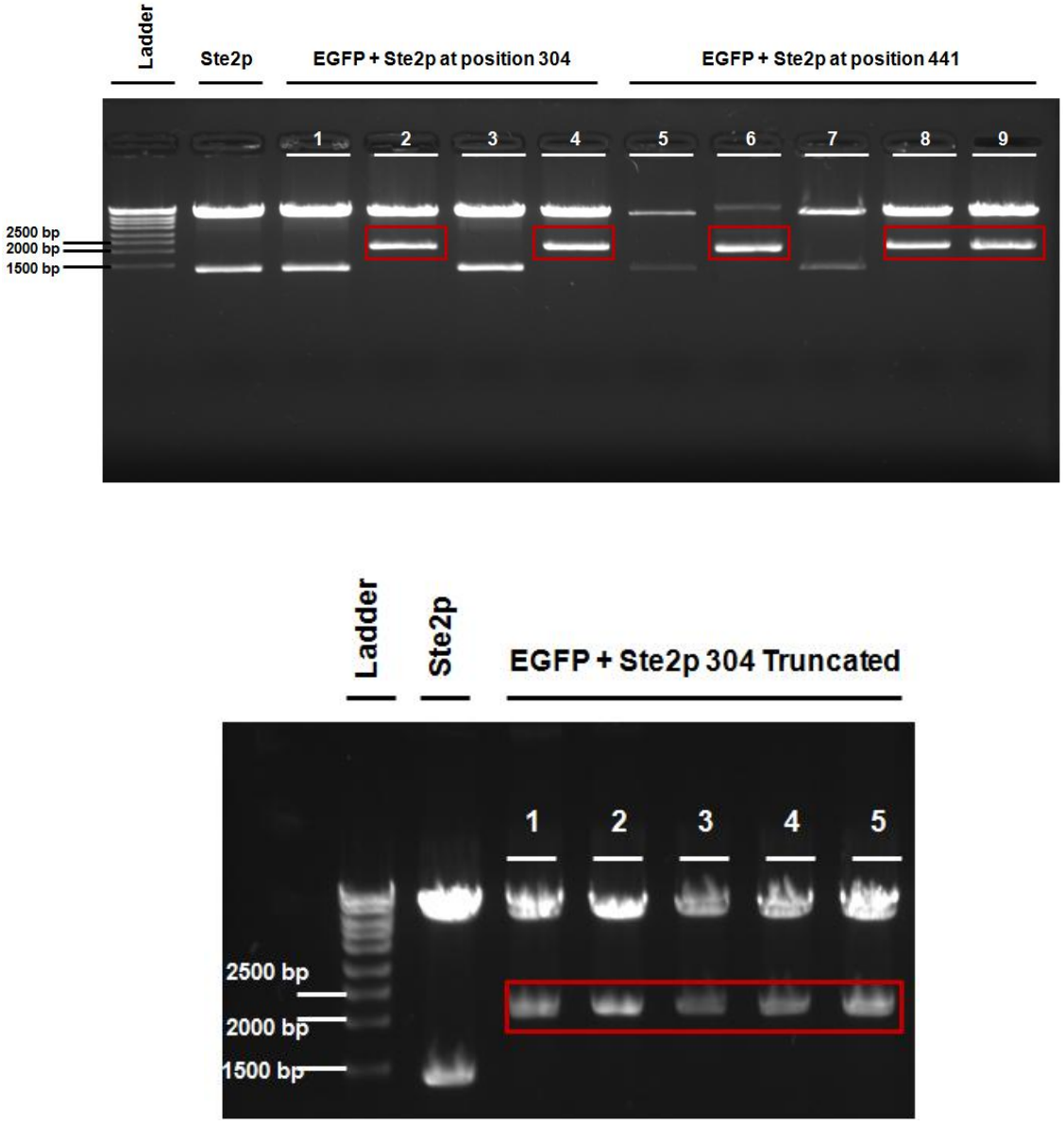

Figure 3.10 Agarose gel photo of BamHI and EcoRI digestion of EGFP tagged Ste2p receptors.

The constructs which had the correct sizes are shown in red boxes. One plasmid was selected from each construct and these constructs were transformed into DK102 yeast cells for observation of fluorescence using laser scanning confocal microscope.

2.4 Growth Arrest (Halo) Assay

It was important to determine whether the labeled Ste2p receptors still had biological activity or these sizable tags interfere to the function of receptor. For that reason, the ability of the α-factor pheromone to activate α-factor receptor (Ste2p) was determined with an assay termed as growth arrest assay or halo assay. For this purpose, filter disks were impregnated with 10 µl of α-factor solutions in various concentrations and placed onto the petri dishes containing DK102 cells which were expressing the fusion Ste2p receptors. The plates were incubated at 30 ^{o}C for 24 hours. After incubation clear zones which are also called halos were observed due to the fact that *Saccharomyces cerevisiae* MATa cells have arrested in G1 phase of the cell division cycle in response to the mating pheromone, alpha factor. The halos around the filter disks were measured and biological activity of each fusion receptor was determined by comparing halo size with the halos formed by wild type receptor.

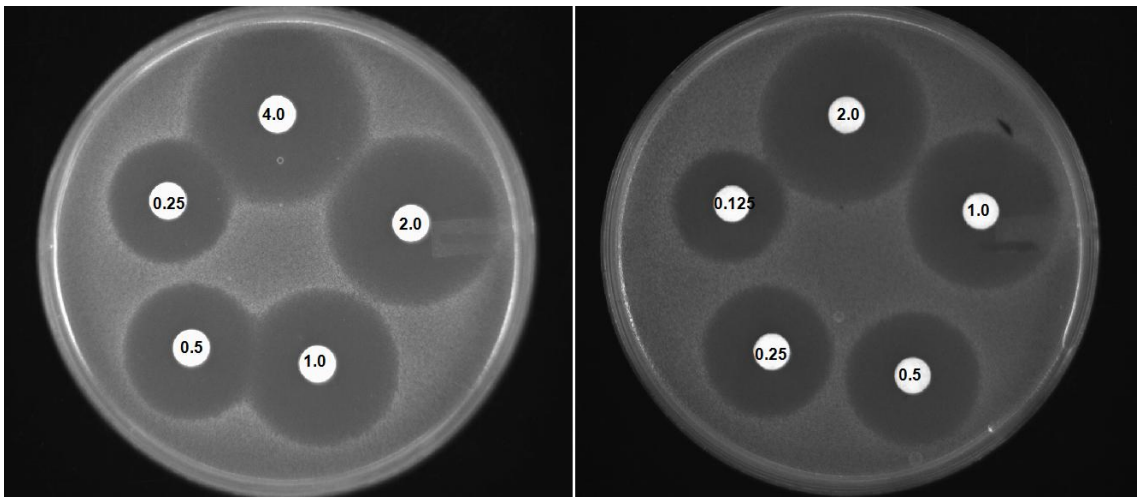

Figure 3.11 Representative images of growth inhibition zones (halo) in growth arrest assay. Various amounts of pheromone (0.125 – 0.4 µg) was absorbed onto filter disks.

2.4.1 Growth Arrest Assay for the plasmids pGNU304, pGCT304, pGNT441 and pGCU441

Growth arrest assay was performed for the Ste2p receptors that were labeled with EGFP fragments whose dissection site was 128th residue. Each pGCT304 and pNT441 receptors which originated from pBEC1 vector were analyzed together and diameters of halos compared with WT pBEC1 vector. Halo assay was repeated at least for three times for each of the plasmids and diameters of halos were plotted against peptide concentration. The reproducibility of the test the difference in inhibition zones was always kept within 2 mm for a given concentration. The results of the halo assay are shown below in Fig. 3.12.

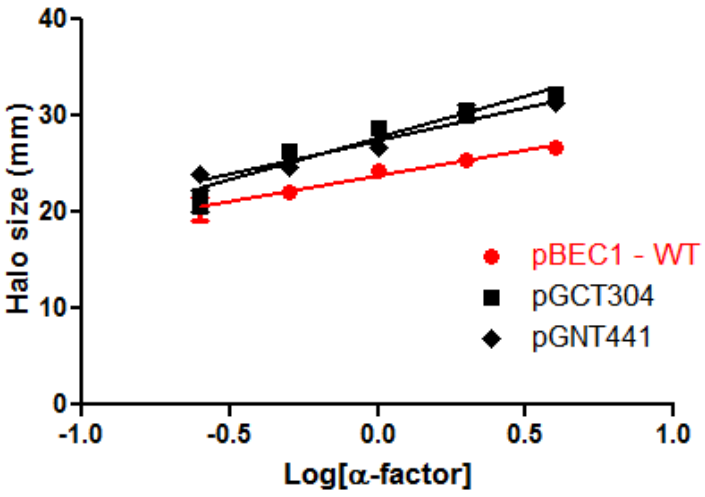

Figure 3.12 Biological assay for pGCT304 and pGNT441 constructs

In the same manner, pGNU304 and pGCU441 was analyzed together with pCL01 parental vector which indicated with red color in the graph. The graph of the results is given in Fig. 3.13.

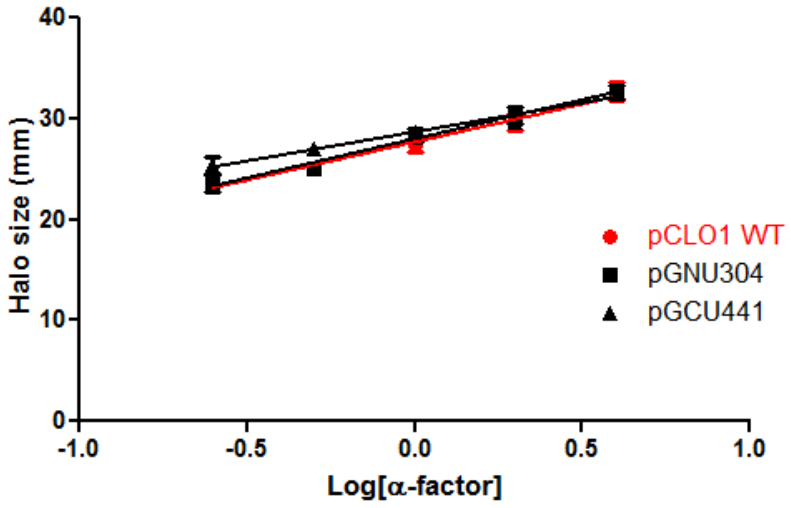

Figure 3.13 Biological assay for pGCT304 and pGNT441 constructs

The results of halo assay showed that, all transformants produce similar zones when compared the wild type receptor which indicates EGFP fragment tagged Ste2p receptors are biologically active.

2.4.2 Growth Arrest Assay for the plasmids pKNT304f, pKCU304f, pKNT304t, pKNT304t

Similar to the previous analysis Ste2p receptors which were labeled with EGFP fragments splited from 158th amino acid, are subjected to growth arrest assay to determine the biological activity. The results of the halo assay for the split EGFP couple plasmids pKNT304f and pKCU304f are shown below on Fig 3.14. The necessity to use different vectors for the fusion receptors, halo assay was carried out for wild

type with two different vector to have a reliable comparison, thus the data was shown in different graphs.

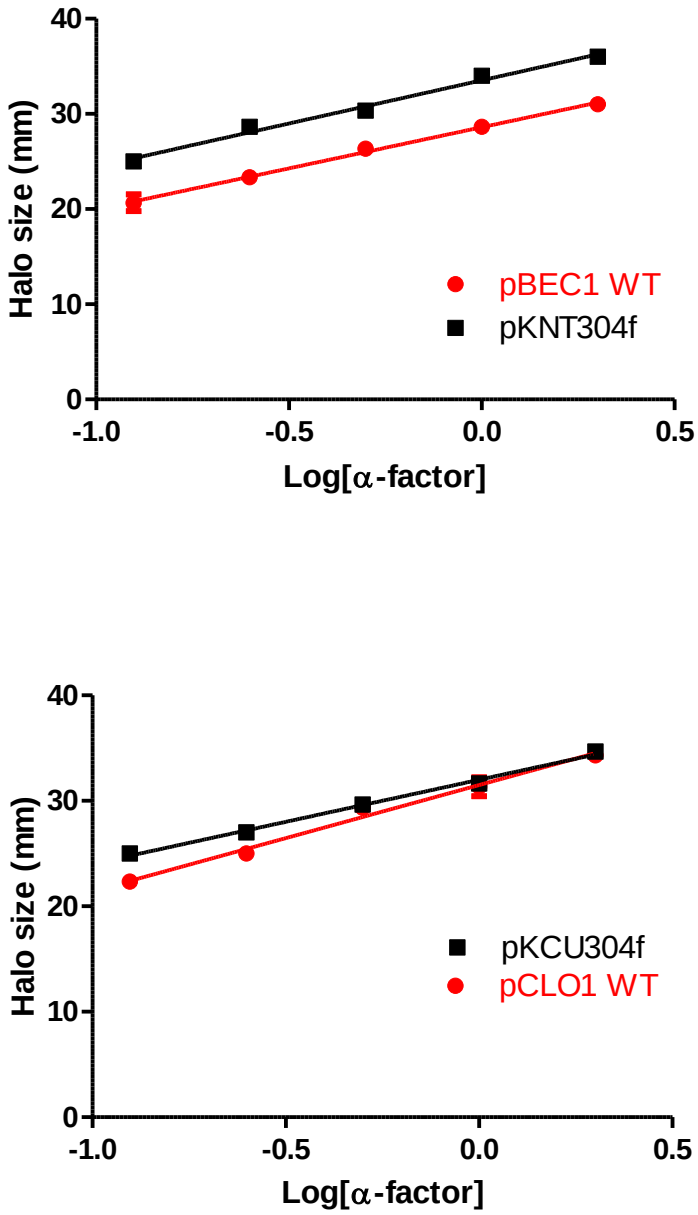

Figure 3.14 Biological assay for split EGFP couple; pKNT304f and pKCU304f

Consistent with the previous results insertion of fluorescent protein fragments to the receptor did not disturb the biological activity of Ste2p. The constructs did not show significant difference when compared to wild type.

In contrast to full length receptors, DK102 yeast cells expressing truncated receptor partners (pKNT304t and pKNT304t) show a different pattern in halo assay. Ligand binding and G-protein activation are not affected by C-tail truncation however these yeast cells form approximately 10 fold wider inhibition zones. The reason of obtaining extended zones is increased sensitivity to α-factor pheromone, due the fact that the C-terminal domain of the receptor contains phosphorylation sites (residues 392-431) which regulate the receptor sensitivity to pheromone (Dosil, Schandel, Gupta, Jenness, & Konopka, 2000). In this assay, the α-factor pheromone concentration reduced with a 1/10 dilution factor to enable measuring of diameter of halo sizes. Because of the all WT vectors contain full length Ste2p, full length EGFP labeled Ste2p was used for comparison.

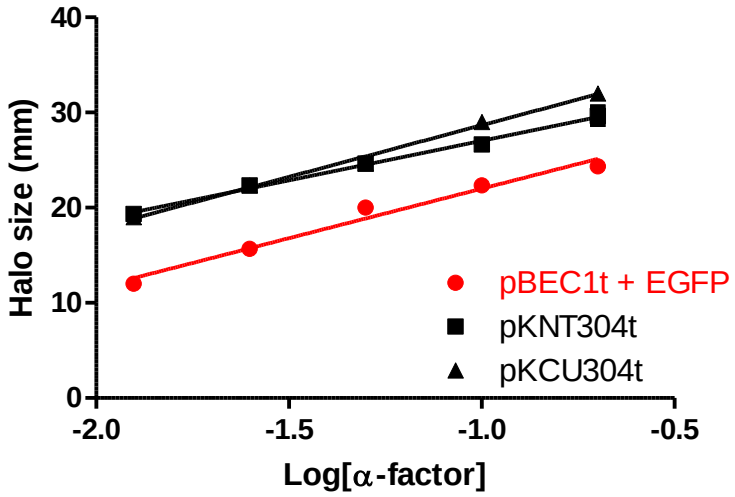

Figure 3.15 Biological assay for split EGFP couple; pKNT304t and pKCU304t

2.4.3 Growth Arrest Assay for the full length EGFP labeled Ste2p receptors

As seen below in Fig. 3.16, all of the fusion proteins tested, including EGFP + Ste2p at 441st position and EGFP + Ste2p at 304th position, were fully functional.

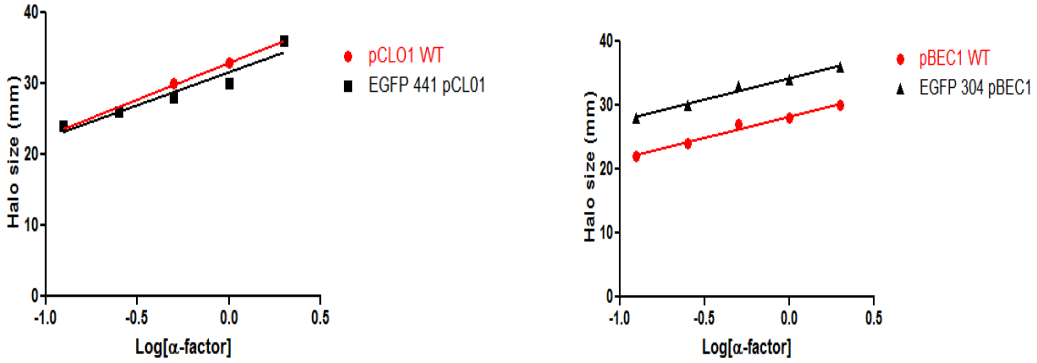

Figure 3.16 Biological assay for EGFP labeled Step receptors at position 304 and 441

2.5 EGFP reassembly and visualization of EGFP signal

Each of the constructed fusion receptors were shown to be fully functional with the biological activity assays. For co-expression of split EGFP partners, double transformation to yeast cells was performed. These partners are shown in Fig. 3.17.

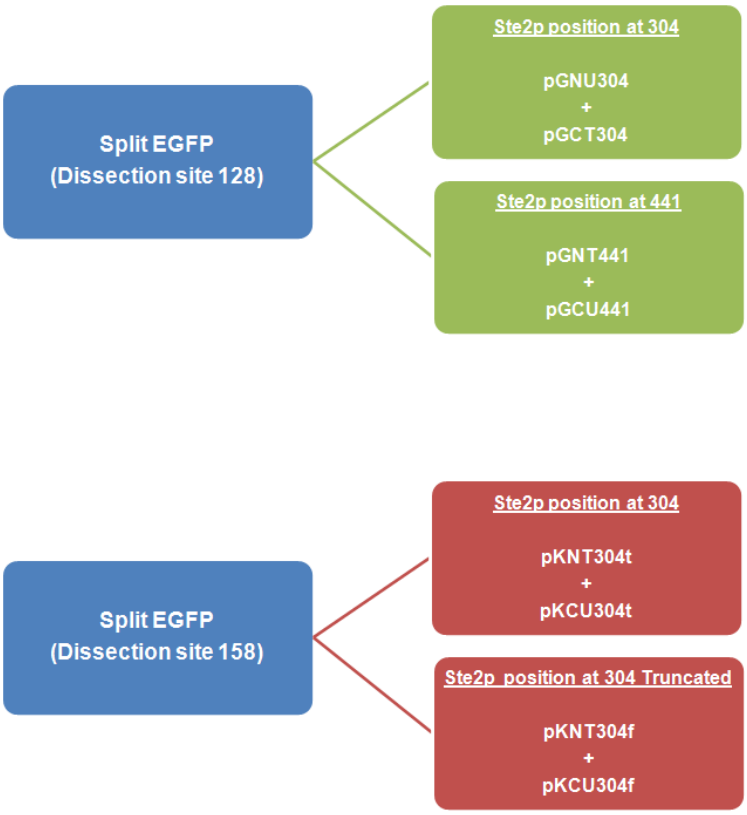

Figure 3.17 List of the Split-EGFP couples that were co-expressed in DK102 yeast cells

For imaging of the split-EGFP partners which was shown in Fig. 3.17, yeast cells were grown overnight and without any fixation, live cell imaging was carried out. Two control groups of each partner were used. One group with WT receptor without any fluorescent tags and the other group with the full EGFP tagged receptor. Cells were observed in single track configurations, see below on Fig. 3.18. As seen in the figure samples were excited at 488 nm and emission was collected in the 505-550 nm range.

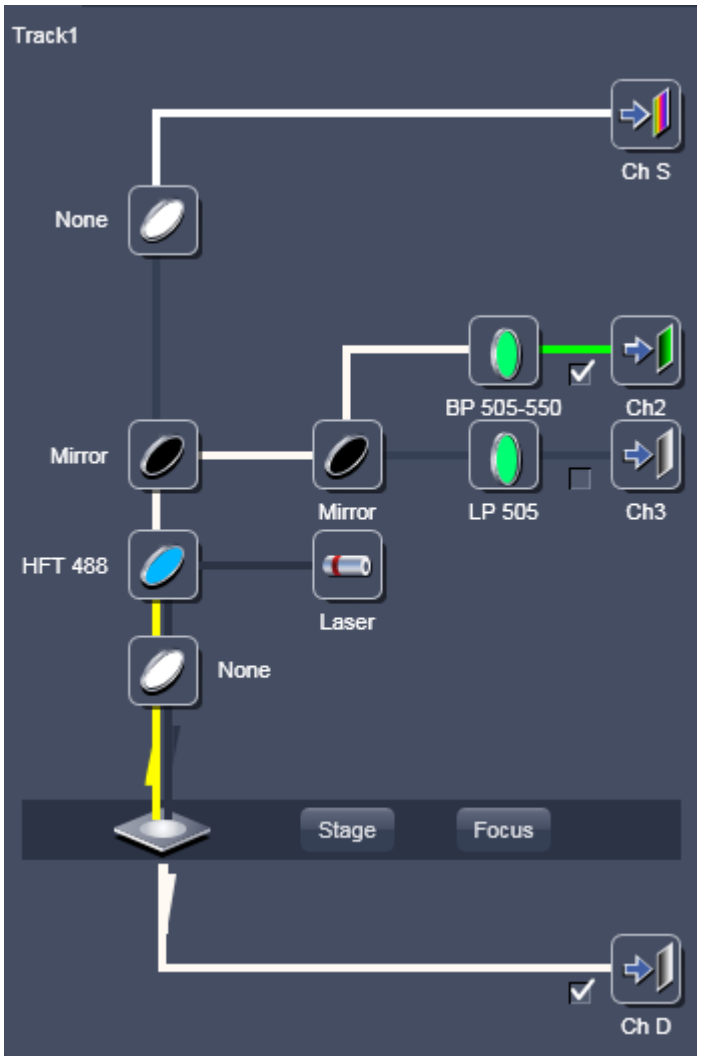

Figure 3.18 Configuration settings of laser scanning confocal microscope.

Split-EGFP couple which were splitted from 128th amino acids and inserted into Ste2p between the residues 304-305th and 441-442nd was analyzed under laser scanning confocal microscope (see Fig 3.19 and Fig. 3.20). Cells were observed under fluorescence and white light conditions. First row shows WT, DK102 yeast cell with empty vectors, second row shows DK102 yeast cells with full length EGFP in Ste2p

(304), third row shows DK102 yeast cells with N-EGFP and C-EGFP fragment inserted in to STE2p (304).

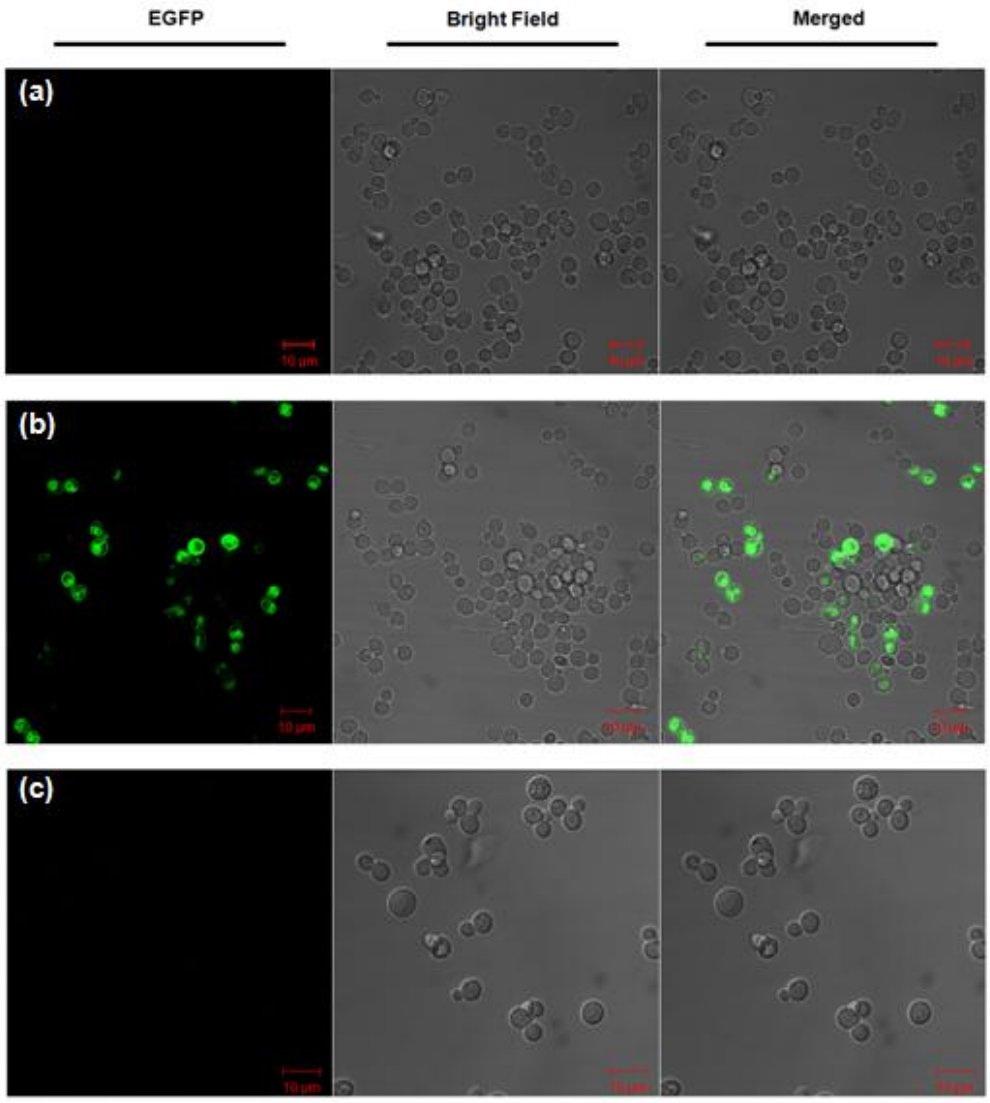

Figure 3.19 Cells which express labeled Ste2p were excited by the 488 nm laser line. (a) shows WT, DK102 yeast cell with empty vectors, (b) shows DK102 yeast cells with full length EGFP in Ste2p (304), (c) shows DK102 yeast cells with N-EGFP (1-128) and C-EGFP(129-239) fragment inserted in to STE2p (304).

Control group with unlabeled receptors did not have fluorescence signal, as expected whereas full length EGFP containing receptors represented in second row have fluorescence signal approximately in 50% of the cells.

Although the pGNU304 and pGCT304 constructs were biologically active, no signal was gathered from the cells which were co-expressing N-EGFP (1-128) and C-EGFP (129-239) fusion proteins labeled at position 304 (see Fig. 3.18 third row).

As the formation of fluorescence is influenced by the steric effect, the position of fragments should be favorable. In other words, labeled receptors should not prevent the association of EGFP fragments. Moreover, moving fluorescent label from 304 to 441 acts like a linker region, and therefore provides flexibility to increase the possibility of association of fluorescent fragments. Therefore, the labeling of receptor from different position in C-tail was examined, and N-EGFP (1-128) and C-EGFP (129-239) fragments were shifted to between 441-442nd residues. However, cellular fluorescence is not gained by this modification on position of the fragment.

Concerning the lower signal levels, master gain and laser power were increased up to 800 and 24%, respectively. The split-EGFP couples which were divided at 128th amino acid were observed under both low and high settings regarding laser power and gain. Images were acquired from same area at two different setups (see Fig 13.20). First row shows DK102 yeast cells expressing Ste2p fusion receptors (EGFP splitted from 128; and Ste2p labeled from 441) with a master gain 650 and laser power 15%. In second row, an image of the same area is shown with increased master gain (800) and laser power (24%). Unfortunately, these alterations in settings did not result with fluorescence signal in the plasma membrane from the receptors. However we observed auto-fluorescence signal received from the cytoplasmic region of some cells along with an increase in background noise. Cells with fluorescence shown to be granulated which indicates unhealthy states or non-living cells, see Fig 13.20, in red boxes. (Meissel, Medvedeva, & Poglazova, 1961; Wolinski et al., 2009).

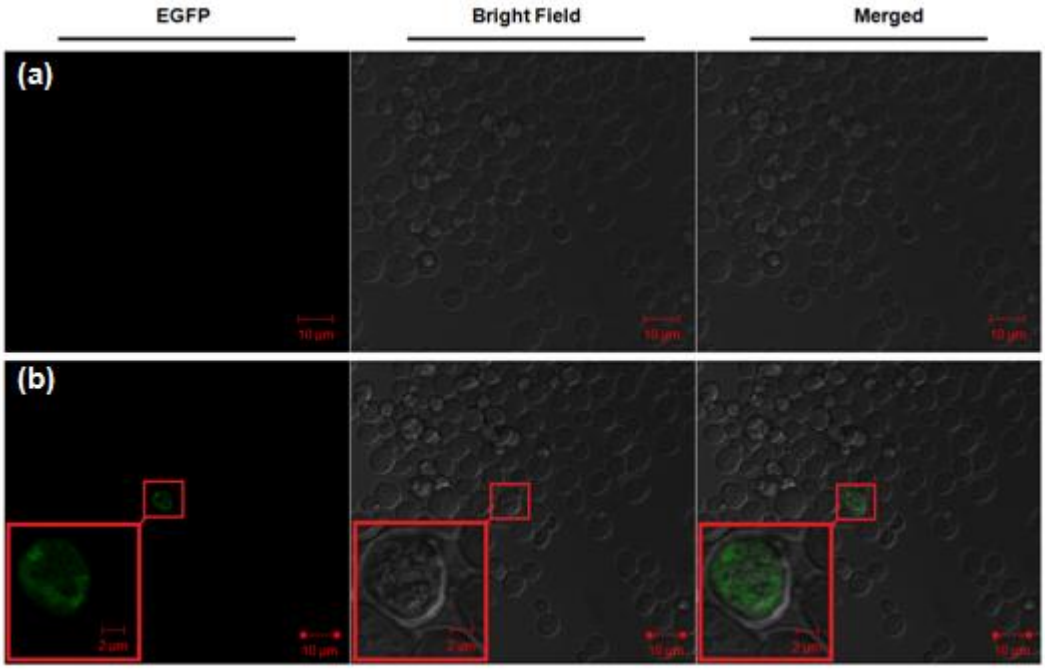

Figure 3.20 DK102 yeast cells which express N-EGFP (1-128) and C-EGFP (129-239) fragment labeled Ste2p (441) observed in different laser power and master gain conditions. (a) lower master gain and laser power, (b) higher master gain and laser power.

To rule out false-negatives, resulting from low expression levels of fusion receptors in yeast cells, western blot analysis was carried out for the plasmids pGNU304, pGCT304, pGNT441 and pGCU441. For this purpose, membrane preparation of modified Ste2p receptors was carried out, and anti-FLAG antibody (Eastman Kodak Co.) was used for immunobloting. High molecular weight bands were observed compared to WT Ste2p, as it was expected. Result of western blot is shown in Appendix F.

To eliminate the possibility that results are caused by topological limitations, dissection point in EGFP was also changed and fluorescent molecule was splitted between 158-159th residues. Additionally to increase the fluorescent signal, the C-tail truncation of

Ste2p was executed due to its effect on trafficking. The new dissection point (158) was successfully applied and fusion proteins were tested in terms of biological activity. Afterwards, split-EGFP partners pKNT304f/pKCU304f and pKNT304t/pKCU304t were co-expressed in DK102 yeast cells. Cells were observed under confocal microscope to test cellular fluorescence (Fig. 3.21 and Fig. 3.22).

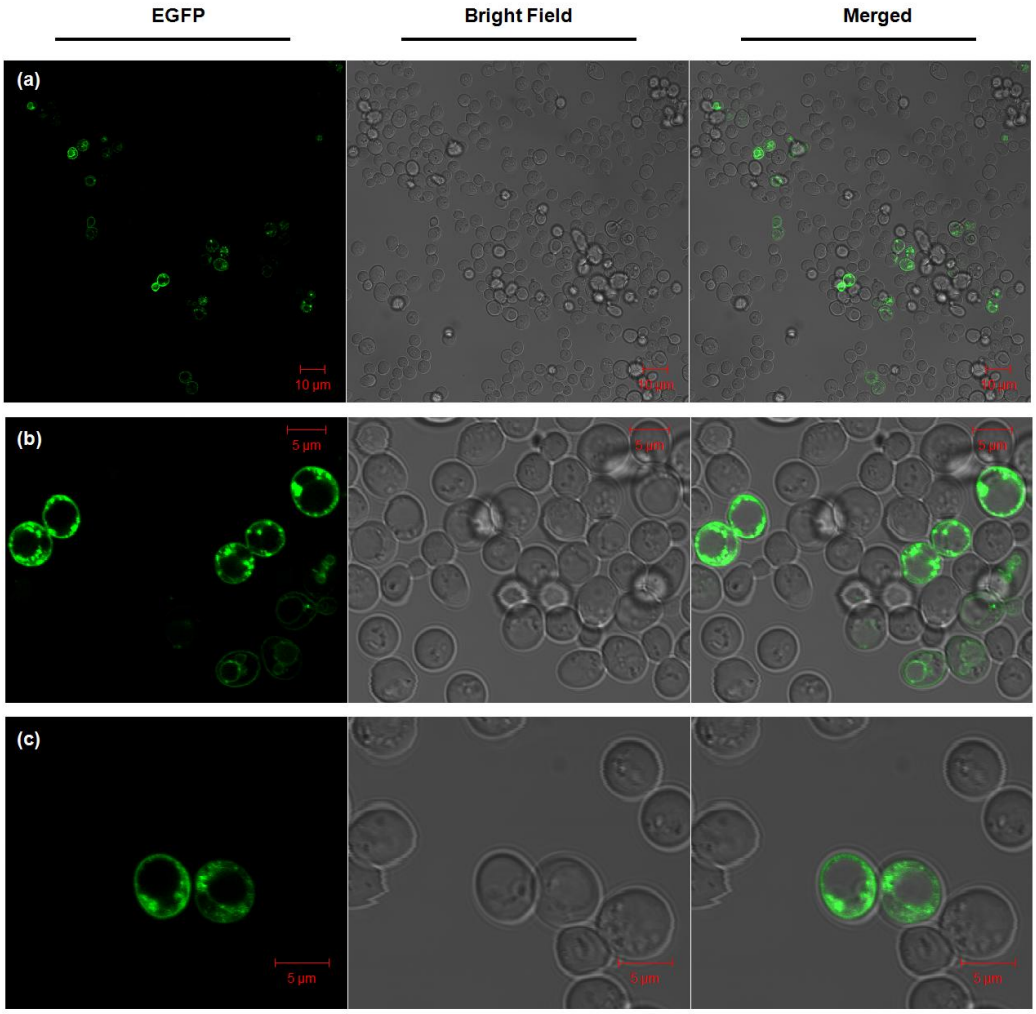

Figure 3.21 DK102 yeast cells which co-express N-EGFP (1-158) and C-EGFP (159-239) labeled Ste2p fusion receptors (a), (b) and (c) show DK102 yeast cells with N-EGFP (1-158) and C-EGFP(159-239) fragment inserted in to Ste2p (304).

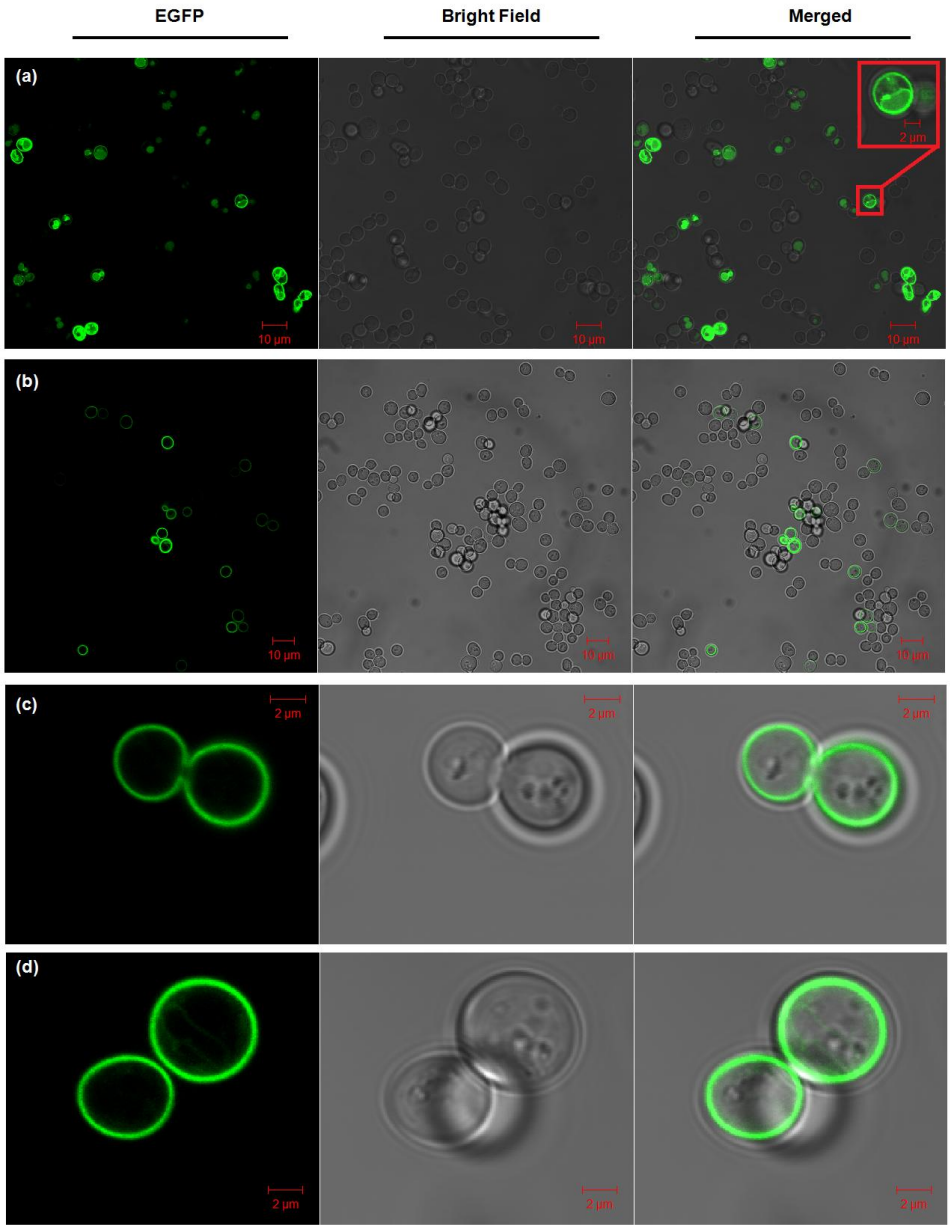

Figure 3.22 DK102 yeast cells which co-express N-EGFP (1-158) and C-EGFP (159-239) labeled Ste2p fusion receptors (truncated from 304). (a) shows DK102 yeast cells with full length EGFP in truncated Ste2p (304), (b), (c) and (d) show DK102 yeast cells with N-EGFP (1-128) and C-EGFP(129-239) fragment inserted in to truncated Ste2p (304), respectively.

In *Saccharomyces cerevisiae,* several other protein-protein interactions have been revealed using BiFC technique. A combination of split-GFP and yeast two hybrids system was applied to detect interaction between Gal4p and Gal11p (Park et al., 2007). Another group utilized homologous recombination to label target proteins in chromosomal level. Sis1p in *MAT***a** yeast cells and and Ssa1p in *MATα* yeast cells were labeled with YFP (Yellow Fluorescent Protein) fragments, and fluorescence signal was observed in diploid yeast cells (Sung & Huh, 2007). In a different study a plasmid based system, resembling to our approach, was used to study protein-protein interactions in different subcellular compartments including mitochondria and nucleus (Barnard, et al., 2008). In this study we demonstrated that labeling of Ste2p with EGFP fragments, which were splited from 158th residue, results in cellular fluorescence signal both for full length and truncated receptors. Fluorescence signal acquired which in fact generated through the reassembly of fragments directed by the dimerization of yeast α-pheromone receptor Ste2p. Previous dimerization studies done with Ste2p have been carried out with truncated receptors (Gehret, et al., 2006; Overton & Blumer, 2000). Due to previous literature claiming that the signal from full length labeled receptors is too low to detect, we tried truncated receptors in our study and signal from truncated receptors was obtained in the plasma membrane. C-tail truncation of Ste2p receptors from 304th residue does not show an impact on cell surface targeting and high fluorescence signal is observed in plasma membrane indicating possible increase in the accumulation of receptor. This is most probably caused by the fact that the signal sequences for the internalization of receptor were found in the cytoplasmic C-tail of the pheromone receptor and with C-tail deletion endocytosis was inhibited (Kim *et al.*, 2012). Therefore, studying dimerization interfaces with truncated receptors is possible due to the increase cell surface signal. However, it will cause complications regarding localization and where dimerization processes take place. This is the reason why we used both full length and truncated receptors for studying Ste2p dimerization in live cells.

The regional signal difference between truncated and full-length receptor split-EGFP partners are distinguished in which truncated receptor expressing cells give higher

fluorescence level in plasma membrane where as the full-length receptor expressing cells has internal signal. Additionally, in full-length receptor split-EGFP samples (Fig. 3.21) the higher fluorescent intensity dots were observed which may indicate transport vesicles in the cell. The observation of these dots were attained not in truncated receptor expressing cells but only in full-length receptor expressing yeast cells showing that these dots might be related with endocytosis processes rather than biosynthesis. Another interesting finding supporting this idea is that; as opposed to truncated split-EGFP couples (Fig. 3.22.b, c and d), the yeast cells which were expressing full-length EGFP labeled truncated Ste2p receptors give internal signal (Fig 3.22 a). In the light of these information it may be claimed that dimerization of Ste2p receptor take place in plasma membrane. However for the justification of this hypothesis further analysis were needed.

It should also be noted that spontaneous reassembly of N-EGFP and C-EGFP fragments is highly unlikely because of the affinity between fragments is too low when they are co-expressed together (Magliery, *et al.*, 2005).

The possible combinations of EGFP partners attained with dimerization of Ste2p are; N-EGFP/N-EGFP, N-EGFP/C-EGFP or C-EGFP/C-EGFP. Only the N-EGFP/C-EGFP interaction will give split-EGFP positive results therefore it is important to note that, dimerization of receptors may result in random association of the same EGFP fragments causes loss of fluorophore reconstitution. The highest fluorescence level attainable with homodimer pair is only half that possible in comparison with a heterodimer.

CHAPTER 4

CONCLUSION

The aim of this study was to design and construct fusion proteins for the implementation of split-EGFP techniques which is a bimolecular fluorescence complementation technique to detect and visualize the GPCR interactions in yeast cells.

At the end of the study,

- The PCR integration method was optimized for cloning of EGFP gene fragments to the Ste2p receptor. The enhanced green fluorescent protein fractionated into two fragments at genetic level, and Ste2p receptor was successfully labeled with these fragments from its C-terminal.

- Sequences of the constructs was verified, and the Ste2p receptors labeled with EGFP fragments have been examined by biological activity test and it has been shown that all labeled receptors showed wild type like biological activity. It was demonstrated that, inserts within the range of 81-158 bp does not alter the Ste2p function.

- Different dissection sites were examined to increase the fluorescence signal and it was concluded that dissection of EGFP molecule from the 158^{th} amino acid enable the EGFP molecule to reassemble and regain its fluorescence function.

- It has been established that split-EGFP technique is able to detect interactions between two membrane proteins only if the proteins possess cytosolic domains. We showed the direct visualization of Ste2p receptor homodimerization in live cells using Split-EGFP method for the first time.

- Although the EGFP fragments which were dissected from 128th amino acid was biologically active and expressed well, no signal was gathered from imaging analyses. Furthermore, different insertion site (441) was also examined since the formation of fluorescence was affected from steric effect however no increase in signal was detected.

- The high fluorescent intensity dots were observed in cytosolic region of yeast cells expressing full length receptors however the signal in yeast cells which expressing truncated receptors was only localized in the membrane. According to the results it is tempting to speculate that these dots were vesicles of endocytosis, and dimerization take place in plasma membrane thus Ste2p receptor internalize as dimers.

- The split-EGFP fusion proteins which were constructed in this study can be used for further analysis of localization of the interaction.

- Additionally, for dimerization, effects of post translational modifications or agonist and/or antagonist binding can be examined using split-EGFP constructs. Also, dimer interfaces between receptors can be mapped by random mutagenesis in Ste2p.

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APPENDIX A

REPRESENTETIVE IMAGE OF NANODROP MEASUREMENT FOR DNA CONCENTRATION

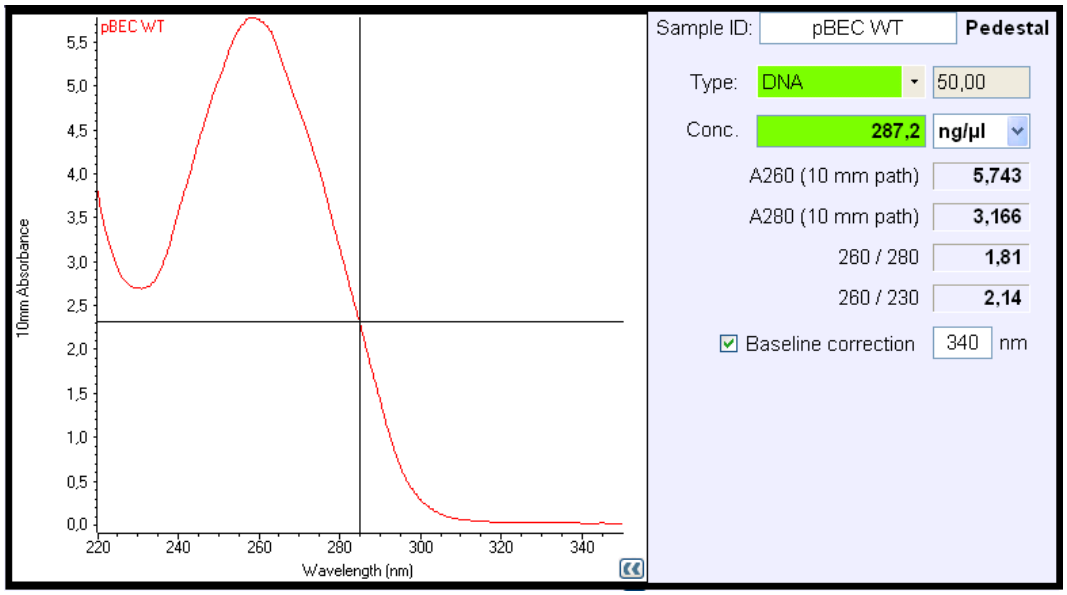

Figure A.1 Representative image of nano-drop measurement for DNA concentration

APPENDIX B

YEAST MEDIA PREPARATION

Table B.1 Composition of dropout mix (stock) for Media Lack of Tryptophane, Uracil,Tryptophane and Uracil (MLT, MLU and MLTU)

Component	**Final concentration (g/L)**
Adenine Sulfate	0.058
Arginine HCl	0.026
Asparagine	0.058
Aspartic Acid	0.14
Glutamic Acid	0.14
Histidine HCl	0.028
Isoleucine	0.028
Leucine	0.083
Lysine	0.042
Methonine	0.028
Phenylalanine	0.69
Serine	0.52
Threonine	0.28
Tyrosine	0.042
Tryptophane*	0.03
Valine	0.21
Uracil*	0.028

***** These aminoacids were cancelled from the formulation of dropout mix one by one or together due to the intended selectivity.
All ingredients are weighed and combined in a dark bottle and mixed completely.

Medium composition

20 g/L	Glucose
10 g/L	Casamino Acids
6.7 g/L	YNB without Aminoacids
1.8 g/L	Dropout mix

15 g/L Agar is added to the media after autoclave sterilization for solid agar plate preparation.

YEPD Media Composition

10 g/L	Yeast extract
20 g/L	Peptone
20g/L	Glucose

For YEPD agar 15g/L agar is added to the mixture and sterilized by autoclaving.

APPENDIX C

BACTERIAL MEDIA INGREDIENTS

Luria-Bertani (LB) Medium

10 g/L Tryptone

5 g/L Yeast extract

5 g/L NaCl

15 g/L Agar is added to the media after autoclave sterilization for solid agar plate preparation.

Super Optimum Broth with Catabolite repression (SOC)

20 g/L Tryptone

5 g/L Yeast Extract

0.5 g/L NaCL

0.186 g/L KCl

0.952 g/L $MgCl_2$

2.408 g/L $MgSO_4$

3.603 g/L Glucose

pH adjusted to 7.0 and medium was sterilized by autoclaving at 121 °C for 20 minutes.

APPENDIX D

SOLUTIONS AND BUFFERS

6X DNA Loading Dye Ingredients

10 mM Tris-HCl (pH7.6)

0.03% Bromophenol Blue

0.03% Xylene Cyanol FF

60% Glycerol

60 mM EDTA

10X Tris-Borate-EDTA (TBE) Buffer

108 g/L Tris Base (890 mM)

55 g/L Boric Acid (890 mM)

40 ml/L EDTA (20 mM)

All ingredients was added to 800 ml of dH_2O and volume was adjusted to 1L. For gel electrophoresis, the solution was diluted 1:10.

1X NEB Buffer 4

20 mM Tris-acetate

50 mM Potassium acetate

10 mM Magnesium acetate

1 mM Dithiothreitol

pH 7.9 at 25 °C

Single-stranded Carrier DNA (2 mg/ml)

200 mg of salmon sperm DNA (DNA Sodium Salt from salmon testes, Sigma D1626) was dissolved in 100 ml of TE buffer (Sigma, #93283) on a magnetic stirrer for 3-5 hours. 500 µl aliquots of the solution were prepared and stored in -20 °C.

1.0M Lithium Acetate Solution

Prepared as stock solution in distilled deionized water (ddH_2O). The solution was filter sterilized and final pH was adjusted to 8.4 – 8.9. Required dilution was made to obtain 100 mM Lithium acetate solution.

Polyethylene glycol (PEG 50% withv)

50 mg of the polyethylene glycol (PEG) (Sigma, #P3640) was mixed with 35 ml of ddH_2O on a magnetic stirrer until it solved completely. Volume was adjusted to 100 ml and the solution sterilized by autoclave. For long term storage the cap of the bottle sealed with parafilm to prevent evaporation of water and increase in PEG concentration.

APPENDIX E

CODING SEQUENCES OF FUSION PROTEINS THAT CONSTRUCTED IN THIS STUDY

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCAC
AATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAGA
TTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATAA
TATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGTAT
TGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAATGGTGAGCAAGGGCGAGGAGCT
GTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGGCG
AGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTAC
AAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCACAAACACAAT
TACTTCAGACTTTACAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTAGCTTTCAAACTGATAGTATCAACAA
CGATGCTAAAAGCAGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATCGGATAAACATT
CGGAAAGAACTTTTGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCCACACCTACGAGT
TCAAAAAATACTAGGATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCGTCGACATGTACA
CTCCCGATACGGCAGCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTTAGACTACAAGGACGA
CGATGACAAGACCGGTGTGCCGCGCGGCAGCGGCAGCAGCCATCATCATCATCATCATAGCAGCGGCTAA

Figure F.1 Coding sequence of N-EGFP (1-128) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to N-EGFP (1-128) fragment coding sequence. Insertion site is between 304-305th residues.

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCA
CAATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAG
ATTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATA
ATATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGT
ATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAATCGACTTCAAGGAGGACGGCA
ACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCAGAAGAACG
GCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGA
ACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAA
GACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGA
CGAGCTGTACAAGACAAACACAATTACTTCAGACTTTACAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTA
GCTTTCAAACTGATAGTATCAACAACGATGCTAAAAGCAGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGG
AAGGAAACAACATCGGATAAACATTCGGAAAGAACTTTTGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTT
TTATCAGTTGCCCACACCTACGAGTTCAAAAAATACTAGGATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGA
GAAGTTGAACCCGTCGACATGTACACTCCCGATACGGCAGCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGAT
AATAATAATTTAGACTACAAGGACGACGATGACAAGACCGGTGTGCCGCGCGGCAGCGGCAGCAGCCATCATCAT
CATCATCATAGCAGCGGCTAA

Figure F.2 Coding sequence of C-EGFP (129-239) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to C-EGFP (129-239) fragment coding sequence. Insertion site is between 304-305th residues.

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCAC
AATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAGA
TTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATAA
TATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGTAT
TGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAACAAACACAATTACTTCAGACTTTA
CAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTAGCTTTCAAACTGATAGTATCAACAACGATGCTAAAAGC
AGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATCGGATAAACATTCGGAAAGAACTTT
TGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCCACACCTACGAGTTCAAAAAATACTAG
GATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCGTCGACATGTACACTCCCGATACGGCA
GCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTTAGACTACAAGGACGACGATGACAAGACCG
GTATGGTGAGCAAGGGCGAGGAGCTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAAC
GGCCACAAGTTCAGCGTGTCCGGCGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCCTGAAGTTCATCTG
CACCACCGGCAAGCTGCCCGTGCCCTGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCC
GCTACCCCGACCACATGAAGCAGCACGACTTCTTCAAGTCCGCCATGCCCGAAGGCTACGTCCAGGAGCGCACCA
TCTTCTTCAAGGACGACGGCAACTACAAGACCCGCGCCGAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCA
TCGAGCTGAAGGGCGTGCCGCGCGGCAGCGGCAGCAGCCATCATCATCATCATAGCAGCGGCTAA

Figure F.3 Coding sequence of N-EGFP (1-128) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to N-EGFP (1-128) fragment coding sequence. Insertion site is between 441-442nd residues

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCAC
AATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAGA
TTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATAA
TATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGTAT
TGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAACAAACACAATTACTTCAGACTTTA
CAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTAGCTTTCAAACTGATAGTATCAACAACGATGCTAAAAGC
AGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATCGGATAAACATTCGGAAAGAACTTT
TGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCCACACCTACGAGTTCAAAAAATACTAG
GATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCGTCGACATGTACACTCCCGATACGGCA
GCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTTAGACTACAAGGACGACGATGACAAGACCG
GTATCGACTTCAAGGAGGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATAT
CATGGCCGACAAGCAGAAGAACGGCATCAAGGTGAACTTCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCA
GCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGA
GCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCC
GCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGGTGCCGCGCGGCAGCGGCAGCAGCCATCATCATCATCA
TCATAGCAGCGGCTAA

Figure F.4 Coding sequence of C-EGFP (129-239) fragment inserted Ste2p fusion protein. Black sequences belong to Ste2p and highlighted parts belong to C-EGFP (129-239) fragment coding sequence. Insertion site is between 441-442nd residues

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCA
CAATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAG
ATTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATA
ATATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGT
ATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAATGGTGAGCAAGGGCGAGGAG
CTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCCGAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACA
AGACCCGCGCAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA
GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTTATATCATGGCCGACAAGCAG
ACAAACACAATTACTTCAGACTTTACAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTAGCTTTCAAACTGAT
AGTATCAACAACGATGCTAAAAGCAGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACATC
GGATAAACATTCGGAAAGAACTTTTGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCCA
CACCTACGAGTTCAAAAAATACTAGGATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCGT
CGACATGTACACTCCCGATACGGCAGCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTTAGAC
TACAAGGACGACGATGACAAGACCGGTGTGCCGCGCGGCAGCGGCAGCAGCCATCATCATCATCATCATAGCAGC
GGCTAA

Figure F.1 Coding sequence of N-EGFP (1-158) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to N-EGFP (1-158) fragment coding sequence. Insertion site is between 304-305th residues.

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCA
CAATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAG
ATTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATA
ATATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGT
ATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAAAGAACGGCATCAAGGTGAACT
TCAAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGC
GACGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAA
GCGCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTA
AACAAACACAATTACTTCAGACTTTACAACATCCACAGATAGGTTTTATCCAGGCACGCTGTCTAGCTTTCAAACTGA
TAGTATCAACAACGATGCTAAAAGCAGTCTCAGAAGTAGATTATATGACCTATATCCTAGAAGGAAGGAAACAACAT
CGGATAAACATTCGGAAAGAACTTTTGTTTCTGAGACTGCAGATGATATAGAGAAAAATCAGTTTTATCAGTTGCCC
ACACCTACGAGTTCAAAAAATACTAGGATAGGACCGTTTGCTGATGCAAGTTACAAAGAGGGAGAAGTTGAACCCG
TCGACATGTACACTCCCGATACGGCAGCTGATGAGGAAGCCAGAAAGTTCTGGACTGAAGATAATAATAATTTAGA
CTACAAGGACGACGATGACAAGACCGGTGTGCCGCGCGGCAGCGGCAGCAGCCATCATCATCATCATCATAGCAG
CGGCTAA

Figure F.2 Coding sequence of C-EGFP (159-239) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to C-EGFP (159-239) fragment coding sequence. Insertion site is between 304-305th residues.

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCA
CAATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAG
ATTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATA
ATATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGT
ATTGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAATGGTGAGCAAGGGCGAGGAG
CTGTTCACCGGGGTGGTGCCCATCCTGGTCGAGCTGGACGGCGACGTAAACGGCCACAAGTTCAGCGTGTCCGG
CGAGGGCGAGGGCGATGCCACCTACGGCAAGCTGACCGAAGTTCATCTGCACCACCGGCAAGCTGCCCGTGCCC
TGGCCCACCCTCGTGACCACCCTGACCTACGGCGTGCAGTGCTTCAGCCGCTACCCCGACCACATGAAGCAGCAC
GACTTCTTCAAGTCCGCCATGCCCGAGGCTACGTCCAGGAGCGCACCATCTTCTTCAAGGACGACGGCAACTACA
AGACCCGCGCAGGTGAAGTTCGAGGGCGACACCCTGGTGAACCGCATCGAGCTGAAGGGCATCGACTTCAAGGA
GGACGGCAACATCCTGGGGCACAAGCTGGAGTACAACTACAACAGCCACAACGTCTATATCATGGCCGACAAGCA
GTAA

Figure F.3 Coding sequence of N-EGFP (1-158) fragment inserted Ste2p fusion protein. Black sequence belongs to Ste2p and highlighted parts belong to N-EGFP (1-158) fragment coding sequence. Insertion site is after 304th residue and Ste2p receptor is truncated after insertion.

ATGTCTGATGCGGCTCCTTCATTGAGCAATCTATTTTATGATCCAACGTATAATCCTGGTCAAAGCACCATTAACTAC
ACTTCCATATATGGGAATGGATCTACCATCACTTTCGATGAGTTGCAAGGTTTAGTTAACAGTACTGTTACTCAGGC
CATTATGTTTGGTGTCAGATGTGGTGCAGCTGCTTTGACTTTGATTGTCATGTGGATGACATCGAGAAGCAGAAAAA
CGCCGATTTTCATTATCAACCAAGTTTCATTGTTTTTAATCATTTTGCATTCTGCACTCTATTTTAAATATTTACTGTCT
AATTACTCTTCAGTGACTTACGCTCTCACCGGATTTCCTCAGTTCATCAGTAGAGGTGACGTTCATGTTTATGGTGC
TACAAATATAATTCAAGTCCTTCTTGTGGCTTCTATTGAGACTTCACTGGTGTTTCAGATAAAAGTTATTTTCACAGG
CGACAACTTCAAAAGGATAGGTTTGATGCTGACGTCGATATCTTTCACTTTAGGGATTGCTACAGTTACCATGTATTT
TGTAAGCGCTGTTAAAGGTATGATTGTGACTTATAATGATGTTAGTGCCACCCAAGATAAATACTTCAATGCATCCAC
AATTTTACTTGCATCCTCAATAAACTTTATGTCATTTGTCCTGGTAGTTAAATTGATTTTAGCTATTAGATCAAGAAGA
TTCCTTGGTCTCAAGCAGTTCGATAGTTTCCATATTTTACTCATAATGTCATGTCAATCTTTGTTGGTTCCATCGATAA
TATTCATCCTCGCATACAGTTTGAAACCAAACCAGGGAACAGATGTCTTGACTACTGTTGCAACATTACTTGCTGTAT
TGTCTTTACCATTATCATCAATGTGGGCCACGGCTGCTAATAATGCATCCAAAAAGAACGGCATCAAGGTGAACTTC
AAGATCCGCCACAACATCGAGGACGGCAGCGTGCAGCTCGCCGACCACTACCAGCAGAACACCCCCATCGGCGA
CGGCCCCGTGCTGCTGCCCGACAACCACTACCTGAGCACCCAGTCCGCCCTGAGCAAAGACCCCAACGAGAAGC
GCGATCACATGGTCCTGCTGGAGTTCGTGACCGCCGCCGGGATCACTCTCGGCATGGACGAGCTGTACAAGTAA

Figure F.4 Coding sequence of C-EGFP (159-239) fragment inserted Ste2p fusion protein. Black sequences belong to Ste2p and highlighted parts belong to C-EGFP (159-239) fragment coding sequence. Insertion site is after 304th residue and Ste2p receptor is truncated after insertion.

APPENDIX F

MEMBRANE PREPARATION AND WESTERN BLOT ANALYSES FOR THE DETERMINATION OF EXPRESSION LEVELS OF pGNU304, pGCT304, PGNT441 and pGCU441

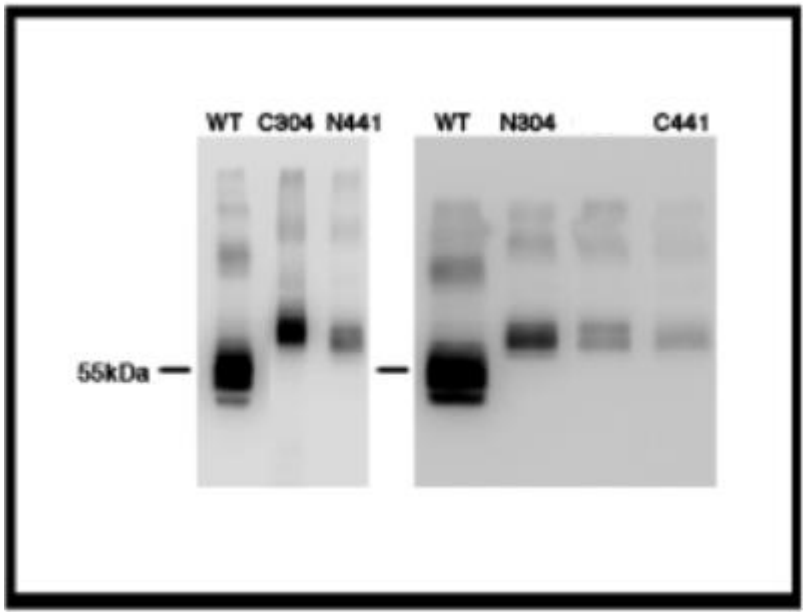

Figure F.1 Verification of EGFP labeled receptor expression in protein level. Total membrane protein was analyzed by SDS-PAGE and immunoblotted with anti-FLAG antibody.

APPENDIX G

PREPARATION OF SDS-PAGE SOLUTIONS

1.5mM Tris pH 8.8, 0.4% SDS was prepared by adding 18g Tris Base and 0.4 g SDS in 100mL distilled H_2O, pH was adjusted to 8.8 with HCl.

0.5M Tris-HCL pH 6.8, 0.4% SDS was prepared by adding 6.055g Tris and 0.4 g SDS in 100 mL distilled H_2O and pH was adjusted to 6.8 with HCl.

10% Ammonium persulfate prepared by adding 1g APS in 10mL H_2O

Table G.1 The composition of 10X SDS Running Buffer

Components	**Amount**
25mM Tris	30.3 g
20mM Glycine	188 g
SDS (1%)	10g

All the components were added to 1 L of distilled H_2O pH was adjusted to 8.3

Table G.2 The composition of 6X Loading Buffer

Components	**Amount**
0.5 M Tris HCl (pH 6.8)	1.2 mL
Glycerol	4.7 mL
SDS	1.2 g
Bromophenol Blue	6 mg
Distilled H_2O	2.1 mL
β-Mercapto ethanol	50 µL (freshly added for 950µL)

Table G.3 The composition of SDS Polyacrylamide ge**l**

Components	**Amounts in 12% Gel**	**Amounts in 6% stacking Gel**
Distilled H_2O	2.6 mL	2.6 mL
30% Bis-Acrylamide	3.2 mL	1 mL
Tris	2.2 mL 1.5 M TRIS pH 8.8	1.25 mL 0.5 M TRIS pH 6.8
10% SDS	80 µL	50 µL
10 % APS	80 µL	50µL
TEMED	8 µL	5 µL
Total volume	8mL	5mL

Table G.4 The composition of Comassie Gel stain and destaining solution

Components	**Staning solution**	**Destaining Solution**
Coomassie Brilliant Blue R-250	2.5 g	-
Ethanol	500 mL	165 mL
Ultrapure H_2O	400 mL	785 mL
Acetic Acid	100 mL	50 mL