ALTERNATIVE ROLES FOR STE2P AND AN α-ARRESTIN IN *SACCCHAROMYCES CEREVISIAE* MATING

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ABSTRACT

Ste2p and Ste3p are well-characterized yeast pheromone G-protein Coupled Receptors (GPCR) those are involved in the signaling of mating responses that lead to cell fusion. Their signaling–associated interactions with G-protein/MAPK signal transduction machinery are well established, homologous to those in mammalian systems, and serve as a simplified model system in GPCR research. While the arrestin-mediated biased signaling mechanism of mammalian GPCR has not been discovered for the pheromone receptors, a recent demonstration of α-arrestins being involved in the internalization of the pheromone GPCR, Ste2p was reported. The present study was designed to reevaluate and extend the alternate functionality for pheromone receptors and to determine the role of yeast arrestins in the yeast mating. Specific residues in the TM6 of Ste2p exhibiting strong mating and constitutive MAPK signaling were combined and investigated in terms of their effect on MAPK signal transduction leading to cell cycle arrest as well as their impact on downstream mating projection formation and zygote formation events. Our findings indicate that Ste2p possess as specific residues that govern its relative bias for mediating MAPK signaling or mating events. Relative dose response experiments accounting for systemic and observation bias for these mutations yielded evidence of mutational-derived functional biases for Ste2p and further validated the alternate pheromone dependent functionalities for Ste2p.

Further, arrestin knockout and knock-in studies showed that Art1 (Ldb19) is selectively involved in the regulation of zygote formation but not MAPK signal transduction following the binding of ligand to Ste2p receptors. In addition, ligand stimulated selective localization of Art1 (Ldb19) to the mating projection, implicating it in the regulation of downstream mating functionalities. Overall, while leaving the full mechanism of alternate/biased Ste2p signaling to be elucidated, these results highlight the possibility of continued relevance of the yeast pheromone-mating pathway as a simplified model for GPCR research in the context of arrestin-mediated biased GPCR signaling.

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blessings from heaven that kept me going during all the hard time that I faced in my life. During your critical illness, your calmness and bravery gave me a new meaning of life. Mom, I really appreciate your courage during the toughest time in our life. Thanks for caring, loving and being with me all time.

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DEDICATION

I would like to dedicate this thesis to my father Dr Ramdev and my mother Mohini, for their endless love, support and encouragement.

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LIST OF ABREVIATIONS

AP2: Adaptor protein 2

AT1A: Angiotensin II type 1A

$AT_{1A}R$: Angiotensin type 1A receptor

BAD: Bcl-2-associated death promoter

CA: Constitutive active

CaM: Calmodulin

CAM: Constitutive active mutant

CBD: Carboxy terminal binding domain

CCA: Cell cycle arrest

CCPs: Clathrin coated pits

CCR7: Chemokine receptor

D1A: Dopamine 1A

DAG: Diacyl gltcerol

DOR: Delta opioid receptor

E.coli: *Eschericia coli*

ECL: Extracellular loop

EIF4E: Eukaryotic translation initiation factor 4E

Endoplasmic reticulum: ER

GAP: GTPase accelerating protein

GDP: Guanidine diphosphate

GFP: Green fluorescent protein

GPCRs: G protein coupled receptors

GRKs: G-protein coupled receptor kinases

GTP: Guanidine triphosphate

HOG: High osmolarity glycerol

ICL: Intracellular loop

IP3: 1,4,5 inositol triphosphate

IRS-1: Insulin receptor substrate

MAPK: Mitogen activated protein kinase

MKK: MAPK kinase

MEK: MAP/extracellular signal-regulated kinase

MKKK: MAPK kinase kinase

MEKK: MEK: MAP/extracellular signal-regulated kinase kinase

MPF: Mating projection formation

MPR: μ-opioid receptor

MVB: Multivesicular bodies

NEB: New England Biolabs

NRC: National research council

ONPG: 2-nitrophenyl β-D-galactopyranoside

PAK: p21 activated protein kinase

PI3: Phosphoinositide kinase

PIP2: Phosphatidyl-inositol 4,5-biphosphate

PKA: Protein kinase A

PKC: Protein kinase C

PLCβ: Phospholipase C-β

RGS: Regulators of G protein signaling

RI media: Rich induction media

RTK: Receptor tyrosine kinase

S. cerevisiae: *Saccharomyces cerevisiae*

SAM: Sterile alpha motif

SH2: Src homology domain 2

SH3: Src homology domain 3

SOS: Son of sevenless

TMDs: Trans-membrane domains

TRH: Thyrotrophin releasing hormone

WT: Wild type

α1bAR: α1b adrenergic receptor

β_{1A}R: Beta 1 adrenergic receptor

β_{2A}R: Beta 2 adrenergic receptor

CHAPTER 1

1 REVIEW OF LITERATURE

1.1 G-protein coupled receptors

The hepta-helical transmembrane receptors known as G-protein Coupled Receptors (GPCRs) comprise one of the largest superfamilies of membrane receptors (Figure 1.1) (Kenakin and Christopoulos, 2013) and represent a major target of pharmaceutical drugs (Takeda *et al.*, 2002; Lagerstrom and Schioth, 2008). GPCRs play crucial roles in the transduction of extracellular signals (such as those of light, calcium, amines, peptides, nucleotides and hormones) to intracellular responses through conformationally mediated interactions with downstream heterotrimeric G proteins (Sprague, 1992; Bourne, 1997).

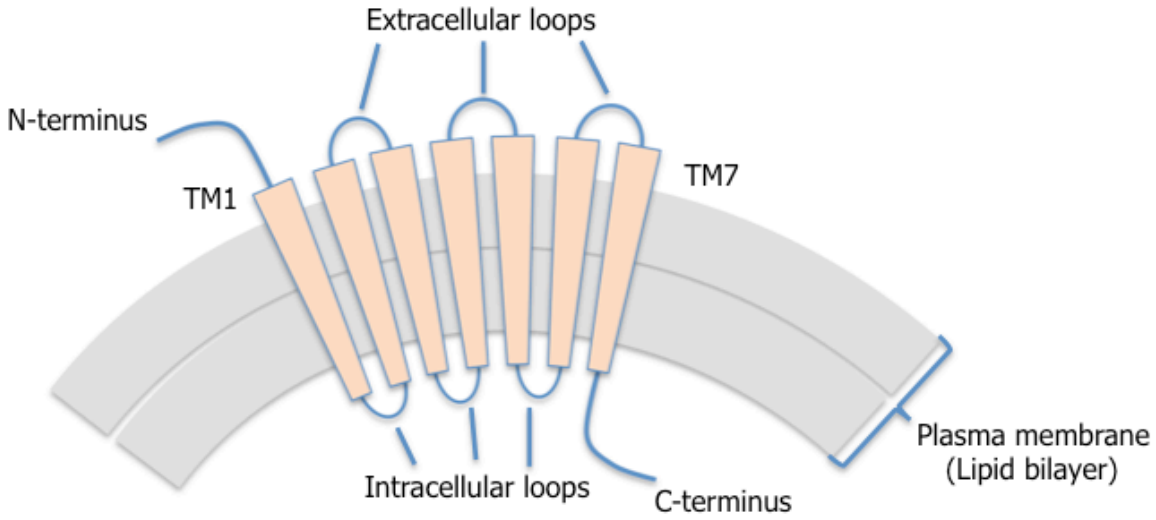


Figure 1.1 The seven trans-membrane domain structure of G-protein-coupled receptors. GPCR has 7 trans-membrane domains that are connected through extracellular and intracellular loops.

1.1.1 GPCR subfamilies

The GPCR superfamily includes almost 1000 members and comprises almost 2% of the human genome (Lander *et al.*, 2001; Bjarnadottir *et al.*, 2006; Almen *et al.*, 2009). GPCRs are exclusively present in the eukaryotic group including the primitive eukaryotes like fungi, to

higher organisms like humans (King *et al.*, 2003). Various classification systems have been used to categorize the GPCRs based on the sequence identity, function and evolutionary origin. However, it has been a challenging task as there is very little sequence similarity amongst the various families and even within the same family. Despite the sequence dissimilarities between various GPCRs, structurally all members of this superfamily share a common seven trans-membrane (TM) spanning domain (including 25-30 residues long each TM helix), interconnecting extracellular (EC) and intracellular (IC) loops (Figure 1.1).

Further, all the GPCRs share an ability to interact with G-proteins to activate a signal transduction pathway (Baldwin, 1993). One of the most primitive classification systems is based upon sequence identity and functional similarities. It divides the GPCRs among 6 major classes (A-F) and further into subclasses (Table 1.1) (Attwood and Findlay, 1994; Kolakowski, 1994). This A-F system includes all GPCRs in both vertebrates and invertebrates.

Table 1.1 The classification of GPCRs

GPCRs major classes	Alternative name
Class A (or 1)	Rhodopsin like
Class B (or 2)	Secretin Family receptor
Class C (or 3)	Metabotrophic/Glutamate
Class D (or 4)	Fungal Mating Pheromone receptor
Class E (or 5)	Cyclic AMP receptor
Class F (or 6)	Frizzled/Smoothened

The class A or Rhodopsin class, is the largest and includes almost 85% of all receptors that includes light, hormone and neurotransmitter receptors and is further divided into 4 groups (α, β, γ and δ) and 16 subgroups (Fredriksson *et al.*, 2003; Nordstrom *et al.*, 2011). Many of the members of receptor families are not present in the Human genome such as Class D and Class E receptors as well as various members of Class A receptors. A more recent classification system based upon phylogenetic analyses categorizes only the human GPCRs into major 5 classes - GRAFS, Glutamate (Class C), Rhodopsin (Class A), Adhesion (Class B), Frizzled (Class F) and Secretory receptors (Class B) (Fredriksson *et al.*, 2003). In the human genome, Rhodopsin is the largest family comprising 683 members, while Adhesion is the

second largest with 33 members. The GRAFS classification system has further divided these into many levels including subfamily, sub-sub family, sub sub-subfamily, sub-sub sub-subfamily and subtypes (Gao *et al.*, 2013). Phylogenetic evaluation of the GPCR's reveals the presence of a common ancestor for the major classes of GPCR's. This is the cAMP receptor family that is the GPCR of primitive eukaryotes like slime molds. All of the Rhodopsin, Adhesion, Frizzled and Secretin major classes have been proposed to evolve from this cAMP receptor. In the evolutionary tree, Adhesion and Frizzled receptors have been proposed to originate from the cAMP before the split of unikonts. Unikonts are common originators of both opisthokonts and amoebozoa. The Rhodopsin class of GPCR splits from the cAMP family at the opisthokonts level. Opisthokonts are common ancestor for fungi and metazoan. The Secretin class of GPCR have been proposed to have evolved from the adhesion receptors in the metazoan lineage (Nordstrom *et al.*, 2009).

1.1.2 GPCR signal activation and G-protein dependent transduction

The members of the GPCR superfamily serve as important modes of communication between the external environment and the intracellular components of the cell based on their surface localization. Upon binding of different ligands, GPCRs elicit a variety of physiological responses (Gether, 2000; Lefkowitz, 2000; Muller *et al.*, 2008a). The binding of a specific ligand elicits conformational changes in the GPCR, converting it from an inactive to an active state. Following ligand dependent activation, the active GPCR receptor binds to the inactive heterotrimeric G-protein (Guanine nucleotide binding Protein) causing its activation. The inactive state of the G-protein is attached to guanidine diphosphate (GDP) and this exchanges for guanidine triphosphate (GTP) following activation. For this reason, GPCRs are often termed GEFs (Guanidine nucleotide exchange factors). Once GDP is exchanged for GTP, the heterotrimeric G protein dissociates into Gα and Gβγ subunits, which further interact with various effector molecules and generate second messengers. The Gα, due to its inherent GTPase activity, causes hydrolysis of GTP to GDP (accelerated by various regulators of G protein signaling) and the Gβγ subunit is then re-attached to the Gα subunit to make the inactive trimeric complex (Gilman, 1987). The association and dissociation cycle of the G-protein is illustrated in Figure 1.2.

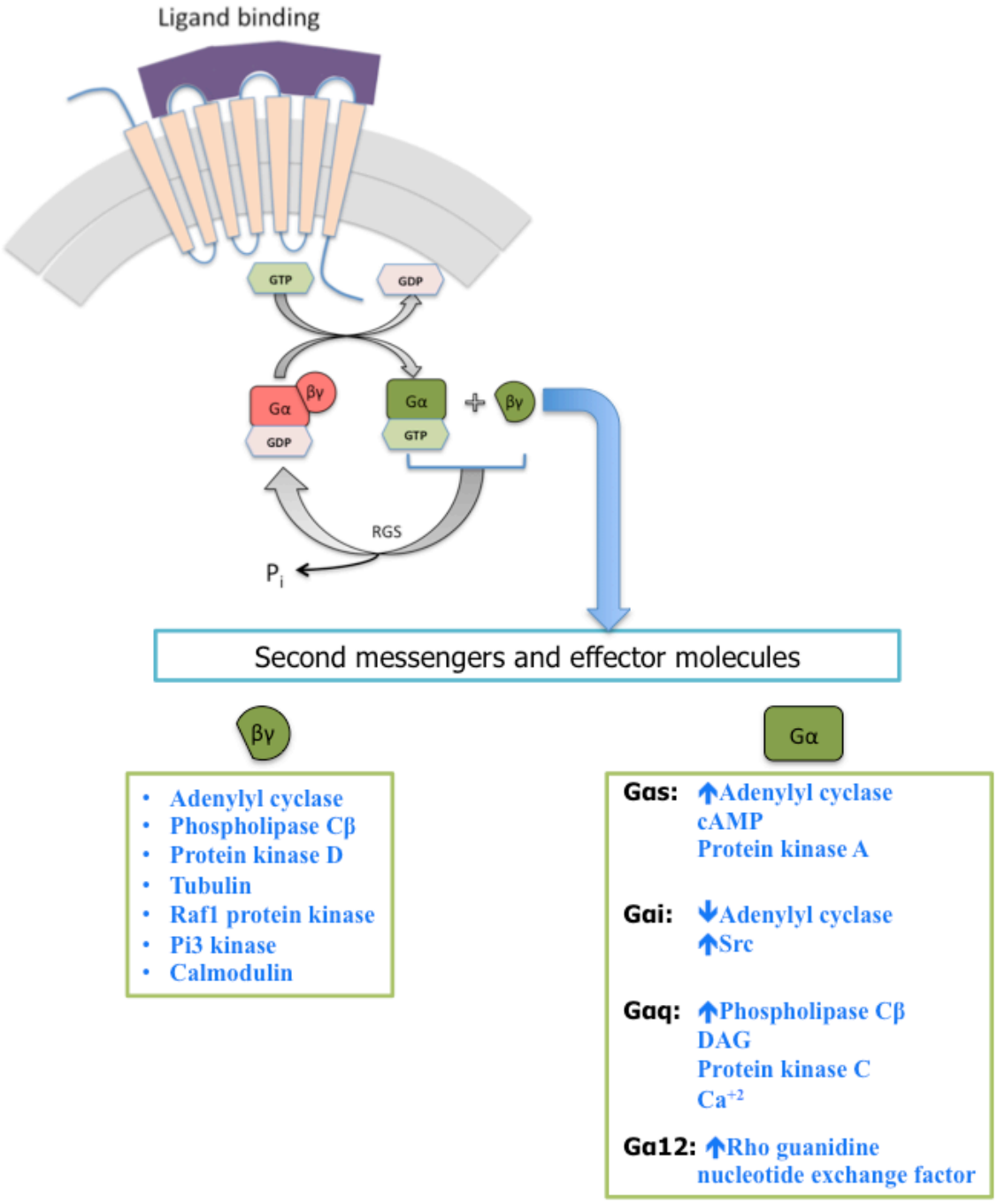


Figure 1.2 G protein activation-deactivation cycle and downstream effector molecules. The heterotrimeric complex is bound to GDP in the inactive state. Upon GPCR stimulation by its ligand, GTP is exchanged for GDP and heterotrimeric G complex dissociates into Gα and Gβγ subunits. Dissociated Gβγ subunit activates effector molecules and initiates G protein dependent signaling. RGS proteins binds to the GTP bound Gα subunit and stimulate GTP hydrolysis and subsequently the re-association of the G protein complex [adapted from (Cabrera-Vera *et al.*, 2003)]. Images reprinted with permission from Endocrine society.

1.1.3 Heterotrimeric G-proteins: structure and function

The important and critical roles of G proteins in various cellular processes were identified in the early 1970's (Gilman and Nirenberg, 1971; Rodbell *et al.*, 1971). Heterotrimeric G proteins have been shown to have Gα, Gβ and Gγ subunits that are coded by 16 genes, 5 genes and 12 genes, respectively. Classically, G-proteins have been sub-divided into four subfamilies based upon the type of Gα subunit, including Gαs, Gαi, Gαq and Gα12 that is further divided into subtypes.

The availability of crystal structures of inactive, active and transition state G protein has provided a basic understanding of the structure and mechanism of action of G proteins. The Gα subunit has a GTPase domain that binds to GTP and a flexible helical domain that has a central long helix surrounded by 5 shorter helices. Following the binding of GTP to the GTPase domain, the Gα subunit looses its flexibility (Lambright *et al.*, 1996). The Gβ subunits consist of a β-propeller fold that is made up of seven beta-sheet structures and an alpha helix in the N-terminal region. The Gβ subunit binds to the GTPase domain as well as the N-terminal residue of the α subunit while the Gγ binds only to the helical N-terminal of the β subunit to form a tight heterotrimeric G-protein (Lambright *et al.*, 1996). Limited information is available about the extreme amino- and carboxy- terminus of the Gα subunit as these parts were either removed or disordered in the crystal structures. However, various biochemical studies suggest that the extreme C-terminus and N-terminus of the Gα subunit (Conklin and Bourne, 1993; Bourne, 1997; Wess, 1997) (Taylor *et al.*, 1996; Blahos *et al.*, 1998; McIntire *et al.*, 2001) play important roles in receptor-G protein binding specificity, as various points of contact have been observed between GPCRs and the N- as well as C-termini of Gα subunit.

Two models have been proposed for the mechanism of interaction of GPCR with the G-proteins. In the Collision-Coupling model it was proposed that an agonist-receptor complex was able to interact with the G protein to initiate downstream events (Orly and Schramm, 1976; Tolkovsky and Levitzki, 1978). On the other hand, the pre-coupling model suggested that a GPCR-G protein complex is already located at the cell surface, where ligand binding causes a conformational change in the G-protein to facilitate binding of GTP to the G-protein (Neubig *et al.*, 1988; Chidiac *et al.*, 1994; Nobles *et al.*, 2005; Gales *et al.*, 2006).

1.1.3.1 Effectors of Gα subunit

Once the G protein-GTP complex is dissociated from the heterotrimeric complex it activates several effector molecules that in turn modulate the second messenger concentrations/levels. The types of activator molecules that are activated are specific to each of the four main families of Gα subunits that bind to GTP. The Gαs type subunit has been shown to activate adenylyl cyclase, a protein that is integral to the plasma membrane and converts ATP into second messenger cAMP molecules (Berlot and Bourne, 1992; Sunahara *et al.*, 1997). The cAMP molecules have been observed to activate the serine threonine protein kinase A (PKA) that catalyzes the phosphorylation of other proteins to modulate a variety of processes. The PKA has two identical regulatory and two identical catalytic domains. The auto-inhibitory regulatory domains occupy the active binding site of the catalytic domains and keep it in an inactive state. When cAMP binds to the regulatory domain it is no longer able to inhibit the catalytic properties of the PKA molecule. In contrast to the Gαs type subunit, the Gαi type subunit has been shown to bind to the catalytic domain of the adenylyl cyclase to inhibit its activity thereby reducing cAMP production and thus promoting inhibition of PKA and downstream effectors. In addition, the Gαi subunit can activate other proteins like receptor tyrosine kinase Src and rap1 and other ERK1/2 pathways (Mochizuki *et al.*, 1999).

The type Gαq subunit was observed to activate phospholipase C-β (PLC β) and cause degradation of phosphatidyl inositol-4,5-biphosphate (PIP2) into two second messengers, diacyl glycerol (DAG) and 1,4,5-inositol triphosphate (IP3). The IP3 causes the release of Ca^{2+} from the endoplasmic reticulum into the cytoplasm. The Ca^{+2} binds to the calcium modulated protein, Calmodulin (CaM) that has been observed to activate the CaM kinase that in turn phosphorylates the other target molecules (Valant *et al.*, 2012). The other second messenger, DAG (released by type Gαq subunit) and Ca^{+2} both activate protein kinase C that phosphorylates members of the ERK pathways (Bence *et al.*, 1997; Gutkind, 2000). The type Gα12/13 subunits interact with the Rho specific guanosyl exchange nucleotide factor (Rho GEF) effector molecules to activate the small GTPase Rho that would cause cytoskeletal remodeling (Hart *et al.*, 1998).

1.1.3.2 Effectors of the Gβγ subunits

Several effector molecules are common to the Gα and Gβγ subunits. Their effects might be independent, additive or antagonistic. However many other specific novel effectors for Gβγ subunits have been identified. These are protein kinase D, tubulin, raf1 protein kinase, Pi3 kinase and CaM (Stephens *et al.*, 1994; Tang and Downes, 1997; Jamora *et al.*, 1999) (Figure 1.2).

1.1.3.3 Inactivation of G protein signaling

As mentioned previously, the intrinsic GTPase activity of the G-protein is responsible for the hydrolysis of the GTP and the attenuation of the G-protein dependent signaling. The Hydrolysis rate of the GTP is variable within the subfamilies of Gα subunit (Fields and Casey, 1997). The variability in the GTP hydrolysis rate among Gα subfamilies depends upon a divergent helical domain as the GTPase binding domain is conserved in the Gα subfamilies (Rens-Domiano and Hamm, 1995; Sprang, 1997).

The intrinsic GTPase activity occurs at a very slow rate and therefore cannot account for the much faster recycling rate of G-proteins. Various GAPs (GTPase acceleration proteins) were identified that increase GTP hydrolysis. The Gα-effectors PLCβ and γ subunit of phosphodiesterase (Pγ) were the earliest identified GAPs (Arshavsky and Bownds, 1992; Berstein *et al.*, 1992; Biddlecome *et al.*, 1996). In addition to the feedback effectors, various regulators of G-proteins (Mathew *et al.*, 2011) were identified that could enhance the GTPase activity of the G-protein. RGS proteins bind with the transition state of the G-protein and enhance the reaction rate by over two orders of magnitude. Approximately 30 RGS proteins have been identified in mammals that share a common RGS box for its activity (De Vries *et al.*, 2000; Ross and Wilkie, 2000; Hollinger and Hepler, 2002).

The function of G protein is regulated by various covalent modifications such as lipid modification and phosphorylation. Addition of lipid to the Gα subunit is required for trafficking to the plasma membrane and attachment with the Gβγ subunits and other proteins. Palmitoylation, myristoylation and isoprenylation are major lipid modifications identified for the G-protein. Isoprenyl units are attached to the Gγ subunit, which determines its interaction with effector molecules (Milligan and Grassie, 1997; Wedegaertner, 1998; Chen and Manning, 2001).

Phosphorylation of the G-proteins regulates the duration and strength of the G-protein signal. Phosphorylation of Gα subunit by protein kinases prevents its association with Gβγ dimer and increases the duration of the signal. However, phosphorylation of Gγ subunit increases its interaction with Gα and decreases its interaction with effector molecules thereby decreasing the signal (Zick *et al.*, 1986; Moyers *et al.*, 1995; Kozasa and Gilman, 1996).

1.1.4 G-protein signaling: mitogen activated protein kinase (MAPK) pathways

Different GPCRs have been shown to play a critical role in biological functions such as glucose metabolism, photoreception, chemoreception, responses in stress conditions, blood pressure control, neurotransmission, chemotaxis and in platelet functions (Dohlman *et al.*, 1996). In addition, GPCRs modulate cell proliferation, mitogenesis and angiogenesis through the MAPK pathways, an aspect that was not appreciated until the nineties. The MAP kinase pathway is a three-tiered protein kinase cascade pathway, which includes serine/threonine protein kinases that are largely conserved from yeast to human. In the three-tier model, the tyrosine and threonine residues of MAPK are phosphorylated by dual kinases called MAPK kinases (MKK). The MAPK kinases are further phosphorylated by MKK kinases (MKKK/MEKK). Based on the sequence similarities and functions, the 12 known MAPK have been categorized into five families namely, ERK1/2, ERK 3/4, ERK 5, JNK1/2/3, and P38 α/β/γ/δ. So far, seven MKK and 14 MKKK have been identified in mammalian systems. The general description of a MAPK pathway is illustrated in Figure 1.3.

While scaffolding proteins hold the different components of the MAPK pathway in proximity and thereby determine the specificity of the pathway at the MAPK level, cross talk between different MAPK pathways can occur. Once activated, MAPKs phosphorylate transcription factors, other kinases as well as regulatory enzymes to influence gene expression (Su and Karin, 1996; Gutkind, 1998; Widmann *et al.*, 1999). MAPKs shuttle from cytoplasm to nucleus in mitogenic response and influence gene expression.

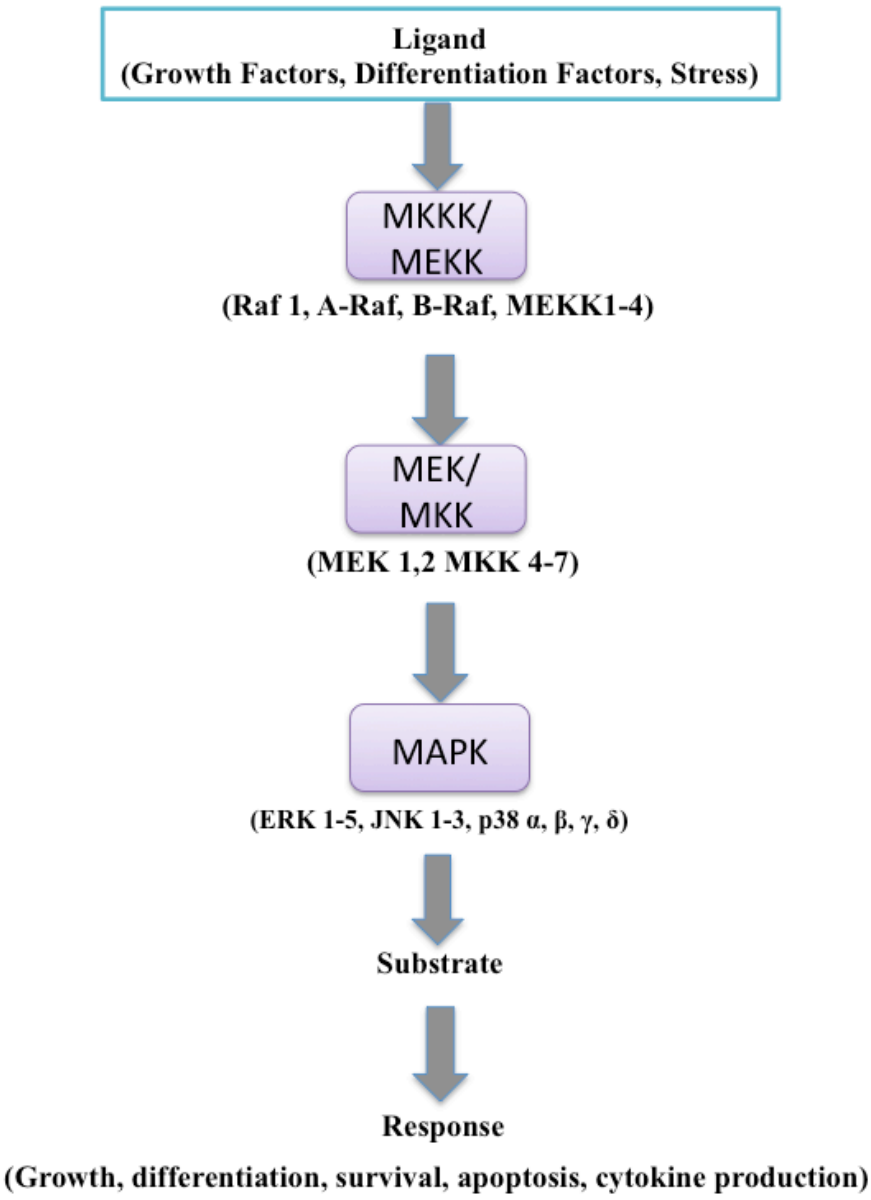


Figure 1.3 Overview of mitogen activated protein kinases (MAPK's). MAPK pathway consists of 3 kinases, MEKK/MKKK, MEK/MKK and MAPK. MKKKs are the target of growth factors, differentiation factors and stress molecules. MKKKs phosphorylate the MKKs at serine and threonine residues. MAPK are the final kinase in three-kinase module and once activated, shuttles into the nucleus, where it phosphorylates many transcription factors and ultimately influence the expression of various genes

The classical MAPK pathway: Initially the MAPK cascade system was identified in the context of the receptor tyrosine kinases (Bos *et al.*, 1997) that constitute a large family of plasma membrane proteins with intrinsic kinase activity. The ligand-binding and kinase domains of RTK are located on the surface and the cytoplasmic face of the plasma membrane, respectively. The RTK family includes epidermal growth factor receptors, platelet derived growth factor receptor, insulin receptors and Eph receptors (Cooper and Hunter, 1981, 1983).

Binding of growth factors to its receptor causes auto-phosphorylation on a tyrosine residue in the cytoplasmic domain. The auto-phosphorylated cytoplasmic domain then catalyzes the phosphorylation of other cytoplasmic domains and these phosphorylated residues act as a docking site for adaptor proteins (Schlessinger, 1993). These adaptor proteins are phosphorylated by the receptor and facilitate binding with other members of the pathway. In the case of insulin receptors, phosphorylated receptors bind with the insulin receptor substrate1 (IRS-1) and Grb2 protein (Sun *et al.*, 1991). Grb2 does not have any intrinsic kinase activity, but it has SH2 (Src homology domain 2) and SH3 (Src homology domain 3) domains that bind to the IRS 1 and SOS protein, respectively, to bring them together (Pawson, 1995; van der Geer *et al.*, 1995). The SOS protein acts as a guanidine nucleotide exchange factor for the Ras protein. The Ras is a member of the small G-protein family that is able to bind to GDP and GTP in inactive and active states, respectively. Activated Ras phosphorylates Raf, the first member of the MAP kinase cascade. The other two members of the kinase pathways are MKK and ERKMAPK. The phosphorylation at tyrosine and threonine residues of MKK and ERKMAPK activates these kinases. Once activated ERK enters into the nucleus and phosphorylates transcription factors modulating the expression of many genes (Davis *et al.*, 1993) (Figure 1.3).

Linkage between GPCR and the MAPK pathway: The connection between GPCRs and the MAPK pathway remained poorly understood until the mid 1990's when the G-protein βγ subunits were shown to activate the MAPK (Ras-Raf) pathway (Crespo *et al.*, 1994; Faure *et al.*, 1994). The GPCR and βγ subunits were shown to promote the phosphorylation of Shc (adaptor protein in Ras-Raf pathway) stimulating formation of a Shc-Grb2 complex. Several non-receptor tyrosine kinases, Src-like kinases, were identified to initiate this response. The involvement of Src-like kinases was demonstrated in mediating the phosphorylation of Shc protein by GPCR (Luttrell *et al.*, 1996). However, the activation of Src kinases itself was not

fully understood initially. The components of the endocytic machinery of GPCRs (arrestin, clathrin coated pits and dynamin 1) play an important role in stimulating the MAPK by GPCR (Ahn *et al.*, 1999; DeGraff *et al.*, 1999; Luttrell *et al.*, 1999). In addition, GPCR stimulation was shown to activate certain RTK directly, that might either occur through the GPCR mediated activation of Src kinases (Luttrell *et al.*, 1997) or through proteolytic cleavage of a latent agonist of RTK (Massague and Pandiella, 1993). Another candidate molecule to link between the GPCR's and MAPK pathway is the Ras Guanidine nucleotide releasing factor (Ras GRF), whose activation could be increased by the overexpression of the GPCR and Gβγ subunits (Mattingly and Macara, 1996). The PI3K (Phosphoinositide 3-kinase) might also relay the MAPK activation by GPCR's as the PI3K inhibitor wortamanin also was shown to decrease the MAPK activation (Hawes *et al.*, 1996). The PI3K is reported to function downstream of Gβγ but upstream of Src-like kinases (Lopez-Ilasaca *et al.*, 1997).

The GPCR mediated modulation of the MAPK pathway can also be relayed by different subunits of the G-protein. For example the Gαq subunit has been shown to activate the PLCβ protein, protein kinase C (PKC) and the Ras-Raf protein of the MAPK pathway (Kolch *et al.*, 1993). On the other hand, the Gαs subunit might stimulate the MAPK pathway by regulating the levels of Ras like GTPase, Rap1. Rap1 protein has dual functionality to control the MAPK. It can block the pathway by competing with the Ras protein for binding to the Raf1 and A Raf (Kitayama *et al.*, 1989; Bos *et al.*, 1997). On the other hand, it can stimulate the MAPK by activating B Raf. EPAC has been identified as a Guanine nucleotide exchange factor for Rap1 that is shown to be activated by cAMP. Other subtypes of Gα protein (Gαo, Gαi, Gαz) can directly regulate the activity of Rap1 as Gαo and Gαz have stimulatory effect while effect of Gαi is inhibitory on Rap1 activity (Jordan *et al.*, 1999; Meng *et al.*, 1999; Mochizuki *et al.*, 1999). In addition to the activation of Ras-Raf MAPK, the JNK-MAPK and P38-MAPK pathways have also been linked to GPCR activation (Coso *et al.*, 1995; Widmann *et al.*, 1999). However, the upstream regulators of these pathways have not been yet identified.

Overall, the activity of GPCR's can affect cytoplasmic signaling through highly interconnected pathways. The formation of second messengers controls the activation of the protein kinases that affect the activity of the enzymes or proteins. As well, GPCRs can signal through the MAPK pathway. The Gα and Gβγ subunits are directly involved in the interconnection of GPCRs and the MAPK pathways as shown in Figure 1.4.

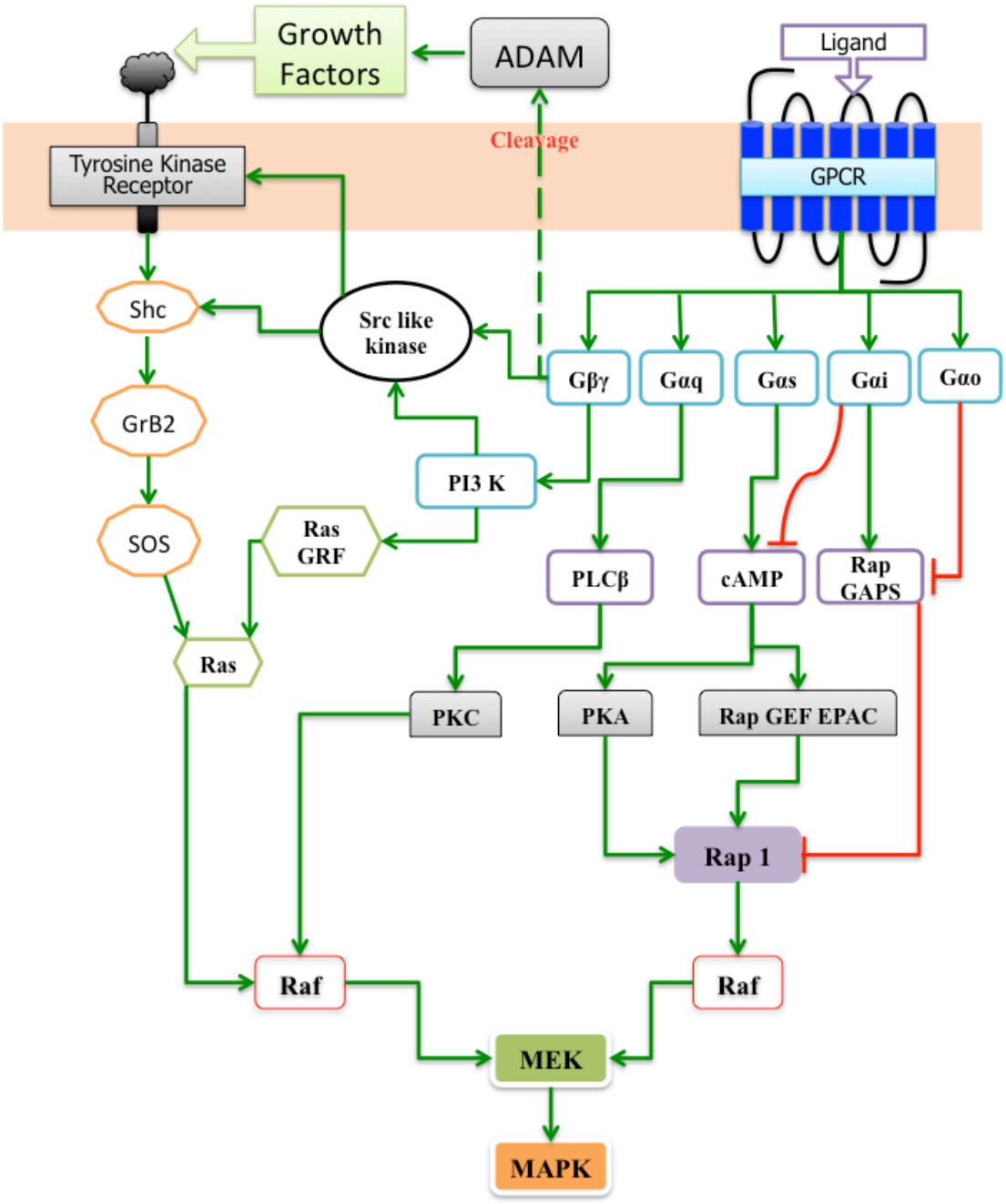


Figure 1.4 Multiple pathways to connect the GPCR's to MAPK pathway. Biochemical routes activate the Ras and Raf molecules and novel pathways activate the Rap1 molecule to integrate into the MAPK pathway. Arrows indicate positive stimulation and blocked lines indicate inhibition [adapted from (Gutkind, 2000)]. Image reprinted with permission from AAAS.

1.1.5 Termination /desensitization of GPCR signals

The homeostasis between activation of signals in response to environmental clues and signal termination/desensitization is very important to maintain the normal physiological state of the cell. Termination/desensitization of the signal is achieved through various regulatory mechanisms. One such regulation occurs at the G-protein level as the intrinsic GTPase activity of the G-proteins in conjunction with the regulators of G protein signaling (RGS proteins) has been observed to cause termination of the signaling activity of the G-proteins themselves (De Vries *et al.*, 2000). Another point of regulation is at the GPCR level itself as the receptors desensitize within seconds after the occupancy of the ligands. Receptor endocytic trafficking is another important mechanism to regulate the GPCR signaling. Endocytic trafficking entails the transport of the receptors from the endocytoplasmic reticulum (ER) to the cell surface and then its removal from the cell surface. From here, the receptors can follow recycling or degradative pathways. Following GPCR synthesis, and before they are expressed on the cell surface, GPCR undergo different posttranslational modifications that include N-linked glycosylation (Davis *et al.*, 1995; Deslauriers *et al.*, 1999) and palmitoylation (Marchese *et al.*, 2008; Chini and Parenti, 2009). These modifications occur within the ER and different chaperones have been shown to play an important role in the trafficking and maturation of the receptors before they are expressed on the cell surface (McLatchie *et al.*, 1998). On the other side, phosphorylation, ubiquitination and arrestins play an important role in the desensitization/internalization of the receptor.

1.1.5.1 Role of phosphorylation and arrestins in the desensitization and internalization of the receptor

Endocytosis refers to the reversible or irreversible removal of the receptor from the cell surface, causing receptor desensitization/internalization. The phosphorylation of receptors at serine and threonine residues of the C-terminal tail plays a crucial role in their endocytosis. Desensitization of receptor can be homologous or heterologous, depending upon the presence or absence of ligand and the type of kinases that phosphorylate the receptor. In homologous/ligand bound desensitization, the activated receptor is phosphorylated by the G-protein coupled receptor kinases (GRKs) (Krupnick and Benovic, 1998). To date, seven types

of GRKs (GRK 1-7) have been identified that phosphorylate different types of GPCRs. Phosphorylation at the C-terminal and third intracellular loops of the GPCRs by GRKs have been shown to result in the binding of other proteins and eventually the internalization of receptor. On the other hand, the heterologous desensitization of GPCRs is ligand independent and requires second messenger dependent kinases (PKA and PKC). PKA and PKC phosphorylate the serine and threonine residues of the C-terminal tail and cytoplasmic loops of the GPCRs to uncouple G-proteins from the GPCR, causing further desensitization of the receptor (Chuang *et al.*, 1996).

The dissociation of GPCR from the G-protein is effected by the recruitment of β-arrestin to the cytoplasmic domain of the receptor following GPCR phosphorylation (Pfister *et al.*, 1985; Lohse *et al.*, 1990). The binding of β-arrestin to the GPCR creates steric hindrance and results in the physical as well as functional separation of the G-proteins from the GPCR. Arrestin is a 48 kDa cytoplasmic protein and so far, four different types have been identified. Arrestin -1 and -4 are called visual arrestins, as they are present in the rods and cones of the photoreceptors (Luttrell and Lefkowitz, 2002). Arrestin -2 and -3 are non-visual arrestins that are involved in the desensitization of different GPCRs (Gurevich and Gurevich, 2006). Arrestins have N- and C- terminal domains that are connected through polar core residues (Granzin *et al.*, 1998; Hirsch *et al.*, 1999). The central polar core residue of arrestins acts as a phosphate sensor region. The link between the polar central core and C- terminal tail keeps the arrestins in an inactive state. Upon binding to the phosphorylated receptor, arrestins undergo a conformational change that exposes various sites in their C-terminal domain, which in turn allow arrestin to act as adapter molecules for the endocytic machinery. The exposed C-terminal sites of arrestins allow them to bind to the adaptor protein 2 (AP2) and clathrin molecules, which together assist in the formation of clathrin-coated pits (CCPs) (Goodman *et al.*, 1996; Laporte *et al.*, 1999). Once the GPCR is taken into the CCP, the pits are pinched off from the plasma membrane with the help of the large GTPase dynamin (McNiven, 1998). The internalized GPCR are then transported by CCP to the tubular early endosome wherein receptors are sorted out into the recycling vs. degradative late endosomes. The GPCR in the recycling endosomes are processed back to the cell surface and get resensitized again following dissociation of arrestin before receptor dephosphorylation.

Based upon the binding affinity of GPCR to the arrestins, the receptors have been classified into two major groups Class A and Class B. GPCRs that bind transiently with the arrestin molecules and are recycled back to the cell surface are termed Class A receptors. On the other hand, the Class B GPCRs bind with arrestin strongly and persistently. These GPCR-arrestin complexes are transported to the lysosome for degradation (Oakley *et al.*, 2000; Shenoy and Lefkowitz, 2003). Examples of class A GPCR are beta 2 adrenergic receptor (β_2AR), μ-opioid receptor (MOR), dopamine 1A (D1A), and α1b adrenergic (α1bAR) receptors. The Class B receptors include angiotensin II type 1A (AT1A), vasopressin (V2) and thyrotropin-releasing hormone (TRH).

1.1.5.2 The role of ubiquitination in endocytic trafficking

Several studies have demonstrated that ubiquitin, a 78 amino acid protein, has an important role in endocytic trafficking of mammalian GPCRs (Marchese and Benovic, 2001; Shenoy *et al.*, 2001). Initially it was proposed that ubiquitin plays an indirect role, however recent studies have demonstrated a more direct influence of ubiquitination in GPCR endocytosis (Wolfe *et al.*, 2007). Phosphorylation of receptor and subsequently it's binding to the arrestin, are the prerequisite steps for the ubiquitination of receptor

Ubiquitin binds covalently to the amino group of a lysine residue in the C-terminal tail of GPCRs and the length of the ubiquitin chain determines the fate of the receptor. Polyubiqitinated receptor is degraded through the 26S proteasomal degradation machinery system, while mono-ubiquitinated protein is directed towards lysosomal degradation. Ubiquitination is a dynamic and reversible process that requires E1 (Ubiquitin activating), E2 (ubiquitin conjugating) and E3 (ubiquitin ligase) enzymes (Somesh *et al.*, 2007). The E3 enzyme consists of a HECT domain in its Ring finger domain that recognizes and binds to GPCRs. The endosomal-sorting-complex required for transport (ESCRT) machinery assists in the assembly of ubiquitinated cargo and formation of intraluminal vesicles called multivesicular bodies (MVB) (Katzmann *et al.*, 2001). These MVB containing ubiquitinated proteins are directed from early endosomes towards lysosomes where they fuse with the lysosome and get degraded by lysosomal enzymes. Protein gets deubiquitinated before entering into the MVB's. Further, vacuolar protein sorting 4 (VPS4) is required for the disassembly of ESCRT machinery and entry of receptor cargo into the MVB's (Babst *et al.*, 1998). Many mammalian GPCR such

as β_2AR and CXCR4 require ubiquitin and ESCRT machinery for sorting into the lysosome system for their degradation (Marchese and Benovic, 2001; Shenoy *et al.*, 2001).

On the other hand, other GPCRs such as delta opioid receptor (DOR) are independent of ubiquitination and ESCRT machinery for their trafficking for lysosomal degradation. Mutation of the lysine residues in the C terminal tail of DOR does not inhibit its degradation, indicating ubiquitin independent machinery for degradation. However, GPCR associated sorting protein (GASP) has been shown to be involved for its delivery to the lysosome (Tanowitz and Von Zastrow, 2002).

1.1.6 GPCR structural studies

Several studies have been carried out with an aim to determine the structural details underlying the mechanisms of action of different GPCRs. Such studies have been helpful in understanding the conformational changes in the GPCRs following ligand/agonist or antagonist binding that led to downstream signaling events.

1.1.6.1 Crystal structures and conformational changes

In a first ever report for GPCRs, the crystal structure of rhodopsin in its inactive form was published in 2000 (Palczewski *et al.*, 2000). Following that it took another seven years to get the structural details for a second human GPCR, β_2AR (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007). The reason for the delay in solving the β_2AR structure is that β_2AR (along with most GPCRs) continues to oscillate in its inactive form, while the rhodopsin receptor is quite stable in its inactive form (Kobilka and Deupi, 2007). In order to stabilize the inactive β_2AR, an inverse agonist was employed to decrease the constitutive activity of the receptor (Rasmussen *et al.*, 2007). Constitutive active (CA) receptors are active even in the absence of the agonists and show some basal activity that can be blocked by the binding of inverse agonists. Crazolol is an inverse agonist that shifts the equilibrium towards an inactive conformation of β_2 AR. In addition to stabilizing the inactive form by using inverse agonists, the flexibility of the C-terminal domain and 3rd ICL of the β_2AR were reduced by truncation and a conformation specific antibody, respectively (Cherezov *et al.*, 2007; Rasmussen *et al.*, 2007; Rosenbaum *et al.*, 2007). Alternatively, lysozyme has also been used to fix the conformation of the third intracellular loop of β_2AR. Furthermore, the authors

reduced the hydrophobicity of the β_2AR by utilizing lipid cubic phase or bicelles composed of the lipid dimyristoyl phosphatidylcholine. Later on, thermo stabilization of mutants was identified as another method to increase the stability of the receptors (Warne *et al.*, 2008). Together, all the strategies mentioned above made it possible to ascertain the structure of human β_2AR at 2.4 Å resolutions. Since then, the crystal structures of other receptors such as human A_{2A} adenosine, β_1AR, squid rhodopsin and various intermediates of photo-activated bovine rhodopsin were determined using similar strategies (Massague and Pandiella, 1993; Jaakola *et al.*, 2008; Tate and Schertler, 2009; Lebon *et al.*, 2012; Palczewski, 2012). The structure of the human A_{2A} adenosine receptor was obtained at 1.8 Å resolutions, which is the highest resolution that have been achieved for a GPCR (Liu *et al.*, 2012b). Presently, a total of 21 unique GPCR structures are available (Katritch *et al.*, 2013; Vaidehi *et al.*, 2014).

Despite having low sequence similarity between GPCRs, the members of this superfamily share a major hallmark of the family, the seven TM spanning domains, connected through ICL and ECL loop regions. Both rhodopsin and β_2AR receptors are very important members of Class A GPCRs involved in the visual sense and flight and fight response of the sympathetic nervous system respectively. Despite their dissimilar sequences, the crystal structure analysis of rhodopsin and β_2AR receptors revealed that they have strong structural similarities in their TMDs along with a similar topographical view (Figure 1.5). The closeness in the structural design of these two receptors was indicated by lower root mean square deviations of 1.6 Å and 2.3 Å for trans membrane domains and overall structure respectively (Lefkowitz *et al.*, 2008). Some structural dissimilarity between rhodopsin and β_2AR receptors was also noticed related to the positioning of the 2nd ECL. In rhodopsin, the 2nd ECL forms a β sheet that is buried in the TMDs to form a lid for the ligand-binding pocket. On the other hand, the 2nd ECL in the β_2AR receptors was shown to exist as a short helix with an outward direction that was proposed to shape the route of the entry of the ligand into the ligand-binding pocket. Furthermore, an ionic lock due to interaction of hydrogen bonds and ionic interactions between the conserved motif (E/DRY) of TM 3 and the residues of TM6 have been indicated to keep the rhodopsin in an inactive form (Palczewski *et al.*, 2000; Yao *et al.*, 2006). Interestingly, such an ionic lock disruption is not sufficient for full activation of β_2AR structure.

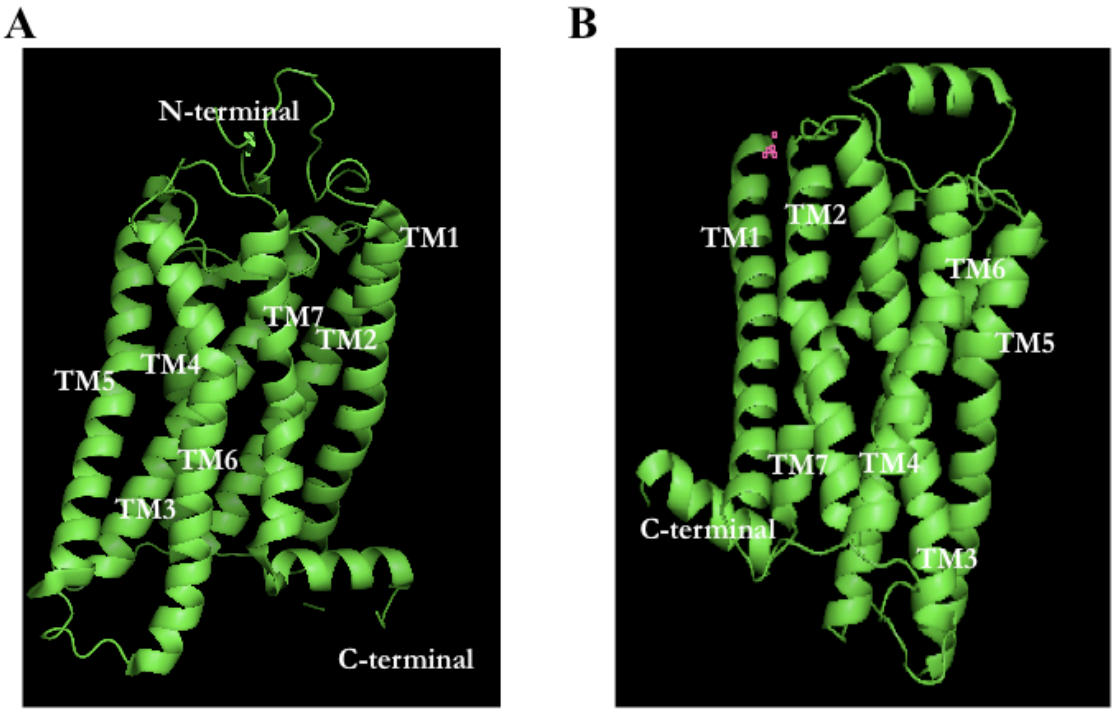


Figure 1.5 Topographical view of A) rhodopsin (3C9L) and B) β_2AR (2RH1) crystal structure. Images were made using Pymol softwares taking coordinates for rhodopsin from (Stenkamp, 2008) and for β_2AR from (Cherezov *et al.*, 2007)

Another important structural rearrangement occurs in the TM6 during activation of the rhodopsin receptor. The conserved proline residue introduces a kink in the domain and its interaction with other aromatic residues determines the kink angle in the domain. The kink angle was observed to change following agonist binding, a phenomenon that has been termed rotamer toggle switch (Shi *et al.*, 2002; Schwartz *et al.*, 2006). Interestingly, the activated form of the rhodopsin (meta rhodopsin) did not exhibit large conformational changes upon activation (Salom *et al.*, 2006; Lodowski *et al.*, 2007). Meta rhodopsin II has been observed to undergo a 2-8 Å structural shift after the activation due to major changes in the cytoplasmic ends of TM5 and TM6 domains.

One of the most significant outcomes of the GPCR crystallography was to understand the structure of activated human β_2AR. The ternary complex of β_2AR in a complex with Gs protein and agonist was crystallized (Rasmussen *et al.*, 2011). The structural analysis revealed that the amino- and carboxyl- ends of the Gs protein were interactig with the receptor. The observed major conformational changes were in the cytoplasmic ends of TM5 and TM6 that showed an

alpha helix extension and 14 Å outward movement respectively. Amongst the chemokine receptors, the crystal structure of CXCR4 revealed large differences from the structure observed for β_2AR and other GPCRs. These included major differences in the position and rotation of TM1, 2 and 6 as well as in the length of the TM5 and TM7 helices (Wu *et al.*, 2010).

Overall, it can be inferred from the structural studies conducted so far on GPCR that the extracellular domains are much more variable compared to the intracellular loops. However, binding of ligand induces more significant conformational changes in the intracellular loops compared to the extracellular loop region. Another important feature of the GPCR structure is the presence of a proline kink near the middle of TM6 that demarcates extracellular and intracellular regions of the receptor. Though there is not much difference in the inactive and active state for many receptors, TM5 and TM6 seem to be particularly important domains showing major conformational changes in the active state of the receptor.

1.1.7 G-protein independent signaling, biased agonism and functional selectivity

Contrary to early findings that GPCRs induced downstream events only through activation of G proteins, more recent reports identified GPCR signaling pathways independent of G-proteins. The β-arrestin molecule previously mentioned as an effector of GPCR desensitization and internalization of the receptor has in particular been observed to act as a scaffold protein for the components of the alternate signaling pathway.

1.1.7.1 β-arrestin as a signal transducer

The early proof of the existence of a G-protein independent GPCR signaling mechanism came from studies using the AT_{1A} receptor as a model. In the classical G-protein mediated signaling pathway, binding of ligand AngII to the AT_{1A} causes Gq protein activation that leads to the production of IP3 and DAG. Both of these second messengers further activate PKC that connects the AT_{1A} receptor to the ERK1/ERK2 MAPK pathway. The AT_{1A} receptor mutant DRY/AAY as well as stimulated by synthetic analogues of AngII (Sar1, IIL4, IIL8) lack the ability to bind G-protein and therefore could not elicit the production of IP3 (Wei *et al.*, 2003). Despite their inability to activate the Gq protein, the mutant as well as synthetic analogues of AngII caused a robust increase of ERK1/ERK2 production (Seta *et al.*, 2002). These findings led to studies with an aim to identify the potential candidates that could relay G-protein-

independent signaling of GPCRs. Along those lines, a study reported that down regulation of β-arrestin 2 expression by siRNA transfection abolished the ERK1/ERK2 expression in both mutant receptors stimulated by AngII and in the wild type receptors stimulated by synthetic analogues of AngII (Wei *et al.*, 2003). It is worth noting that the physiological consequences of G-protein-dependent signaling and β-arrestin-dependent signaling could be totally different. The G-protein activated ERK1/2 can translocate to the nucleus where it can phosphorylate different transcription factors (Pierce *et al.*, 2001). On the other hand, the β-arrestin activated ERK1/2 remains in the cytoplasm to phosphorylate various targets (Tohgo *et al.*, 2002). The latter pathway has been shown to mediate protein translation and antiapoptotic effects (DeWire *et al.*, 2008; Ahn *et al.*, 2009). In addition, the G-protein and β-arrestin mediated pathways are temporally distinct as in the case of $AT_{1a}R$ the former's signaling maximum is reached within 2 min while the latter's signaling peaks after 30 min.

In addition to linking to the ERK pathway, β-arrestin can also act as a scaffold for the JNK (McDonald *et al.*, 2000; Miller *et al.*, 2001), P38 (Bruchas *et al.*, 2006) and Akt pathways (Povsic *et al.*, 2003). The Akt pathway stimulation leads to the deactivation of glycogen synthase kinase and dopaminergic behavior of the dopamine receptor (Beaulieu *et al.*, 2005). The comprehensive view of β-arrestin mediated signaling and its physiological functions are shown in Figure 1.6.

The activity of β-arrestin is regulated by both dephosphorylation and ubiquitination mechanisms. β-Arrestin exists in the cytoplasm in a constitutive phosphorylated state that gets dephosphorylated upon binding of the activated GPCR. This GPCR dependent dephosphorylation of arrestin is crucial for the recruitment of clathrin and other molecules for receptor endocytosis (Lin *et al.*, 1997). Ubiquitination of β-arrestin determines its binding affinity to the receptor. As discussed earlier, receptors can be classified into loose binding (Class A) and tight binding (Class B) based on the binding affinity of receptor to β-arrestin. The phosphorylated status of the receptor and attachment of ubiquitinin to the arrestin are the two determinants of receptor affinity towards β-arrestin (Shenoy and Lefkowitz, 2005). The β-arrestin dependent ERK activation is stronger and sustained in case of Class B receptors compared to the Class A receptors.

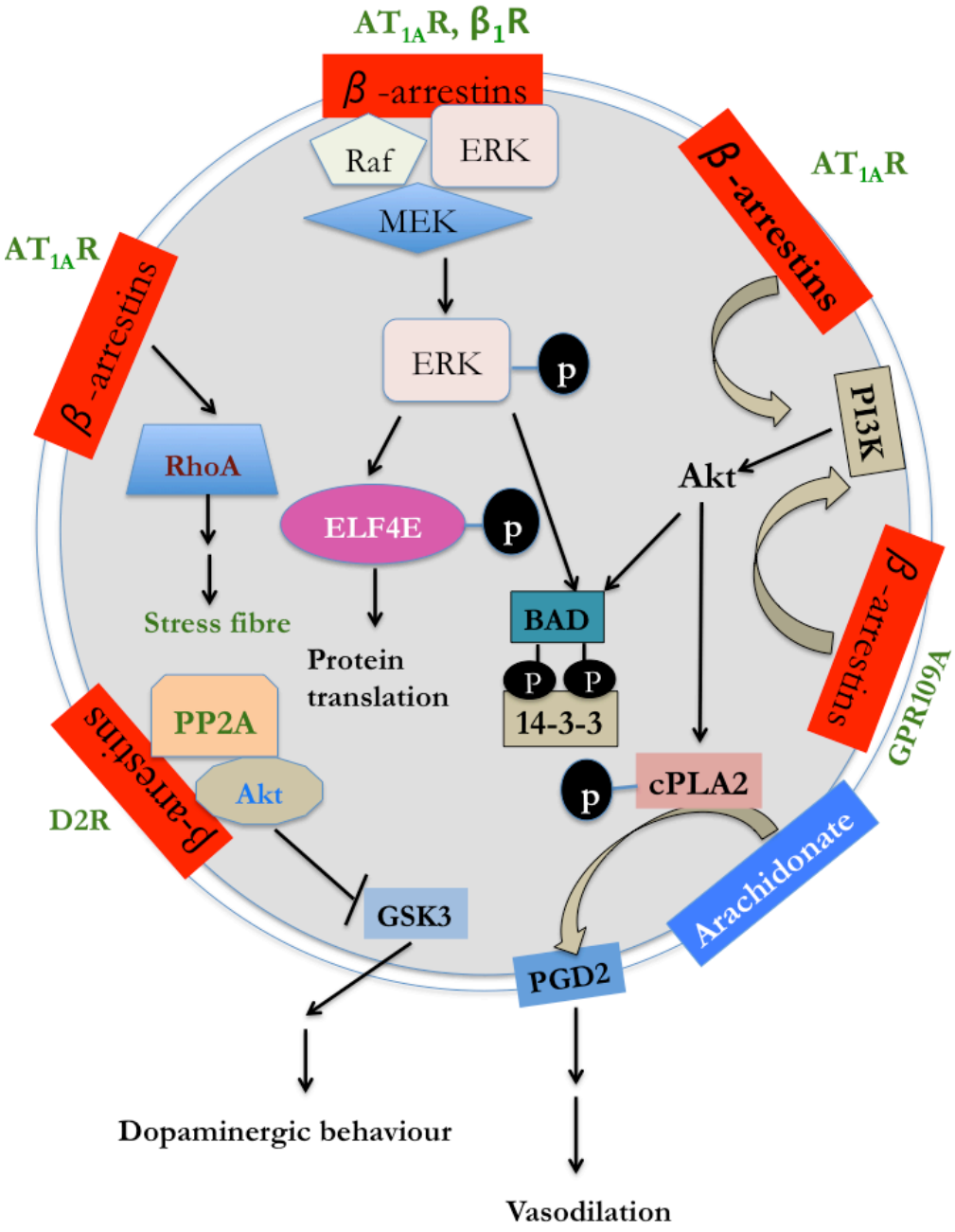


Figure 1.6 β-arrestin dependent signaling at GPCR. It includes RhoA dependent stress fibre formation (Barnes *et al.*, 2005); protein phosphatase 2A (PP2A)-mediated dephosphorylation of Akt, which leads to the activation of glycogen synthase kinase 3 (GSK3) and dopaminergic behavior (Beaulieu *et al.*, 2005); extracellular signal-regulated kinase (Lander *et al.*, 2001)-dependent induction of protein translation and antiapoptosis (DeWire *et al.*, 2008; Ahn *et al.*, 2009); phosphatidylinositol 3-kinase (PI3K)-mediated phospholipase A2 (PLA2) induction and increased vasodilation through GPR109A activation (Walters *et al.*, 2009)) [(Adapted from (Reiter *et al.*, 2012)]. Image reprinted with permission from Annual reviews.

1.1.7.2 Functional selectivity/ biased agonism

The concept of biased agonism or functional selectivity of a receptor arose from the observed ability of GPCRs to activate various signaling pathways in response to different ligands (Figure 1.7).

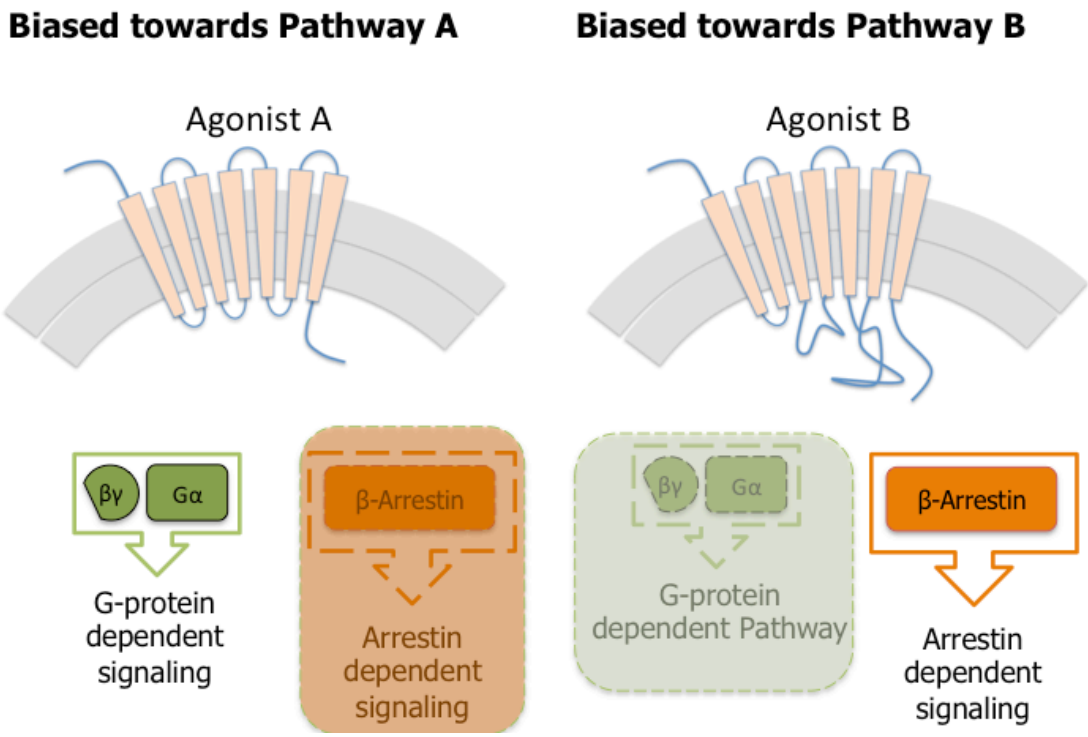


Figure 1.7 Biased signaling in GPCR. Agonist A produces biased stimulus for a specific cellular signaling pathway (G protein dependent signaling), whereas agonist B produces another conformation that selectively induces another signaling pathway (arrestin dependent signaling) [adapted from (Kenakin and Christopoulos, 2013)]. Image reprinted with permission from Nature publishing group.

Ligand properties can be defined by three distinct parameters, affinity, efficacy and potency. Affinity measures the tightness of the receptor ligand interaction while the efficacy defines the ability and extent of a ligand to activate a certain pathway (Stephenson, 1956). Potency is described as the amount of ligand that is required for producing half maximal response and it is a complex interplay of both the binding affinity and the efficacy. Based on these properties a ligand is termed a full agonist, partial agonist, inverse agonist or an antagonist. The full or partial agonist stabilizes the active conformation of a receptor while an

inverse agonist stabilizes an inactive conformation of a receptor. On the other hand an antagonist does not favor any conformation, but does inhibit ligand binding to the receptor (Urban *et al.*, 2007; Violin and Lefkowitz, 2007). In a simplistic concept, all the agonists are believed to have a linear efficacy for all the possible pathways related to a receptor, often termed as collateral efficacies (Kenakin, 2005; Galandrin *et al.*, 2007). However, there is increasing evidence that all ligands/agonists of a receptor don't activate the different downstream pathways with the same efficacy. Like other receptors, some ligands of GPCRs activate one downstream pathway while inhibiting another pathway. The phenomenon of a ligand's specific ability to selectively activate one downstream signaling pathway over another is termed 'functional selectivity' or 'biased agonism'. In the case of perfect bias, a ligand bound to its receptor can maximally activate G-protein dependent signaling and inhibit the internalization and arrestin dependent signaling or vice versa. However, this is not the case for most ligands as they often activate one pathway maximally while also partially activating the other downstream pathway (Holloway *et al.*, 2002).

1.1.7.3 Molecular mechanisms of functional selectivity

Conformation ensemble model: In the case of GPCRs, the functional selectivity of a ligand can be explained by specific changes in the conformation of the TMs of the activated GPCR. A ligand biased toward a particular downstream pathway can selectively bring about a unique GPCR conformation that modulates a specific signaling pathway. Several studies have directly measured the distinct conformational changes for different ligands related to a particular receptor (Swaminath *et al.*, 2004; Liu *et al.*, 2012a). A given unique ligand induced conformational change in the GPCR is tied to a specific signaling pathway through "GRK mediated phosphorylation bar codes", as the recruitment of β-arrestin to the receptor is influenced by the phosphorylation status of the receptor (Gurevich and Benovic, 1993; Lefkowitz and Shenoy, 2005). There are seven known members of the GRK family that can phosphorylate different receptors and thus regulate the specific functions of receptors. In the case of chemokine receptor (CCR7), which has two endogenous ligands CCL19 and CCL21, where CCL19 binding induces receptor phosphorylation by GRK-3 and -6 to activate desensitization, internalization and beta arrestin mediated signaling. On the other hand, CCL21 binding induces conformational changes to cause CCR7 phosphorylation through GRK6 only to

activate the G-protein dependent ERK pathway activation (Zidar *et al.*, 2009). In the case of β_2AR, the ligand carvidilol induces a conformational state of the receptor that can be phosphorylated only by the GRK6. On the other hand, isoproterenol induces structural changes in receptors to enable phosphorylation by both GRK 2 and GRK6.

1.1.7.4 Physiological importance and therapeutic applications of biased ligands

As mentioned earlier, both G protein mediated and β-arrestin mediated pathways have different physiological consequences. β-arrestin mediated pathways in MOR have been shown to cause side effects like constipation and respiratory suppression while the G-protein pathways have been shown to relay the analgesic effect of MOR activation. Morphine, an agonist of the MOR, has strong analgesic properties, but lesser side effects due to its weak ability to cause MOR phosphorylation and to recruit β-arrestin (Zhang *et al.*, 1998). In order to further reduce the side effects of MOR, activation by selective activation of G-protein pathways, a perfect biased agonist, herkinorin, was identified (Groer *et al.*, 2007).

As for $AT_{1A}R$, it regulates electrolyte homeostasis and therefore maintains blood pressure. Several antagonists (ARB) have been identified that block the G-protein linked pathways leading to lower blood pressure, but they have been also shown to inhibit the cytoprotective effects of β-arrestin pathways. However, one synthetic analog SII, linked to a different pathway, has been reported to reduce the arterial pressure while maintaining the cardio protective effects hence improved cardiac performances and stroke volume in rats (Violin *et al.*, 2010). It would be worthwhile to identify other SII like compounds to treat elevated blood pressures in humans.

Another example of β-arrestin mediated adverse effect has been shown for GPR109A receptor. Niacin, which is used to decrease triglyceride levels and increase high-density lipoprotein levels, works through G-protein mediated signaling via the GPR109A receptor. Niacin binding to GPR109A also stimulates the β-arrestin pathway that leads to the generation of arachidonate, which is responsible for the flushing of face and trunk in humans. This side effect has resulted in limited use of Niacin in humans to treat elevated cholesterol levels (Semple *et al.*, 2008; Walters *et al.*, 2009).

Another GPCR, βAR has been shown to increase blood pressure and heart rate in response to various catecholamine compounds (epinephrine and norepinephrine) through G-protein

signaling pathways. However, β-arrestin mediated downstream events following β AR internalization have positive anti-apoptotic effects. β-blockers, such as carvidilol, reduce blood pressure through inhibition of G-protein signaling and at the same time encourage cardio-protective β-arrestin signaling, providing added therapeutic benefit in the treatment of heart failure (Noma *et al.*, 2007; Wisler *et al.*, 2007; Kim *et al.*, 2008). Overall, It is evident from the above discussion that biased agonists have superior therapeutic value in many diseases like heart diseases, kidney conditions and osteoporosis with fewer side effects.

1.1.7.5 Allosteric modulators and their role in functional selectivity

As discussed in the previous section, binding of different ligands can selectively activate one pathway over another due to the stabilization of different conformations of the GPCR. Until the mid 1960's it was believed that binding of biased ligands was orthosteric if they shared the same binding site on the protein as those of the endogenous ligands. It was also proposed that even antagonists and inverse agonists could be orthosteric. In 1965 the concept of allosteric modulation for regulatory enzymes was introduced wherein the modulators of enzymes bind to a site different than that meant for endogenous ligands (Monod *et al.*, 1965). The first evidence of allosteric modulators for GPCRs was reported for muscarinic acetylcholine (Kords *et al.*, 1968; Jepsen *et al.*, 1988). The concept of the allosteric modulation of GPCRs has added complexity to the understanding of GPCR functionality (May *et al.*, 2007). Allosteric modulators of a GPCR might have different consequences on affinity and efficacy of the receptor as they might modulate the binding affinity or efficacy of an orthosteric ligand. Alternatively, they might modulate the binding affinity and efficacy of the G-protein molecule. It has been reported that binding of an orthosteric ligand to the receptor is required for allosteric modulators to exert their effects on that receptor (Christopoulos and Kenakin, 2002) (Figure 1.8).

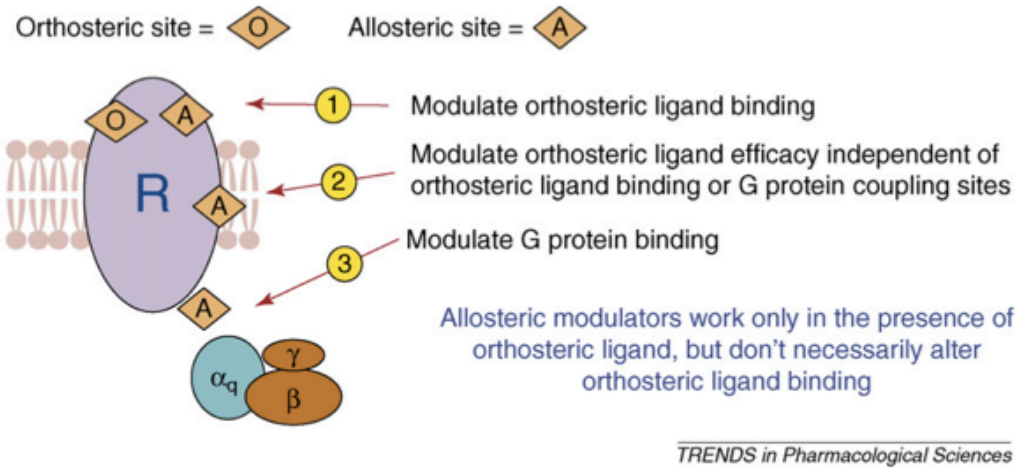


Figure 1.8 Consequences of binding of allosteric modulators. 1) It might modulate the affinity and 2) efficacy of orthosteric ligand binding or alternatively 3) it might modulate the affinity and efficacy of G protein binding [Images taken from (Gilchrist, 2007)]. Image reprinted with permission from Elsevier.

Allosteric modulators have been classified into 3 classes i.e. enhancers, antagonists/inhibitors and agonists/activators. Allosteric enhancers usually increase the orthosteric ligand affinity/efficacy towards the receptor but they do not have any effect on their own. Allosteric antagonists/inhibitors, inhibit the activity of the receptors working through affinity and efficacy of ligands towards their receptors (May and Christopoulos, 2003). On the other hand allosteric agonists or activators are able to activate the receptors even in the absence of orthosteric ligands through binding to receptor sites other than those meant for endogenous agonists. The International Union of Basic and Clinical Pharmacology committee has introduced a refined terminology for these compounds as ago allosteric modulators and allosteric agonists (Neubig *et al.*, 2003). Ago allosteric modulators are compounds that are both allosteric agonist and allosteric modulators (Holst *et al.*, 2005; Schwartz and Holst, 2006), while allosteric agonists are purely allosteric agonists (Langmead and Christopoulos, 2006; May *et al.*, 2007).

Allosteric agonists have been shown to promote the distinct conformational states and a different signaling profile compared to their orthosteric agonist (Kenakin, 2005; Leach *et al.*, 2007). In other terms, allosteric agonists have an effect on their own on the signaling profile and thus create biases in their signaling. As in the case of Adenosine A1 receptor, the allosteric

modulator VCP 520 was observed to not only potentiate the effect of its agonist, but also it influenced the signaling pathway on its own (Sachpatzidis *et al.*, 2003). Similarly in the case of chemokine receptors, the allosteric peptide ASLW (Ala-Ser-Leu-Trp) has been shown to potentiate G-protein signaling without inducing receptor internalization. However, the endogenous agonist (CXCL12) binding to the chemokine receptor was observed to promote both downstream pathways (Valant *et al.*, 2012).

Binding sites for the allosteric enhancers, agonists and ago-allosteric modulators: According to the classical allosteric concept, allosteric enhancers and orthosteric agonists were proposed to have separate binding sites on the receptor (Gao *et al.*, 2003; Schwartz and Holst, 2006). This was shown to be the case with many receptors such as muscarinic acetylcholine receptor. It was observed that the binding sites for the enhancers were located more extracellularly compared to the deep seated orthosteric binding site, such that the enhancer can block the exit of the agonist and decrease its off rate from the receptor (Wess, 1993; Birdsall *et al.*, 1996; Wess, 2005). In family C GPCRs the allosteric and orthosteric sites are located on separate subunits of the receptors. On the other hand in some cases, the allosteric enhancers and orthosteric ligands do have overlapping binding sites.

In the case of ago-allosteric modulators, their binding sites overlap with those for the orthosteric ligands of the particular receptor. As in the case of muscarinic receptor, the allosteric modulator AC-42 shares several interaction points with the endogenous agonist carbachol (Spalding *et al.*, 2002; Spalding *et al.*, 2006). If that is the case, the question arises of how the ago allosteric modulators or allosteric enhancers act as allosteric modulators when they share the binding site with the orthosteric ligand. According to the classical allosteric model, allosteric modulators and orthosteric ligands should bind to the receptor at the same time and that can possibly happen in only a few ways (Schwartz and Holst, 2007). According to the Flip-flop binding model ago allosteric modulators might bind at the site that overlaps with the orthosteric ligand-binding site in the absence of orthosteric ligand. However, in the presence of endogenous ligand ago allosteric modulators may partially occupy a different site (Steinfeld *et al.*, 2007). Another proposed mechanism is the time resolved allosteric model wherein both ago allosteric modulators and orthosteric agonist bind to the receptor at different time points when the receptor is displaying different conformations. Ago allosteric modulators bind with the receptor and change its conformation so orthosteric agonists can bind. However this

proposition does not fit with the other hallmark of allostery, that modulators affect the off rate of the orthosteric agonist. Yet another mechanism has been proposed involving dimerized receptors, where ago allosteric modulators bind to the allosteric protomer which is different from the orthosteric protomer region (Schwartz and Holst, 2006) (Figure 1.9). Allosteric/ orthosteric protomer is the proposed term for the receptor unit in which an allosteric modulator/endogenous ligand binds to a receptor dimer (Schwartz and Holst, 2006).

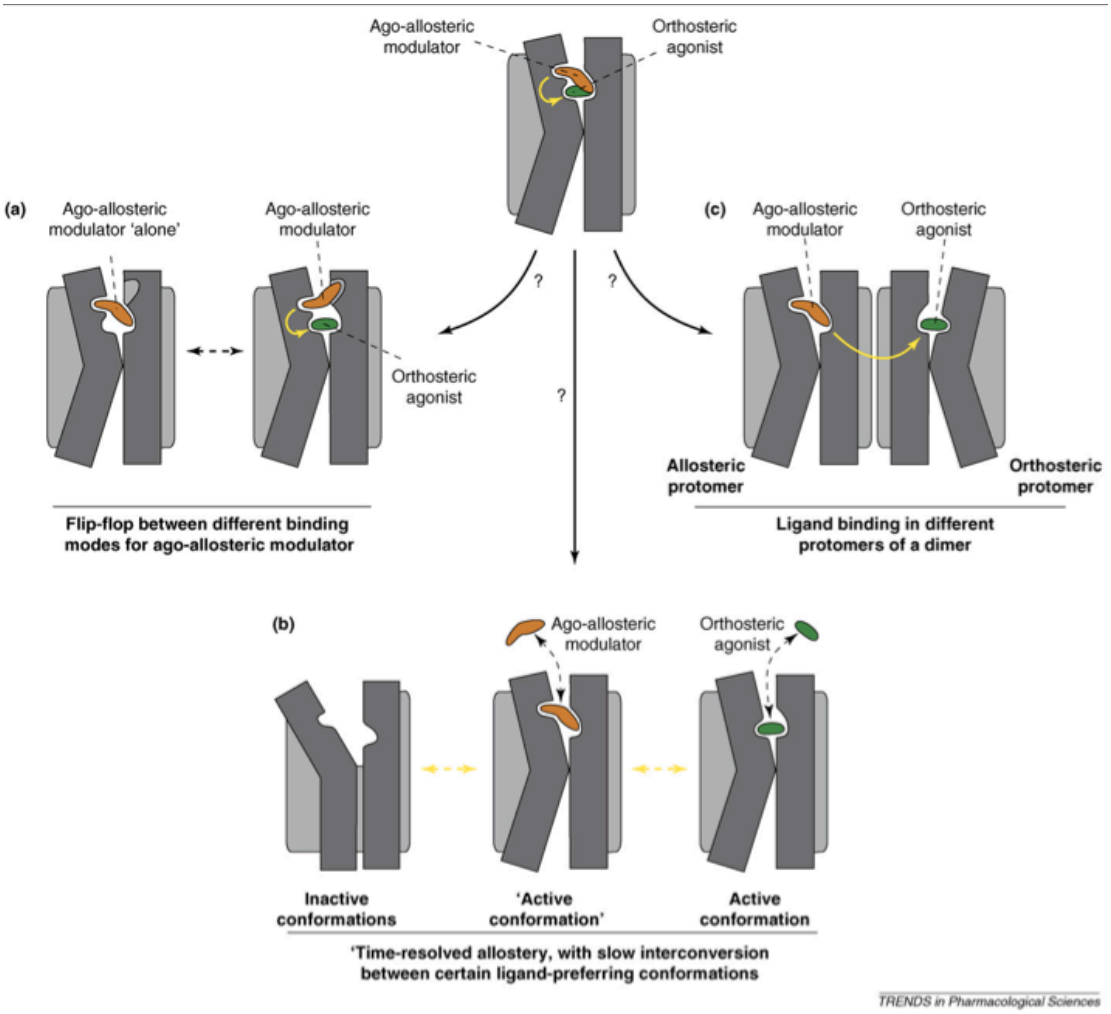


Figure 1.9 Molecular mechanisms of ago allosteric modulators with overlapping binding sites that overlap with the orthosteric binding sites. A. flip flop binding model: In the absence of orthosteric ligand, ago allosteric modulator occupies the overlapping binding site whereas in the presence of ligand it binds at a partially different site. B. Time resolved allostery: Ago allosteric modulator and orthosteric ligand both bind to the receptor at different time points. C: Ligand binding in different protomer in a dimeric receptor: both ago allosteric modulators and orthosteric ligand bind to the orthosteric sites but in different promoters of a dimeric receptor [Images taken from (Schwartz and Holst, 2007). Image reprinted with permission from Elsevier.

1.1.8 Structural correlation of biased signaling

Structural studies on both inactive and active states of different GPCR have imparted valuable information about the receptor. These studies have yielded information about the conservation of important residues within families as well as the conformational changes observed in the TMDs upon ligand binding. In order to understand the conformational changes associated with GPCR G-protein mediated signal transduction versus those that occur due to interaction with the arrestin molecule for G-protein independent signaling/biased signaling, a fluorine 19 (^{19}F) NMR based study was conducted (Liu *et al.*, 2012a). The ^{19}F probe is sensitive to its chemical environment and can reflect the dynamic behavior of an associated receptor. On this basis a ^{19}F probe was attached to the cysteine residues of TM6 and TM7 intracellular ends on β_2AR. It was observed that in absence of ligand for β_2AR, two conformational states related to the movement of TM6 and TM7 domains attain equilibrium. In the presence of the biased agonist, carvidilol, a shift towards the TM7 active state conformation was observed whereas the full agonist formeterol/ isoproterenol elicits a greater shift towards the TM6 domain. In a separate fluorescence spectroscopy based study on human β_2AR receptor structure, the receptor was observed to undergo sequential changes in its conformational states. The endogenous agonist induced activation result in both rapid and slow changes in the receptor conformation that were kinetically distinguishable and represented different signaling cascades of the receptor with regard to G-protein vs. arrestin based signaling. Dopamine induced only the rapid conformational change in β_2AR and therefore activated only the G protein signaling. However, both epinephrine and norepinephrine induced both rapid and slow conformational changes in β_2AR and as a result both G protein dependent and arrestin dependent signaling pathways were upregulated. In the same study, the catechol ring of epinephrine and norepinephrine were found to react with TM3 and TM6 residues of the receptor inducing a rapid phase conformational state. On the other side, the interaction between the chiral hydroxyl group of epinephrine and norepinephrine and TM7 residues of the receptor was shown to be essential for slow phase conformational states in the receptor (Swaminath *et al.*, 2004). In another study the functional selectivity and conformational state of the Ghrelin receptor was correlated using a fluorescent bimane probe attached to the second intracellular loop and third extracellular loop of the receptor (Mary *et al.*, 2012). Following binding of

different biased agonists, the different conformational landscapes were measured by the changes in the bimane fluorescence emission spectra.

Finally, in a structural study on chemokine receptor CCR5, it was suggested that the manipulation of the TM6 and TM7 interface induced biased and constitutive signaling of the Gαi dependent pathway. The CCR5 has been targeted by the drug industry to develop antagonists as it acts as a co-receptor for HIV entry into the cell. Biased agonists that promote the recruitment of arrestin and internalization of receptor and inhibition of Gαi dependent signaling are considered a superior way to inhibit the entry of the virus. The Gly 286 to Phe (G286F) mutation introduces steric hindrance in the receptor structure and affects the positioning as well as rotameric state of the aromatic amino acid Trp248. The G286 F-CCR5 mutant was biased towards G-protein dependent signaling and inhibits β-arrestin dependent signaling. This information could be useful to develop biased agonists towards chemokine receptor (Steen *et al.*, 2013). In the case of the glucagon receptor, the importance of conserved polar residues in TMs 2, 3, 6 and 7 has been recently described (Wootten *et al.*, 2013). Mutation in the conserved polar residues network including the afore-mentioned residues has been shown to induce biased signaling of the glucagon receptor. Thus, the conserved polar residues of class B GPCR are not only involved in the proper folding and expression of the receptor but they also have an effect on the biased signaling of the receptor.

1.1.9 Oligomerization of GPCRs

Traditionally it was considered that GPCRs act as monomeric entities, coupling with the G-protein with 1:1 stoichiometry. The existence of a neuropeptide receptor as dimeric or oligomeric complexes was reported in 1980 when high molecular weight receptor complex bands were observed in a gel filtration column (Agnati *et al.*, 1982). Since then, many other studies have also reported the dimer/oligomer forms of GPCRs such as opioid, adrenoreceptor, chemokine and dopamine receptors (Hebert *et al.*, 1996; Nimchinsky *et al.*, 1997; George *et al.*, 1998; Angers *et al.*, 2000; Jordan *et al.*, 2000; Mellado *et al.*, 2001). The dimerization or oligomerization of GPCRs has been shown to present during synthesis, posttranslational modifications in endoplasmic reticulum, maturation in Golgi bodies, transport to the plasma membrane, ligand binding, G-protein or β-arrestin activation and the endocytosis of the receptor (Bulenger *et al.*, 2005; Milligan, 2008).

A number of techniques such as Bioluminescence resonance energy transfer (BRET), Fluorescence resonance energy transfer (FRET), co-immuno precipitation, atomic force microscopy and X ray crystallography have been used to study the GPCR-GPCR interactions, but these methods have failed to address the functional significance of receptor self-association. As well, questions continue to be raised even over the existence of the dimer/oligomer forms of the GPCR's for the Class A receptors (Chabre and le Maire, 2005). On the other hand, the class C receptors always form dimer/oligomers (Kunishima *et al.*, 2000; Muto *et al.*, 2007). Despite this difference, many receptors from Class A and Class C are able to bind to G-proteins in monomeric forms only. Receptors like rhodopsin, muscarinic M1, somatostatin receptors require a single active receptor to mediate G-protein dependent pathways stimulation, as the presence of another active receptor in the vicinity has been shown to impede signal transduction (Patel *et al.*, 2002; Park and Wells, 2003; Meyer *et al.*, 2006). Contrary to G-proteins, the size and shape of the arrestin molecules allow them to interact with the rhodopsin dimers. The rhodopsin receptors and arrestin molecules interact with each other in a 2:1 stoichiometry to stimulate downstream events (Liang *et al.*, 2003; Fotiadis *et al.*, 2006). Moreover, dimerization of GPCRs helps in the comprehension of ago allosteric modulation of receptor functions (Schwartz and Holst, 2006).

Several studies have shown that GPCRs can exist as heterodimers as well, wherein one type of GPCR makes complexes with other GPCRs. The first evidence of functional heterodimerization came from the study on γ-amino butyric acid receptors ($GABA_BR1$). The study reported that $GABA_BR1$ formed a heterodimer with a $GABA_BR2$ receptor type to facilitate the $GABA_BR1$ trafficking from endoplasmic reticulum to the cell surface. Both receptor types were shown to bind through their trans-membrane domains as well as their N-terminal domains to make a functional unit for intracellular trafficking of $GABA_BR1$ (White *et al.*, 1998; Kuner *et al.*, 1999). Similarly, co expression of β_2AR was reported to enhance the cellular surface expression of α1C as well as α1DAR (Uberti *et al.*, 2005).

Similarly homodimerization and/or heterooligomerization of receptors have been shown to inhibit or facilitate the internalization of receptors and thus effect downstream signaling. As in the case of β_2AR, its oligomeric complexes with the β1AR and β3AR reduced the internalization of β_2AR (Lavoie *et al.*, 2002; Mercier *et al.*, 2002; Breit *et al.*, 2004). As well both β_1AR and β_2AR have been observed to make complexes with the angiotensin receptor type

1 (AT1) to cause cross-inhibition of the receptor signaling by βAR and angiotensin receptor antagonists (Barki-Harrington *et al.*, 2003).

The bonding between different components of GPCR oligomers occurs through covalent and non-covalent interactions including hydrophobic, electrostatic, and disulphide bonds. In the case of the Bradykinin receptor the amino terminal domains are reportedly involved in oligomer formation (Kunishima *et al.*, 2000; AbdAlla *et al.*, 2001), while the carboxyl terminal regions were shown to interact in homo- and hetero-oligomers of the δ receptor as well as cannabinoid receptors (Trapaidze *et al.*, 1996; Wager-Miller *et al.*, 2002). Besides that, the TMDs have been shown to facilitate the interaction of different receptors as the TM1 and 2 of rhodopsin receptor were observed to make contact with the TM5 and 6 of another rhodopsin molecule in dimer formation (Liang *et al.*, 2003). Furthermore, the TM4 domain of rhodopsin was observed to be crucial for the heterodimerization of rhodopsin with those of serotonin and metabotropic glutamate 2 receptors (Gonzalez-Maeso *et al.*, 2008). Despite controversial views, current evidence indicates that GPCRs form homo-oligomers as well as hetero-oligomers to affect their physiological responses. This concept has allowed the drug industry to design drugs that can target the heterooligomers to make use of cross talk between receptors. For example beta-blockers can block the signaling of both βAR and AT1AR receptors, which have been shown to exist in a heterodimerized state.

1.2 *Saccharomyces cerevisiae* as a model to study GPCR structure and function

Yeasts are unicellular eukaryotes that have been classified in the kingdom Fungi as typical heterotrophs, are able to produce spores and maintain similar cell wall structures as other fungus. Among yeasts, *Saccharomyces cerevisiae* (*S. cerevisiae*) also known as baker's or brewer's yeast has been commercially utilized for winemaking, baking and brewing since ancient times due to its ability to generate carbon dioxide and alcohol during carbohydrate metabolism. *S. cerevisiae* has been classified as belonging to the Ascomycota phylum of yeasts and reproduces through both asexual and sexual life cycles. In its asexual life cycle, yeast proliferates through budding or mitosis as it doubles its number every 100 min, producing identical haploid daughter cells. In the sexual life cycle of the yeast, two mating type haploid cells, Mat a and Mat α, fuse to give a diploid zygote (Ydenberg and Rose, 2008). During the fusion of haploid cells types both cellular and nuclear fusion occurs to form a single diploid cell

(zygote), which can further undergo mitosis to produce more diploid cells. Under nutritional deprivation, each zygote undergoes meiotic cell division to produce four haploid cells (Herskowitz, 1988).

Yeast mating haploid cells (Mat a and Mat α) express mating receptors on their surfaces, Ste2p and Ste3p respectively, that belong to the GPCR superfamily. The ligands for Ste2p and Ste3p are mating pheromones a and α, respectively, secreted by opposite cell types. Like other GPCR, both Ste2p and Ste3p were observed to couple with a G-protein mediated MAPK pathway upon pheromone binding (Bender and Sprague, 1989; Slessareva and Dohlman, 2006). Since the early 1990's, the yeast-mating system has served as a model and tool to study GPCR function in humans and other species (Dohlman *et al.*, 1991; Pausch, 1997; Dohlman, 2002; Ladds *et al.*, 2005). The yeast mating system offers several advantages as it has only two GPCRs, one for mating and one for sugar metabolism compared to about 800-1000 GPCR in humans. Thus in yeast, there is no cross talk between receptors and signaling can be studied separately for individual receptors. Most of the components of the yeast-mating pathway have been identified and shown to have homologous counterparts in mammalian system (Panetta *et al.*, 1999; Kong *et al.*, 2002; Ladds *et al.*, 2005). Indeed, many regulatory proteins of the MAPK pathway were first discovered in yeast, and subsequently led to the discovery of similar components in human GPCR pathways as well. As an example, the Sst2 (RGS protein in yeast) protein was first identified in yeast (Dietzel and Kurjan, 1987) and later its homologous protein RGS5 was identified in human (Seki et al,1998). Moreover, the Gβγ mediated signal transduction pathway and Ste5 scaffold protein were first reported in studies on yeast (Whiteway *et al.*, 1989). Despite sequence dissimilarities between the yeast mating receptors and other GPCRs, other GPCRs such as β_2AR (King *et al.*, 1990) have been successfully linked to the yeast-mating pathway. The frizzled receptors (fz1 and fz2) when targeted to the yeast membrane were shown to stimulate the mating pathway as well. Furthermore, introduction of an intracellular part of frizzled receptors into Ste2p receptor not only allowed the Ste2p to retain their responsiveness to the mating factor but also made the Ste2p constitutively active (Dirnberger and Seuwen, 2007).

As such the yeast system has been used in the screening of ligands for orphan receptors that have no known physiological functions, using different adaptations for the expression of heterologous receptor in the yeast system. One such modification was the use of a chimeric

yeast G-protein, where the extreme C-terminus of the yeast Gα is replaced with the equivalent residues of the mammalian Gα that showed an increased affinity towards orphan receptors. Expression of an orphan receptor in the chimeric yeast G-protein system was able to induce the yeast signal transduction pathway (Brown *et al.*, 2000). On the other hand, Ste2p folded and embedded in a mammalian membrane system (HEK293), and was able to activate the Erk1 kinase pathway following stimulation with the α factor ligand (Yin *et al.*, 2005).

In addition to the above-mentioned aspects of the yeast receptor system, there are other general features of yeast cells that make them a versatile and robust model system for studying human GPCR. The yeast cells are non-motile, non-pathogenic microorganisms that can be easily propagated in the laboratory as they have a generation time of only 90 min. Further, the yeast genome is much smaller than human as it has 6000 genes and 12 mega base pairs (Mbp) of DNA on 16 linear chromosomes making it easy to implement genetic and recombinant manipulations. Indeed, yeast is the only model organism where it is possible to track the expression and localization of almost every gene of a signaling pathway thus making yeast an attractive tool to study the complexity of GPCR signal transduction.

1.2.1 Evolutionary origin of Fungal GPCRs

Yeast mating receptors (Ste2p and Ste3p) belong to the Class D GPCRs group based on sequence similarities and physiological function. However, yeast pheromone receptors were not included in the GRAFS classification system due to their unique sequences and novel functions. Over the years, additional GPCRs were identified in fungi that have been classified into six major families including, Ste2p like pheromone receptor, Ste3p like pheromone receptor, carbon/ amino acid receptor, nutrient receptor, cAMP like receptor and microbial opsin receptor, based on their sequence similarities and ligand sensing (Xue *et al.*, 2008).

Initially it was thought that the mammalian GPCRs and fungal GPCRs had different evolutionary origins. However, more rigorous analysis of mammalian GPCR revealed that four of the five main families of mammalian GPCRs, namely Rhodopsin, Adhesion, Glutamate and Frizzled have several homologs in fungi (Krishnan *et al.*, 2012). So far, 142 novel sequences of mammalian GPCR have been identified in fungal organisms. Like all mammalian GPCRs, fungal GPCRs have also emerged from the cAMP receptor. Pheromone sensing receptors (Ste2p and Ste3p) diverged in the evolutionary tree after the split of the Dikarya, which is a

subkingdom of fungi and includes the phyla ascomycetes and basidiomycetes. Interestingly, the Chytridiomycota phyla of fungi that arose early in the evolution tree has only metazoan lineage for its GPCR, that further evolved to become a fungal specific GPCR in *Ascomycetes* and *Basidiomycetes*.

The fungal GPCRs play important roles in fungal-plant interactions as many plant products act as the ligands for various GPCRs. Further, GPCRs from pathogenic fungi have been observed to mediate human and plant diseases (Silveira and Paterson, 2005; Xu *et al.*, 2006; Meersseman *et al.*, 2007).

1.2.2 Yeast mating and mating receptors (Ste2p and Ste3p)

1.2.2.1 Mating in General

Yeast have two haploid mating type cells, 'Mat a' and 'Mat α', that express mating specific surface receptors Ste2p and Ste3p, respectively. The involvement of these receptors in yeast mating has been proven beyond doubt, as sterile mutants did not respond to the pheromones (Hartwell, 1980).

Each haploid cell, 'Mat a' and 'Mat α', secretes a mating specific pheromone, 'a' and 'α', respectively, and expresses a surface localized GPCR (Ste2p or Ste3p, respectively) that can bind to the pheromone secreted by the opposite cell type (Bender and Sprague, 1989; Slessareva and Dohlman, 2006). Upon pheromone binding, these GPCR initiate G-protein mediated MAPK signal transduction events that are homologous to those observed in mammalian systems and are known to lead to G1 cell cycle arrest as well as associated transcriptional changes. Cell cycle arrest in yeast sets the stage for subsequent mating events including projection formation, agglutination, plasmogamy and karyogamy to produce the final diploid zygote. During polarized growth, the haploid yeast cells assume the pear shaped structure, known as "shmoo", which projects towards the opposite mating partner present in close vicinity (Lipke *et al.*, 1976; Tkacz and MacKay, 1979). Two opposite mating types adhere to each other on "shmoo" tips and lead to cell wall dissolution, plasma membrane juxtaposition, remodeling and ultimately membrane and nuclear fusion (White and Rose, 2001) (Figure 1.10).

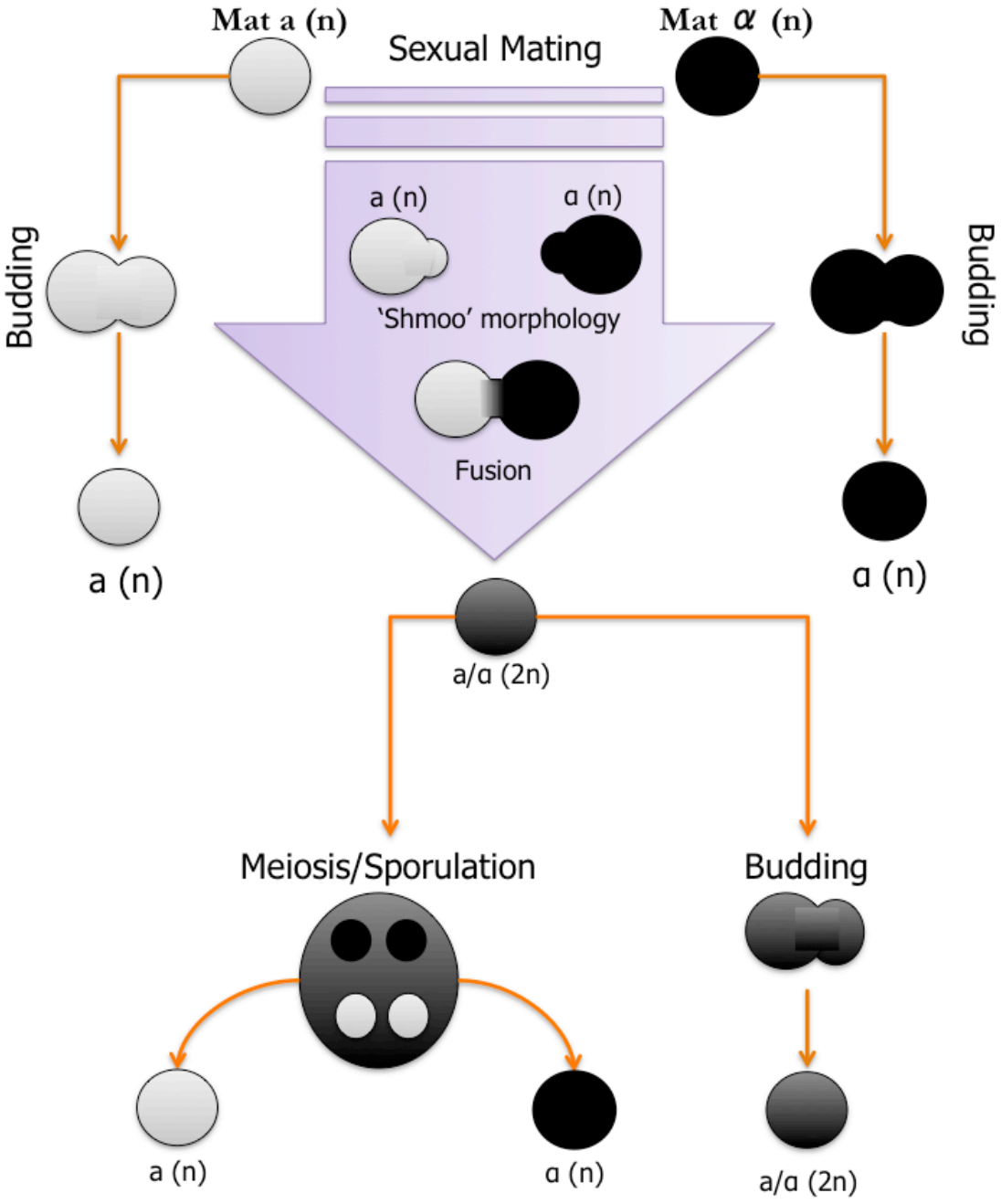


Figure 1.10 Yeast life cycle. Yeast has two phases in the life cycle, the asexual life cycle with budding and a sexual life cycle with mating. During budding, yeast reproduces through mitosis whereas in mating, two haploid cells Mat 'a' and Mat 'α' fuse to produce the diploid zygote. Diploid zygotes are able to reproduce through mitosis and meiosis (sporulation to give the diploid and haploid cells respectively [adapted from (Herskowitz, 1988)]. Image reprinted with permission from American society for microbiology (ASM).

1.2.2.2 Mating pheromones

Mating pheromones, a-factor and α-factor are the primary requirements in mating in addition to the mating receptors. The α-factor is a 13 amino acid (sequence WHWLQLKPGQPMY) long peptide secreted by the Mat α cells, whereas, a-factor is a 12 amino acid peptide (YIIKGVFWDPAC) produced by the Mat a cells that is covalently attached to a lipid farnesyl group and has a carboxy methyl group that is required for its biological activity (Caldwell *et al.*, 1995; Edwards and Ericsson, 1999). The lipid group attached to the a-factor makes it very hydrophobic and very difficult to study in structure function assays. In the case of α factor, the C-terminal residues (10-13) are important in binding of ligand to the receptor whereas the N-terminal residues (1-4) are crucial for receptor activation. The central residues of the α factor have been observed to contain a pro-gly sequence yielding a β turn in its structure that facilitates the orientation of its binding to the receptor. Various mutational and cross-linking studies have been used to understand and propose a model for binding of α factor to the receptor. It has been proposed that C-terminal residues of α factor are bound to the extracellular end of TM1, while the N-terminal residues are bound to the extracellular loop of TM6 and the third ECL of the receptor (Mathew *et al.*, 2011).

1.2.2.3 G Protein dependent Signaling

Binding of mating factors to their respective receptors results in receptor activation indicated by conformational changes as described in the previous sections, for other GPCRs. The activated mating receptors are now able to bind to the heterotrimeric G-protein on their cytoplasmic domains and the basic mechanism of G protein activation is similar in both Ste2p and Ste3p receptors. As in mammalian cases, activated yeast mating receptors act as guanidine nucleotide exchange factors for the Gα subunit of G-proteins and cause dissociation of the G-protein into two subunits, Gα (Gpa1) and Gβγ (Ste4p and Ste18p) subunits (Bender and Sprague, 1986; Blumer and Thorner, 1991). Later studies reported some information in addition to the basic mechanism of G-protein activation by the mating receptors. The Gα is not truly released from the trimeric complex as it may remain loosely bound to the Gβγ complex and receptor itself (Klein *et al.*, 2000). As well Gα has been proposed to play an additional role in signaling besides keeping the Gβγ in the inactive form (Metodiev *et al.*, 2002; Guo *et al.*, 2003); although the Gβγ subunit is again responsible for the flow of the information through the

MAPK pathway to the various targets in the nucleus and cytoplasm.

Cellular responses of G-protein dependent signaling include, growth arrest at the G1 phase of the cell cycle (Wittenberg and Reed, 1996; Oehlen *et al.*, 1998), induction of gene transcription (Sprague, 1991b; Roberts *et al.*, 2000) polarized growth (Madden and Snyder, 1998), and change in nuclear architecture (Stone *et al.*, 2000). Arrest in the growth or cell cycle during yeast mating is required so that both mating partners are at the same stage of the cell cycle (Hartwell, 1973). Subsequently, proteins required for cell adhesion and fusion are synthesized by induction of various genes (Guo *et al.*, 2000; Heiman and Walter, 2000). As the yeast cells are non-motile, they require polarized growth in order to bring the opposite mating partner into close proximity and designate the site of the cell fusion (Dorer *et al.*, 1994). The changes in nuclear architecture have been shown to prepare the nucleus for nuclei fusion and ultimately zygote formation (Rose, 1996).

The Gβγ complex that separated from the activated G-protein transmits a signal to three different effectors in order to elicit the cellular responses. The three effector proteins that are activated in response to Gβγ complex are i) cdc24 ii) Ste20 and iii) Ste5. Ste20, the first effector protein to be activated in the mating pathway is a member of the p21 activated protein kinase family (Lim *et al.*, 1996). Upon pheromone stimulation, Ste20 binds to the Gβ subunit of the released βγ dimer complex, which causes Ste20 kinase to bind to Ste11 protein, the first kinase of the MAPK pathway. Another effector of the pathway, Cdc24, is a guanidine exchange nucleotide factor for the Cdc42 protein (Zheng *et al.*, 1994). In the absence of pheromones, Cdc24 is mainly sequestered in the nucleus; however, it migrates to the cytoplasm as a complex with Far1 upon pheromone stimulation (Nern and Arkowitz, 1999). Far1 is a scaffold protein that joins Cdc24 with the Gβγ dimer to form a complex that has been shown to stimulate GDP-GTP exchange upon Cdc42 (Butty *et al.*, 1998; Nern and Arkowitz, 1999). Far1 protein acts as an adaptor for bringing the Cdc24 and its substrate Cdc42 to the plasma membrane. Cdc42 is a small Rho like G-protein. Once activated, Cdc42-GTP has many targets in the cytoplasm, including activation of many proteins involved in the assembly of actin microfilament in yeast (Kozminski *et al.*, 2000) such as a formin homolog, Bni1 (Evangelista *et al.*, 1997), Gic1 and Gic2 (Brown *et al.*, 1997; Chen *et al.*, 1997; Bi *et al.*, 2000), and Lsb7 (Soulard *et al.*, 2002). The Gβγ-far1-Cdc24-Cdc42 complex localized in the mating projection tip is required for the cytoskeletal rearrangements and polarized growth. Another function of

Cdc42-GTP is to activate Ste20 protein kinase that is otherwise present in a low activity state in the cytoplasm. The N-terminal Cdc42/Rac interaction-binding domain (CRIB) of Ste20 occupies the active site present in its catalytic domain, which is unmasked and available for phosphorylation following binding of Cdc42-GTP to the CRIB domain (Leberer *et al.*, 1992; Ramer and Davis, 1993). Cdc42 is also tethered to the plasma membrane through its covalently attached geranyl-geranyl group and thus keeps Ste20 on the plasma membrane to stimulate the MAPK pathway. The third effector of the pathway is a large multifunctional protein, Ste5, which is a scaffold protein located mainly in the nucleus in the absence of pheromones. Upon pheromone stimulation Ste5 migrates into the cytoplasm where it interacts with the Gβγ dimer and Ste11 protein (Pryciak and Huntress, 1998; Mahanty *et al.*, 1999; Sette *et al.*, 2000; Kunzler *et al.*, 2001). In the cytoplasm, Ste5 is in close proximity to Ste20 that is bound to Gβγ-Far1-Cdc24 complex and Cdc42 protein. This allows Ste20 kinases to facilitate the phosphorylation and activation of Ste11 to initiate the MAPK pathway that in turn results in the induction of many genes and arrest of cell cycle in G1 phase (Figure 1.11).

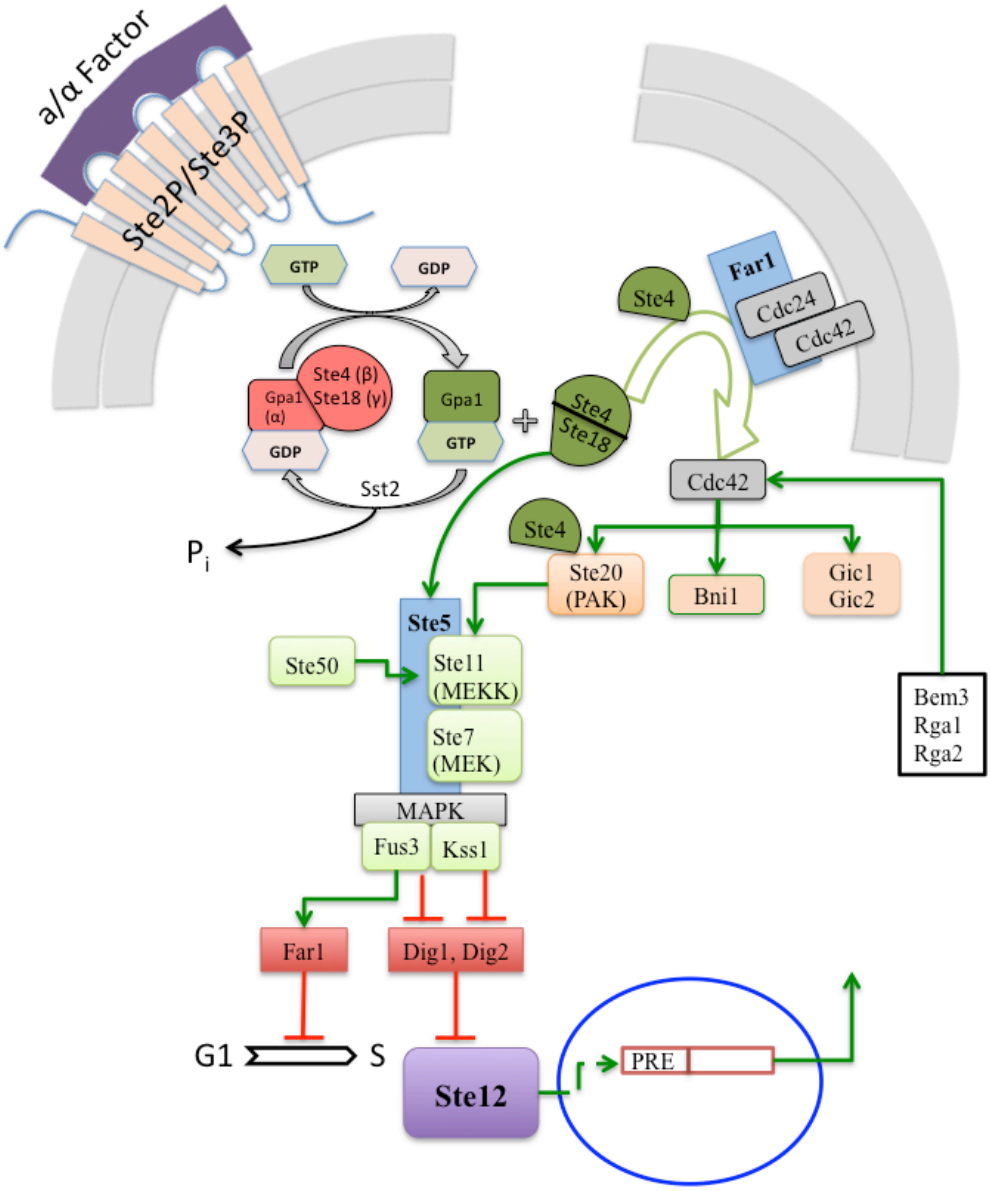


Figure 1.11 Schematic representation of the yeast pheromone mating pathway. The mating factor pheromones bind to a cell surface receptor (Ste2p/Ste3p), which promotes the GTP binding on Gα (Gpa1) subunit and its dissociation from Gβγ (Ste4 Ste18) subunits. Free Gβγ activates multiple effector molecules, including Ste20 protein kinase, an adaptor protein Far1 and a scaffolding protein Ste5. The attenuation of signal is carried out by the GTP hydrolysis and re-association of G protein subunits. An RGS protein (Sst2) accelerates the GTPase activity of Gα subunit. Far1 binds with the cdc24 and Gβγ subunits and this complex activates the GDP-GTP exchange on cdc42 protein. Once activated, cdc42 has many targets including many proteins involved in the assembly of actin microfilaments Bni1, Gic1 and Gic2 and upstream activator of MAPK pathway-Ste20 protein kinase. Scaffold protein Ste5 binds with the kinases of MAPK pathway-Ste11, Ste7 and Fus3 [adapted from (Bardwell, 2005)]. Image reprinted with permission from Elsevier.

1.2.2.3.1 MAPK kinase in Yeast Mating: An Overview

As mentioned previously, MAPK pathways are three-protein kinase cascades and in the case of the yeast mating process include Ste11 (MKKK), Ste7 (MKK) and Fus3 (MAPK). The Fus3 MAPK is closely related to the ERK kinases. With the help of the Gβγ subunits, Far1 and Ste5 protein, all the components of the MAPK machinery are recruited to the plasma membrane. P21 activated protein kinase Ste20 (Shi *et al.*, 2002), binds to the Ste11 (MKKK) and phosphorylates its N-terminal regulatory domain. The target of the Ste11 is the activation loop of Ste7 (MKK), another kinase in the pathway. Ste7 further activates the Fus3 (MAPK) kinase (Figure 1.11). Ste5 has distinct binding domains for Ste11 and Fus 3 proteins thereby assisting in keeping all the components of MAPK together to enhance the signaling and specificity of the pathway (Burack and Shaw, 2000; Elion, 2001; Harris *et al.*, 2001; Ptashne and Gann, 2003). One more component present in the yeast MAPK pathway is Ste50 protein that binds to the Ste11 to assist in its activation (Jansen *et al.*, 2001).

Ste11 (MKKK): Ste11 has N terminal and catalytic domains that govern its function. The N-terminal region is divided into a sterile alpha motif (Pei *et al.*, 1994) domain where Ste50 binds, a carboxy terminal domain (CBD) that maintain the inactivity of the catalytic domain and a Ste5 binding domain between the SAM and CBD regions (Wu *et al.*, 1999; Drogen *et al.*, 2000; Jansen *et al.*, 2001). Ste20 protein phosphorylates the serine and threonine residues of the CBD that antagonizes CBD's ability to inhibit the catalytic domain (Drogen *et al.*, 2000). Further, Ste50 binding to Ste11 accelerates Ste20 phosphorylation of CBD (Wu et al., 1999). Interaction between Ste5 and Ste11 brings Ste11 closer to Ste20 and makes CBD residues more accessible to the Ste20 phosphorylation. Ste5 also binds with Ste7 and helps in the transmission of the signal to the next component of the MAPK pathway (Figure 1.11).

Ste7 (MKK): Like all other kinases, Ste7 consists of an N terminal extension and catalytic domain that is well conserved (Bardwell *et al.*, 2001). Ste11 phosphorylates specific residues in the activation loop of Ste7. Ste7 has great affinity for Fus3 MAPK through its N-terminal extension, and thus phosphorylates the serine and threonine residues of Fus3 (Bardwell *et al.*, 2001).

MAPK Targets*:* The Fus3 MAPK is a proline directed serine threonine kinase that phosphorylates many targets in the nucleus including Ste12/Dig1/Dig2 transcription factor and Far 1 protein. Ste12 is a DNA binding transcriptional trans-activator factor that binds to a

consensus motif region of the DNA promoter known as the pheromone response element (PRE) (Harrison and DeLisi, 2002). The PRE has been observed to regulate the transcription of around 100 genes upon pheromone simulation (Roberts *et al.*, 2000). Dig1 and Dig 2 transcription factors act as negative regulators of Ste12 activity as they keep Ste12 in an inactive form in the absence of pheromone stimulation (Tedford *et al.*, 1997). Following phosphorylation of Dig1/Dig2 by Fus 3 they no longer suppress Ste12 activation. Upon pheromone stimulation, Ste12 activates the transcription of the many components of the mating pathway, including Ste2/Ste3 receptors, pheromones, Far1 and Fus3 as well as many fusion specific genes such as *FUS1, FUS2, FIG1, FIG2* and *AGA1* (White and Rose, 2001).

Far1 is another target that is regulated by the Fus3 kinase. Once activated Far1 participates in projection formation as a complex with Gβγ and Cdc24 and in the pheromone imposed cell cycle arrest as described above (Chang and Herskowitz, 1990). However, the exact mechanism by which it promotes cell cycle inhibition is not clear (Gartner *et al.*, 1998). A few studies have proposed that Far1 might act as an inhibitor of cell cycle dependent kinases (CDK28) as it has been associated with the CDK28 (Tyers and Futcher, 1993; Peter and Herskowitz, 1994).

1.2.2.3.2 Regulation of G-protein dependent Signaling

G-protein signaling in yeast is very fast as maximal activity of the G-protein is observed within seconds after ligand binding to yeast mating receptors (Yi *et al.*, 2003). Further, stimulation of the MAPK pathway is detectable within min of G-protein activation (Sabbagh *et al.*, 2001). All the steps in signal transduction are tightly regulated to maintain the specificity and sensitivity of the pathway.

Regulation of G-protein signaling in yeast mating has been shown to occur at multiple levels. One such regulation occurs at the level of ligand secretion as both 'a' and 'α' factors are exported from the respective cell types through different methods. The 'α' factor is synthesized in the endoplasmic reticulum as a pre-prohormone precursor and thereafter processed in the Golgi bodies to be finally secreted through secretory vesicles (Fuller *et al.*, 1988; Brake, 1989). On the other hand, farnesylated and methylated 'a' factor pheromone is exported directly from the cell by the Ste6 transporter (Kuchler *et al.*, 1993; Schmidt *et al.*, 2000). Both ligands are secreted at the tip of the mating projections resulting in development of pheromone gradients in the mating projections. Both α factor (*MFα1*) and a factor genes (*MFA1* and *MFA2*) are

induced by the pheromones themselves therefore both genes are influenced by the level of pheromones in the mating projections (Sprague, 1992; Roberts *et al.*, 2000). Further, Bar1, a pheromone inducible protease, secreted by the Mat a cells has been observed to control the levels of 'α' factor (Ciejek and Thorner, 1979; MacKay *et al.*, 1991).

Like mammalian G-proteins, yeast Gα subunit has intrinsic GTPase activity for GTP-GDP hydrolysis but it has been reported to occur at a very slow rates. Sst2 protein, one of the first known members of the RGS (Mathew *et al.*, 2011) has been shown to shorten the life span of the active G-protein (G-GTP). Sst2 has been shown to shorten the life span of G-proteins by increasing the GTP hydrolysis rate by at least 20 fold. Furthermore, Sst2 has been observed to accelerate Gα subunit binding with Gβγ complex (Apanovitch *et al.*, 1998). Sst2 is a pheromone inducible protein as its mRNA and protein levels are up regulated after treatment with opposite pheromone (Dietzel and Kurjan, 1987; Dohlman *et al.*, 1996). The stability of Sst2 was observed to increase by phosphorylation at serine residues, in the pheromone-stimulated cells under the influence of Fus3 MAPK (Garrison *et al.*, 1999). The Gpa1 (Gα) subunit of G-protein is reported to undergo several post-translational modifications such as myristoylation and palmitoylation at the N-terminal amino group and internal cysteine residues, respectively (Casey, 1995). Both modifications are important for proper targeting of Gα subunit towards the plasma membrane (Medici *et al.*, 1997). Further, the Ste4 (Gβ) subunit has been shown to undergo phosphorylation (at several residues) upon pheromone stimulation, however the exact role of phosphorylation is yet to be determined. One study has suggested that the phosphorylation might contribute to the attenuation of MAPK signal (Cole and Reed, 1991). In the case of Ste18 (Gγ), isoprenylation and palmitoylation of its residues have been observed to result in tethering of the subunit to the plasma membrane. Other functions that might be governed by such modifications of Ste18 subunit are hetero-trimer complex assembly and G-protein activation (Fu and Casey, 1999).

Small Rho like G-protein, Cdc42 has been shown to promote morphogenesis and regulate the transcription of genes through Ste20 activation. The three GTPase accelerating proteins (GAPs) Bem3, Rga1 and Rga2 were observed to control the GTP-GDP hydrolysis of Cdc42 (Smith *et al.*, 2002). Besides GAPs, many phosphatases have been observed to attenuate steps of signal transduction following ligand binding to the yeast receptors. The kinase activity of Fus3 is regulated by the Ptp1/Ptp2 and Msg5 phosphatases as the former inactivates the Fus3

kinase, while the later one limits the active life span of Fus3. Further, the expression level of Msg5 itself is regulated by pheromone stimulation (Doi *et al.*, 1994; Zhan *et al.*, 1997). The scaffolding protein, Ste5, has an additional role as it was observed to catalytically unlock the Fus3 sites for its phosphorylation by Ste7 kinase (Good *et al.*, 2009). Moreover, Fus3 also phosphorylates the Ste5 scaffold protein at multiple sites that are needed to keep the steady state level of the Fus3-Ste5 complex. The phosphatase, Ptc1 acts on the Ste5 to dephosphorylate it and further dissociate the Fus3-Ste5 complex that is needed to maintain availability of Fus3 for further downstream signaling (Malleshaiah *et al.*, 2010).

1.2.2.3.3 Specificity in the MAPK pathway

As in the case for other eukaryotes, yeast exhibits sharing of the components across different signaling pathways and still maintains signaling specificity through different mechanisms. To date three different MAPK pathways i.e. mating response pathway, filamentous growth pathway and high osmolarity pathway, have been associated with *S. cerevisiae* signal transduction (Posas and Saito, 1997). Despite the observations that many of the components of the three MAPK pathways are shared, the components are capable of transducing distinct pathways and eliciting appropriate cellular responses. Along the evolutionary path, eukaryotic organisms have undertaken different mechanisms to maintain the specificity of the signaling pathway. Sequestration is the one general mechanism by which physical separation of common components of pathways is achieved by expressing in a cell-type specific manner to avoid the cross talk of the pathways and thus maintain specificity (Schwartz and Madhani, 2004). Subcellular compartmentalization is another way to maintain specificity when the shared pathways are expressed in same cell (Schwartz and Madhani, 2004). Scaffolding proteins have been shown to assist in subcellular compartmentalization, as they were observed to tether two or more components of the pathway to accelerate signaling and maintain specificity at the same time (Harris *et al.*, 2001; Park *et al.*, 2003). In combinatorial signaling, the input from two signaling pathways is required to activate one desired pathway and get a cellular response. This type of signaling is needed to avoid spontaneous yet unnecessary signals for a particular pathway. On the other hand, cross-pathway inhibition mechanisms allow the activation of one pathway that causes inhibition of a specific component of another pathway (Schwartz and Madhani, 2004). Usually a high level of regulation is required to ensure activation of pathways in the presence of appropriate stimuli.

In haploid yeast cells, the MAPK pathways of mating and filamentous growth have many shared components that include kinases Ste20 (Shi *et al.*, 2002), Ste11 (MKKK) and Ste7 (MKK) (Elion *et al.*, 2005) as well as upstream activators like Ste50, Cdc42 and transcription factors (Elion *et al.*, 2005). On the other hand, two different MAPK are needed for non-shared pathways as Fus3 is required for mating and Kss1 is needed for filamentous growth (Cook *et al.*, 1997; Madhani *et al.*, 1997). Though Kss1 is produced in both mating and filamentous growth, several studies have shown mechanisms that prevent Kss1 from modulating the mating pathway while activating filamentous growth. Scaffold protein Ste5 is one such method to prevent the erroneous cross talk of the pathways as it binds to Fus3 but not Kss1 (Andersson *et al.*, 2004; Cullen *et al.*, 2004; Maleri *et al.*, 2004). Further, Msg5, activated during filamentous growth, was shown to lower Fus3 levels causing selective activation of filamentous growth (Andersson *et al.*, 2004).

Additionally, MAPKs have unique targets to maintain the specificity of pathways. Fus3 but not Kss1was observed to phosphorylate Far1, but both Fus3 and Kss1 phosphorylate the Ste12 transcription factor. Ste12 was observed to stimulate another transcription factor Tec1, which is required for the expression of filamentous specific genes (Zeitlinger *et al.*, 2003). The levels of Tec1 during mating were negatively regulated by Fus3 due to phosphorylation at various residues (Madhani *et al.*, 1997; Sabbagh *et al.*, 2001; Bao *et al.*, 2004; Chou *et al.*, 2004), but were not affected by Kss1 (Bruckner *et al.*, 2004). Phosphorylated Tec1 can be degraded through ubiquitin dependent proteolysis by SCF ubiquitin ligase to favor specificity of the mating pathway (Bao *et al.*, 2004; Chou *et al.*, 2004).

High osmolarity glycerol (HOG) MAPK is another pathway that has been shown to share the upstream activators Ste50 and Cdc42 with mating and filamentous growth pathways, however the Pbs2 (MKK) and HOG (MAPK) are unique to the HOG MAPK pathway (Posas and Saito, 1997; O'Rourke and Herskowitz, 1998). Pbs2 has been observed to phosphorylate HOG MAPK and scaffold Ste11 and HOG kinases (O'Rourke and Herskowitz, 1998). The HOG MAPK is induced under the condition of high osmolarity in order to stimulate the synthesis of solutes to compensate for osmotic pressure (O'Rourke *et al.*, 2002).

1.2.3 Trafficking of Ste2p/Ste3p receptors

As in the case of several other GPCRs, yeast-mating receptors are synthesized in the ER, processed in the Golgi-bodies and then targeted to the cell surface. Binding of ligand to the surface located yeast mating GPCR stimulates G-protein dependent signal transduction to elicit desired cellular response. Thereupon, the yeast mating GPCR is endocytosed, which terminates the downstream signals. Endocytosis of receptors is possible in the absence of ligands, termed constitutive endocytosis. Though both ligand dependent internalization and constitutive endocytosis have been shown to occur through similar mechanisms, the former occurs at a much greater rate (Jenness and Spatrick, 1986; Davis, 1993; Raths *et al.*, 1993; Schandel and Jenness, 1994). During ligand dependent internalization of yeast mating receptors, ligand mediated conformational changes expose residues in the C-terminal tail to the endocytic machinery (Rohrer *et al.*, 1993; Bukusoglu and Jenness, 1996). The C-terminal residues of the receptors are phosphorylated at various sites including SINDKASS sequence. Three serine residues in the SINDKASS sequence of Ste2 were reported to mediate its constitutive internalization but not the ligand dependent internalization (Rohrer *et al.*, 1993; Hicke *et al.*, 1998). On the other hand, residues in the middle region of the C-terminal tail of Ste2p receptor were found to participate in the ligand dependent internalization (Kim *et al.*, 2009). The phosphorylation of serine and threonine residues in the C-terminal tail is mediated by the yeast casein kinases (Yck1p and Yck2p) for Ste2p receptor (Hicke *et al.*, 1998) and by Fus3 kinase for Ste3p in negative feedback dependent regulation (Feng and Davis, 2000).

Following phosphorylation, the ubiquitination of the lysine residue of the SINDKASS sequence is crucial for internalization of receptor, as mutation of the lysine residue to arginine results in failure of receptor internalization (Rohrer *et al.*, 1993; Hicke and Riezman, 1996). The constitutive phosphorylation and ubiquitination of the lysine residue of SINDKASS sequence are possible events but the presence of ligand was observed to make the process much more efficient (Roth and Davis, 1996). Three enzymes, ubiquitin activating, ubiquitin conjugating and ubiquitin ligase, facilitate the covalent binding of the ubiquitin to the lysine residue of the receptor. In yeast, Rsp5 (yeast homologue of Ned4) acts as ubiquitin ligase in the reaction (Hicke and Dunn, 2003) and both Ste2p/Ste3p receptors bind to a single moiety of ubiquitin prior to their endocytosis (Roth and Davis, 1996).

Ubiquitinated yeast mating receptors are assembled in CCPs for endocytosis through the

dynamic network of endocytic adaptors and clathrin protein (Traub, 2003; Sorkin, 2004). The endocytic adaptors are multi-subunit complexes, which bind to the lipid moiety of the membrane as well as assist in the recruitment of clathrin protein towards the membrane (Wendland and Emr, 1998; Meyerholz *et al.*, 2005; Newpher *et al.*, 2005; Edeling *et al.*, 2006; Maldonado-Baez and Wendland, 2006). Yeast endocytic adaptors, epsins, Ent1 and Ent2, carry functional ubiquitin-interacting motif (Wendland and Emr, 1998; Wendland *et al.*, 1999; Aguilar *et al.*, 2003), whereas the Ede1 (an epsin 15 like protein) has a ubiquitin-binding motif on its C-terminus (Dores *et al.*, 2010). Once the clathrin complex is assembled, it is pinched off from the membrane and enters into the vesicular intermediates. Similar to mammalian cells, the internalized yeast mating receptor is directed towards lysosomal degradation through formation of endocytic vesicles and MVB (Singer and Riezman, 1990; Schandel and Jenness, 1994). A 33kDa vacuole membrane syntaxin homolog (VAM3) was shown to have a role in targeting the receptor bearing vesicles to the lysosomal compartment (Stefan and Blumer, 1999).

Unlike Ste2p receptor, Ste3p has two different processing modes of action for constitutive and ligand dependent endocytosis. The constitutive internalization of Ste3p receptor involves ubiquitination followed by lysosomal degradation. In contrast, ligand dependent endocytosis is not linked to the ubiquitination and degradation of the receptor, as the Ste3p receptor is recycled back to the membrane and reutilized in the presence of its ligand (Chen and Davis, 2002).

1.2.4 Oligomerization of Yeast mating receptors

As mentioned in a previous section, dimerization/oligomerization of GPCRs is important for various functions such as receptor targeting towards the plasma membrane, signaling and endocytosis of the receptor (Park and Wells, 2003; Terrillon and Bouvier, 2004; Overton *et al.*, 2005). Like other GPCRs, yeast-mating receptors were shown to present dimeric or oligomeric forms those are evident in western blots.

In order to identify the specific regions that are involved in dimer/oligomer formation in yeast mating receptors different studies have been conducted. As Ste2p lacks cysteine, the cysteine crosslinking methods were used as a tool to study dimer/oligomer formation. It was observed that the extracellular residues of TM1 and TM4 form contacts (TM1-TM1, TM4-TM4

and TM1-TM4) in Ste2p (Wang and Konopka, 2009). The study also suggested TM1-TM4 contact was observed in oligomers only due to distant location of TM1 and TM4 domains on the Ste2p receptor (Wang and Konopka, 2009). In another study, TM7-TM7 interactions were reported to be involved in the dimer formation in addition to the TM1 and TM4 interactions (Kim *et al.*, 2009). TM1/TM7 domains were proposed to interact with the TM7/TM1 of another receptor in a dimer, whereas TM4-TM4 interactions were proposed possible with a third receptor in an oligomeric structure (Kim *et al.*, 2009). Interestingly, ligand binding decreases the level of dimer formation formed by TM7-TM7 interactions (Kim *et al.*, 2009). In another study, a possible role of α factor ligand was proposed in oligomer formation of Ste2p, although, the study didn't preclude the existence of dimeric forms in the absence of ligand (Shi *et al.*, 2009b).

However, in an earlier study ligand binding was observed to have no effect on the equilibrium of monomer to dimer forms (Overton *et al.*, 2005). It was speculated that ligand might affect the dimer interface but not the total amount of dimer formation. A more recent study has shown that the N-terminal domain residues of Ste2p are involved in dimer formation (Uddin *et al.*, 2012). These residues are part of the putative β strand in the N terminus of Ste2p. The study also suggested that a ligand induced conformational change in the N terminus of the Ste2p receptor (Uddin *et al.*, 2012).

Physiologically, in addition to a role in receptor activation, the homo-oligomeric units of Ste2p receptor were considered functional units for endocytosis (Yesilaltay and Jenness, 2000). Moreover, a specific residue in the TM1 is believed to be involved in oligomerization as well as the expression of the receptor on the plasma membrane. Substitution of amino acids in a GXXXXG motif of TM1 impaired the oligomer formation and eventually inhibited receptor targeting to the cell surface (Overton *et al.*, 2003). In addition, oligomerization of Ste2p receptor was proposed to be very critical for G-protein signaling that can be activated in two ways. G-protein activation may occur due to individual binding of two receptor monomer units to two G-protein units or collective binding of two receptor dimer units to a single G-protein (Overton and Blumer, 2000).

1.2.5 Structure/function analysis of Ste2p

To date no crystal structure of Ste2p has been solved. However, extensive

structure/function studies have been carried out. These studies have addressed the changes Ste2p receptor undergoes upon ligand binding. The third intracellular loop of Ste2p receptor is considered very important in receptor activation and signal transduction as it provides the binding site for Gpa1. The third ICL undergoes conformational changes as shown by an increased sensitivity of the receptor to proteases following ligand binding (Bukusoglu and Jenness, 1996). Constitutive active (CA) receptors have been identified as an important tool to study the activated state of the receptor. Mutations in the TM5, TM6 and TM7 were observed to make the Ste2p receptor active in the absence of ligand, although these Ste2p mutants were still further activated when ligand was added to the media (Sommers *et al.*, 2000). More recent structural analysis of Ste2p suggested that ligand binding induces conformational changes in Ste2p specifically in the cytoplasmic ends of TM5 and TM6 much like other GPCRs (Umanah *et al.*, 2011).

Polar residues that face inwards to avoid the hydrophobic environment of membrane lipids are considered very important to determining the conformational states of GPCRs. The polar residues are able to make several intramolecular contacts to keep the receptor in an inactivated state as mutations of TM6 polar residues in Ste2p were observed to increase the basal level of signaling as well as its affinity to the ligand (Dube and Konopka, 1998). Mutation of highly conserved proline 258, a residue that produces a kink in TM6 of the Ste2p structure, made the receptor CA, further substantiating the importance of the TM6 in receptor activity (Konopka *et al.*, 1996). Similarly, a conserved proline residue in Ste3p receptor is shown to be involved in conformational changes associated with the activation of that receptor (Stefan and Blumer, 1999). The asparagine 205 located in the 2nd ECL makes an important contact with the tyrosine 266 of TM6 in an activated state of the Ste2p receptor. The interaction of these residues appears to be a result of the relative movements of trans-membrane helices during receptor activation as in other GPCRs (Lee *et al.*, 2006). The role of some relative movements of TM5 and TM6 in the activation of the receptor was also demonstrated in another study. Cysteine mutagenesis and genetic screening methods were used to identify the cross-linked dependent constitutive active mutants of Ste2p receptor (Taslimi *et al.*, 2012). Structural mutational studies of Ste2p provide important clues regarding the mechanism of receptor activation. It supports the notion that all GPCRs, despite having extensive sequence diversities, share a common mode of activation upon ligand binding.

1.2.6 Alternate signaling by Ste2p

In mammalian systems, both subunits of the heterotrimeric complex, Gα and Gβγ have been shown to transmit to signaling pathways after their dissociation from the complex. In the past, the Gβγ subunit was considered solely responsible for transmitting to the signal pathway that worked under the regulatory control of the Gα (Gpa1) subunit. The activated GTP-bound form of Gpa1 facilitated the release of the Gβγ unit from the heterotrimeric complex to process the signal transmission, while inactivated Gpa1 hydrolyzes GTP and re-associates with the dimer complex. Contrary to previous reports, relatively recent studies have indicated that Gpa1 is capable of transmitting an alternate mating signal after its dissociation from the heterotrimeric complex (Guo *et al.*, 2003). A constitutive active mutant of Gpa1 (GTPase deficient mutant of Gpa1/ $Gpa1^{Q323L}$) was shown to activate transcription of mating specific genes even in the absence of pheromones. Endosomes rather than plasma membrane have been proposed to relay the alternate Gpa1 signal to the genes. In a gene deletion analysis, two genes Vsp15 and Vdp34 were identified as crucial for the Gpa1 mediated signals (Slessareva *et al.*, 2006). Genes Vsp15 and Vsp34 encode the regulatory and catalytic domains of a second messenger PI3K, respectively (Slessareva *et al.*, 2006). Activated Gpa1 binds with the Vsp34 domain of P13K and induces the production of PI3P. On the other hand, Vsp15 (regulatory domain of PI3K) binds to the inactive GDP-bound form of Gpa1 to modulate its signaling.

Following, PI3K activation, Bem1 binding protein is recruited to the endosome complex (Slessareva *et al.*, 2006). The Bem1 has been observed to act as an adaptor protein for Cdc42 and Fus3 pathways (Lyons *et al.*, 1996), as well as it might bind with other complexes and activate different signals in the endosomes. It has been speculated that regulatory mechanisms may differ for the endosomal pool of Gpa1 versus the plasma membrane pool Gpa1. One proposed mechanism is that the endosomal pool of Gpa1 is not under direct control of RGS proteins (Chen *et al.*, 2003). Evidence from mammalian cell studies as well as yeast suggest that the endosomes are not just an end sink of the cell but that they are also actively involved in cell signaling (Miaczynska *et al.*, 2004).

1.2.7 Evidence of a distinct role for Ste2p/Ste3p in downstream yeast mating events

As mentioned previously, the MAPK signal transduction machinery of the yeast-mating pathway that leads to cell cycle arrest is well established. Cell cycle arrest sets the stage for

subsequent mating events including projection formation, agglutination, plasmogamy and karyogamy to produce the final diploid zygote. However, the mechanisms underlying these subsequent mating events are not fully understood (Figure 1.12). A small number of fusion facilitators that include Prm1p, ergosterol, Fus1 and Fus 2 have been reported to mediate events such as cell-cell contact, positioning of vesicles and opening and closing of fusion pores (Gammie *et al.*, 1998; Heiman and Walter, 2000; Nolan *et al.*, 2006; Jin *et al.*, 2008).

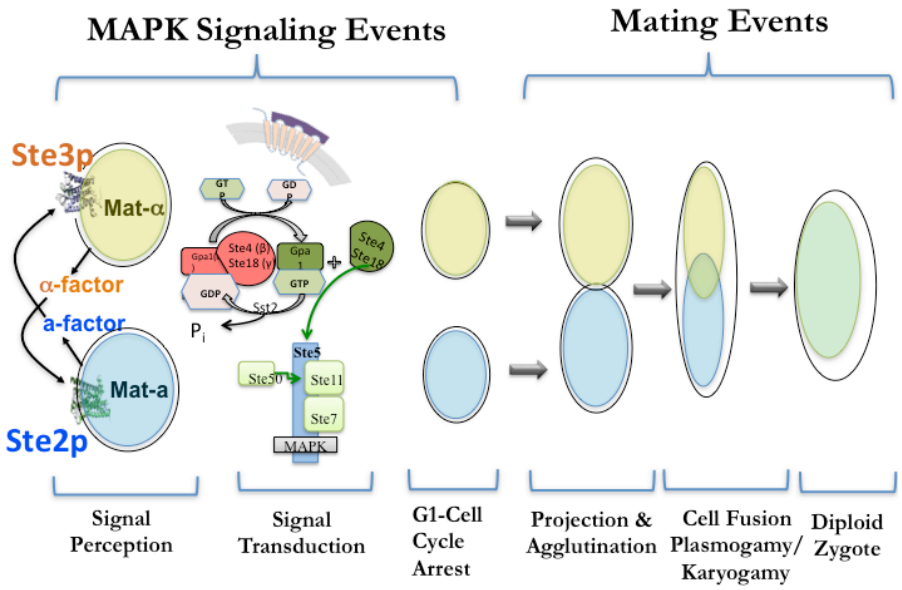


Figure 1.12 The two temporally distinct aspects of pheromone-mediated mating in yeast. We are endeavoring to demonstrate a direct role and associated mechanisms for the yeast mating receptors in the later mating events.

Reports from our lab (Shi *et al.*, 2007) and elsewhere (Moore, 1983; Jackson *et al.*, 1991; Brizzio *et al.*, 1996; Konopka *et al.*, 1996) have yielded evidence supporting the possibility that yeast mating receptors have a dual functionality in i) initial MAPK signal transduction steps leading to cell cycle arrest and then ii) further in downstream mating events. In this context, Ste2p has been reported to regulate mating partner discrimination (Jackson *et al.*, 1991). Interestingly, an immuno-localization study has demonstrated the presence of Ste2p in the projection tip and site of cell fusion (Jackson *et al.*, 1991). Further, Ste2p null yeast mutants that overexpressed a heterotrimeric G-protein subunit maintained MAPK signal transduction

and G1 arrest but displayed only minimal mating efficiency (Whiteway *et al.*, 1990). Moreover, mutation in the TM6 domain of Ste2p resulted in constitutive MAPK signaling activity without the formation of mating projections (Konopka *et al.*, 1996). More recently, *in vivo* yeast genetic studies demonstrated that N-terminal mutants exhibit normal G1 arrest but with diminished mating efficiency (Shi *et al.*, 2007). At the molecular level, part of the Ste2p N-terminus that putatively contains a β-strand was reported to form a dimer interface for Ste2p molecules (Uddin *et al.*, 2012). Dimerization mediated by the N-terminus was affected by ligand binding, indicating a change in the conformation of Ste2p upon receptor activation. Interestingly, specific residues in the putative β-strand loop of the Ste2p N-terminal region were suggested to influence mating events, while having no effect on MAPK signaling events (Shi *et al.*, 2009a). Importantly these studies have shown that substitutions of the residues Pro15, Ill 24, and Ill 29 in Ste2p receptor greatly lowered mating abilities while maintaining normal G1 cell-cycle arrest (Shi *et al.*, 2007; Shi *et al.*, 2009a).

The mechanism by which the mating (versus MAPK signaling) functionality of the yeast mating receptors is carried out remains enigmatic. This is largely due to difficulty in separating the closely linked, as well as sequential nature of MAPK signaling and then mating events governed by the yeast mating receptors. Previously, it was proposed that the dual role of the yeast GPCR is associated with a second lower affinity pheromone binding site and an alternative (as yet unidentified) signaling pathway (Moore, 1983; Jackson *et al.*, 1991; Brizzio *et al.*, 1996; Konopka *et al.*, 1996). According to this model, the low-affinity binding site is filled only if higher concentrations of ligands are present, as observed during cell-cell contact and cell wall degradation steps (Brizzio *et al.*, 1996). Hypothetically, such a binding event may bring about conformational changes in the receptor that could lead to as yet uncharacterized signaling events through alternate pathways resulting in cell wall degradation and fusion events. Alternatively, the low affinity binding sites may serve to present an interface on the extracellular domain formed by the receptor and ligand together, which enables an interaction with another receptor present in the same yeast cell or on the surface of opposite mating type apposed cell (transactivation) leading to an alternate effector signaling pathway or even direct mediation of membrane fusion events (Ritter and Hall, 2009). It is also possible that mating events occur through the involvement of more than one receptor mechanism encompassing conformational change mechanisms, interface presentation and transactivation.

This thesis is concerned with investigating these enigmatic signaling events.

1.3 Overall goal of the thesis

A ligand-based alternate functionality for Ste2*p in yeast mating?*

As mentioned in the previous sections, the existence of important alternate functionalities and associated alternate signaling pathways for many human GPCRs has been identified. These alternate functionalities and signaling mechanisms have been evaluated by application of alternate ligands or select mutations of the receptors, that influence the GPCR conformation such that it preferentially interacts with either β-arrestin or G-proteins. These effects are referred to as ligand and mutational-derived bias, respectively (reviewed in Reiter *et al.*, 2012; Kenakin and Christopoulos, 2013). With the recent demonstration of a role for α-arrestins in internalization of the yeast GPCR, Ste2p, the possibility of alternate signaling follows for the Ste2p receptor (Alvaro *et al.*, 2014). At the same time, a number of mutations in Ste2p as discussed in the previous section, have yielded differential effects on G-protein-mediated MAPK signaling versus diploid zygote formation. Some examples of these include mutations in the N-terminus (I24C and I29C), yielding a phenotype that has strong G-protein dependent MAPK signaling and cell cycle arrest activity, but no actual mating functionality (Shi *et al.*, 2009a). Similarly, mutation S251L, in the central region of TM6, also yielded normal signaling, but a weak-mating phenotype (Dube and Konopka, 1998). In contrast, other TM6 mutations such as S254L produced normal signaling, but exceptionally strong mating, while the double mutation P258L/S259L yielded high levels of basal signaling or constitutive signaling activity, with normal inducible signaling, but weak-mating (Konopka *et al.*, 1996). While these mutations support the possibility that the dual functionalities of Ste2p receptor can be separated and that the different functions are mediated at least in part by different parts/ residues of the Ste2p receptor, their significance remains to be addressed comparatively to account for observational and systemic bias (Kenakin and Christopoulos, 2013).

In contrast, mutational studies evaluating the alternate/biased functionalities of Ste3p receptor are limited due to the commercial unavailability of a-factor ligand. One study did include the TM6 mutation (P222L) and third ICL mutation (L194Q) yielding a constitutive MAPK signaling phenotype for Ste3p receptor, however the mating functionalities were not evaluated for this receptor.

Hypothesis: Distinct, pheromone concentration-dependent, MAPK-signaling and alternate mating functionalities exist for the yeast pheromone receptors.

1.4 Specific objectives:

Objective 1: Test Ste2p for mutational derived functional bias and account for systematic and observational bias.

- Characterize the induced and constitutive signaling, as well as mating activities of select Ste2p and Ste3p mutants.
- Determine α-factor dose-responsiveness of MAPK-signaling and mating functionalities of Ste2p mutants.
- Determine expression and localization of Ste2p mutants in the response to the α-factor ligand.

Objective 2: Test the role of arrestins in the alternate mating functionality of Ste2p.

- Assess involvement of arrestins in pheromone regulated MAPK signal transduction pathway of Ste2p receptor.
- Assess involvement of arrestins in modulation of pheromone-based mating events.
- Determine the localization of α-arrestin (Art1/Ldb19) in response to α-factor ligand.

CHAPTER 2

2 MATERIALS AND METHODS

2.1 Reagents

All reagents were from Sigma-Aldrich, Ontario, Canada otherwise listed in Table 2.1.

Table 2.1 List of reagents (with their suppliers) used in different experiments.

Reagent	Supplier
Bacto-Agar	BD, Ontario, Canada
Bacto-Tryptone	BD, Ontario, Canada
Bacto-Yeast Extract	BD, Ontario, Canada
Dimethyl sulfoxide	Fisher Scientific, Ontario, Canada
Phosphate buffer saline	Invitrogen/Life technologies, California, USA
Formaldehyde	Fisher scientific, Ontario, Canada
Sodium phosphate dibasic	EMD, New Jersey, USA
Sodium phosphate monobasic	EMD, New Jersey, USA
Potassium chloride	Fisher Scientific, Ontario, Canada
CSM-His-Ura	MP Biomedicals, California, USA
CSM-Leu-Ura	MP Biomedicals, USA
CSM-His-Leu-Ura	MP Biomedicals, California, USA
CSM-Lys-Met	Sunrise Science products, California, USA

2.2 Strains, plasmids and media

Strains: *Eschericia coli* (*E.coli*) strains DH5α, one-shot Top10 competent cells and one shot ccdB survival 2T1^{R} competent cells were used as hosts to construct, propagate and amplify the plasmids in different experiments and obtained from Invitrogen/Life technologies, California, USA.

DH5α is ideal for routine cloning of genes into plasmid vectors with >1 x 10^{6} cfu/µg

plasmid DNA transformation efficiency. DH5α cells have the genotype: F–**Φ**80lacZ**Δ**M15 **Δ** (lacZYA-argF) U169 recA1 endA1hsdR17 (rK–, mK+) phoA supE44 **λ**– thi-1 gyrA96 relA1.

One-shot Top10 chemically competent *E.coli* cells provide a very high transformation efficient of 1 x 10^9 cfu/µg plasmid DNA and are ideal for high-efficiency cloning. The genotype of TOP10 is: F– mcrA **Δ**(mrr-hsdRMS-mcrBC) **Φ**80lacZ**Δ**M15 **Δ**lacX74 recA1 araD139 **Δ**(ara leu) 7697 galU galK rpsL (StrR) endA1 nupG.

One Shot *ccd*B Survival 2 $T1^R$ Chemically Competent Cells are high transformation efficiency (1 x 10^9 cfu/µg plasmid DNA cells) and suitable for the propagation of *ccd*B gene containing plasmids like Gateway destination and entry vectors. The genotype of $2T1^R$ is F^- *mcr*A Δ(*mrr-hsd*RMS-*mcr*BC) Φ80*lac*ZΔM15 Δ*lac*X74 *rec*A1 *ara*Δ139 Δ(*ara-leu*)7697 *gal*U *gal*K *rps*L (Str^R) *end*A1 *nup*G *fhu*A::*IS2*. The *S. cerevisiae* used in this study are described in Table 2.2 and Table 2.3 with their relevant genotypes and sources listed.

Table 2.2 Description and source of yeast strains used in different experiments.

Yeast Type	Yeast Strains	Genotype	Gene deletions	Source
Mat α	201389	Mat α *his3 leu2 lys2 ura3*	N/A	ATCC
	SCYO60	Mat α *his3-11,15 leu2-3,112 trp 1-1 ura3-1 kan1-100 ade2-1 are1::Leu-2*	N/A	S. Sturely, California, USA
	1987	Mat α STE3 *(del):: Kan R his3 leu2 ura3 lys2 met15*	*STE3, SST2*	Wu Kunle, Montreal, Canada
	T24D	Mat α STE3 *mfa1:: Leu2 mfa2:: Leu2*	*MFα1, MFα2*	(Kurjan, 1985)
	SY1793	Mat α STE3 *mfa1delta mfa2delta FUS1P::HIS3 ura3-52 leu2-3,112 ade1*	*MFα1, MFα2*	(Davis *et al.*, 1993)
	YKL178C BY4742	Matα *his3delta1 leu2delta0 lysdelta0 ura3 delta0 delta STE3*	*STE3*	ATCC, Virginia, USA
Mat a	SCYO61	Mat a *his3-11,15 leu2-3,112 trp 1-1 ura3-1 kan1-100 ade2-1 are1::his-3*	N/A	S. Sturely, California, USA
	201388	Mat a *his3 leu2 met15 ura3*	N/A	ATCC, Virginia,USA
	YDR461W BY4741	Mat a *his3delta1 leu2delta0 met15delta0 ura3delta0 deltaMFA1*	*MFA1*	ATCC, Viginia, USA
	SM 1229	Mat a *mfa-D1::leu2 mfa2-D1::ura3 trp1 leu2 ura3 his4 can1*	*MFA1, MFA2*	(Michaelis and Herskowitz, 1988)
	SM1458	Mat a *mfa1-D1::leu2 mfa2-D2::lacZ trp1 leu2 ura3 his4 can1*	*MFA1, MFA2*	(Chen *et al.*, 1997)
	SM 2331	Mat a *mfa1-D1 mfa2-D1 trp1 leu2 ura3 his4 can1*	*MFA1, MFA2*	(Chen *et al.*, 1997)
	JKY78	Mat a far1 bar1::hisG ste2::LEU2 lys2::FUS1-lacZ arg4 his3 leu2 lys2o trp1 ura3	*STE2*	(Dube and Konopka, 1998).
	JKY79	Mat a bar1::hisG ste2::LEU2 ste5-3tslys2::FUS1-lacZ ade2-1o ade3 cry1 his4-580a leu2lys2o trp1a tyr1o ura3 SUP4-3ts	*STE2*	(Dube and Konopka, 1998).
	JKY127-36-1	Mat a bar1::hisG far1 sst2-1 ste2D mfa1::LEU2mfa2::his51 ade2 his3 leu2 ura	*STE2, SST2*	(Dube and Konopka, 1998).
	LM102	Mat a bar1 his4 leu2 trp1 met1 ura3 *FUS1 lacZ*:URA3 ste2-dl	*STE2*	(Kim *et al.*, 2012)

Table 2.3 Description and source of yeast strains used in arrestin knockout experiments

Yeast Type	Yeast Strains	Genotype	Gene deletions	Source
Arrestin knockouts				
Mat α	EN06	rod1::G418 rog3::G418 his3 ura3 leu2	Art4, Art7	(Nikko and Pelham, 2009)
	EN07	aly1::G418 aly2::G418 his3 ura3 leu2	Art3, Art6	(Nikko and Pelham, 2009)
	EN08	ecm21::G418 csr2::G418 his3 ura3 leu2	Art2, Art8	(Nikko and Pelham, 2009)
	EN09	ygr068c::G418 rim8::G418 his3 ura3 leu2	Art5, Art9	(Nikko and Pelham, 2009)
Mat a	EN59	ecm21::G418 csr2::G418 bsd2 rog3::natMXrod1 ygr068c aly2 aly1 ldb19 rim8 ylr392c::HIS his3 ura3 leu2	Arrestin null	(Nikko and Pelham, 2009)
	EN60 (9-arrestin)	ecm21::G418 csr2::G418 bsd2 rog3::natMX rod1 ygr068c aly2 aly1 ldb19 ylr392c::HIS his3 ura3 leu2	Arrestin null except Art9	(Nikko and Pelham, 2009)

Table 2.4 Description of plasmids used in different experiments.

Plasmid name	Type of plasmid	Resistance/selection	Source of plasmid
pUC19	Bacterial	Amp	New England Biolabs, Ontario, Canada (NEB)
pSB 234	Yeast, signal transduction	Amp, Uracil	
pENTR/ D-TOPO	Bacterial, Gateway entry vector	Kanamycin	Invitrogen/Life technologies, California, USA
pDONR 221	Bacterial, Gateway donar vector	Kanamycin	
pAG 423 GAL-ccdB	Yeast, Gateway destination vector	Amp, Histidine	Addgene 14149, Massachusetts, USA
pAG 425 GAL-ccdB	Yeast, Gateway destination vector	Amp, Leucine	Addgene 14153, Massachusetts, USA
pAG426GAL-EGFP-ccdB	Yeast, Gatwway destination vector	Amp, Uracil	Addgene 14203, Massachusetts, USA
pYES-DEST 52	Yeast, Gateway destination vector	Amp, Uracil	Invitrogen/Life technologies, California, USA
Arrestin Plasmids			
pEN311	3HA LEU2 CEN Ecm21	Leucine	Hugh RB Pelham, Cambridge, UK
pEN340	3HA LEU2 CEN Rod1	Leucine	
pEN341	3HA LEU2 CEN Rog3	Leucine	
pEN342	3HA LEU2 CEN Aly1	Leucine	
pEN343	3HA LEU2 CEN Aly2	Leucine	
pEN344	3HA LEU2 CEN Ygr068C	Leucine	
pEN345	3HA LEU2 CEN LDB19	Leucine	
pEN346	3HA LEU2 CEN Ylr392c	Leucine	
pEN347	3HA LEU2 CEN Csr2	Leucine	

Plasmids: The different bacterial and yeast plasmids used in the study with their sources are mentioned in the Table 2.4. Plasmid pSB234 has the *FUS1 lacZ* β galactosidase gene that is used to measure the MAPK signal transduction activity of the receptor (Jin *et al.*, 2008).

Arrestin plasmids were based on the YCplac 111CEN LEU2 or YCplac33CEN URA3 vectors. Individual arrestins were expressed from their own promoters in each plasmid. The plasmid map of gateway vectors; pENTR/ D-TOPO, pDONR 221 and pYES-DEST 52 is given in Figure 2.1A, B and C respectively. For addgene vectors, pAG423/pAG425 and pAG426GAL-EGFP-ccdB, maps are given in Figure 2.2A, B and Figure 2.3, respectively.

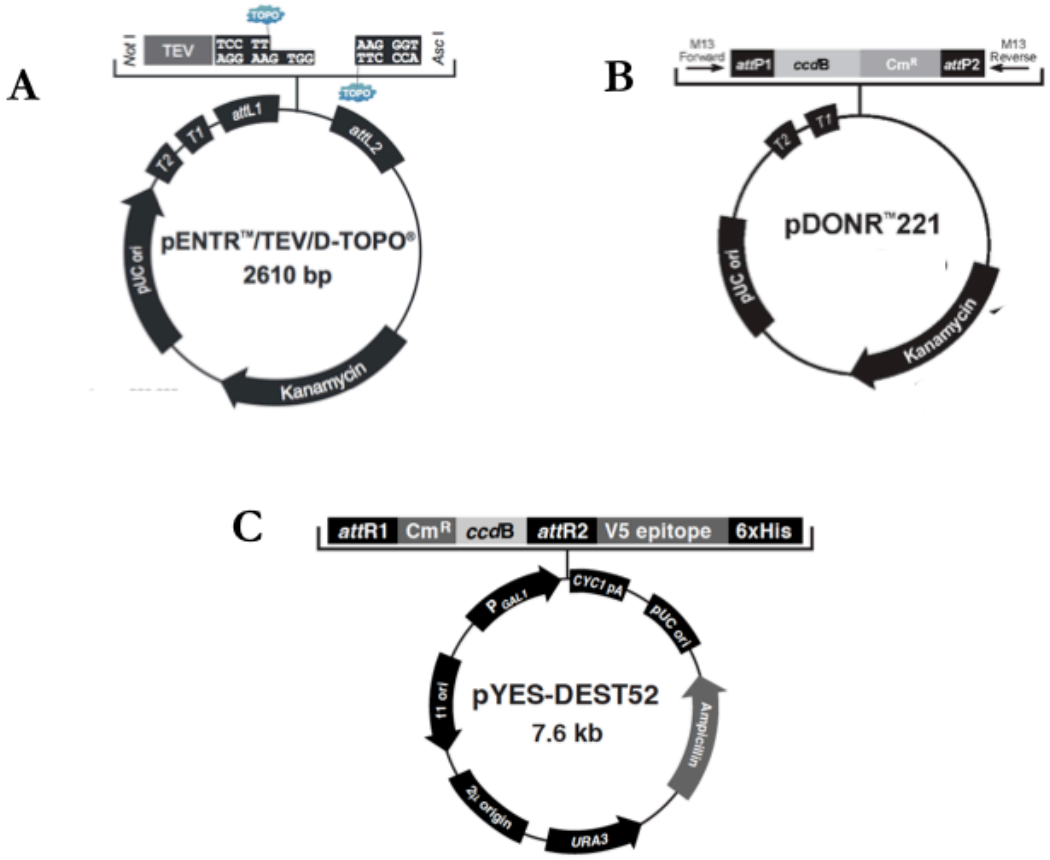


Figure 2.1 Plasmid maps of Gateway entry and destination vectors. A) Gateway entry vector pENTR/D-TOPO is a bacterial plasmid and kanamycin resistance vector. Recombination sites attL1 and attL2 are present between 569-668 and 705-804 nucleotides respectively. B) Gateway adapted vector pDONR 221 has pUC origin (4085-4758) to maintain and replicate plasmid in E. coli. Two recombination sites, attP1 (570-801) and attP2 (2753-2984), for recombination cloning of the gene of interest from a att B PCR product. C) Yeast expression plasmid pYES-DEST52 contains GAL1 promoter to allow inducible expression of gene into vector; attR1 and attR2 recombination site for cloning of the gene of interest fr0m an entry vector; *URA3* gene for selection of yeast transformants in uracil-deficient medium and 2 µ origin for high copy replication in yeast.

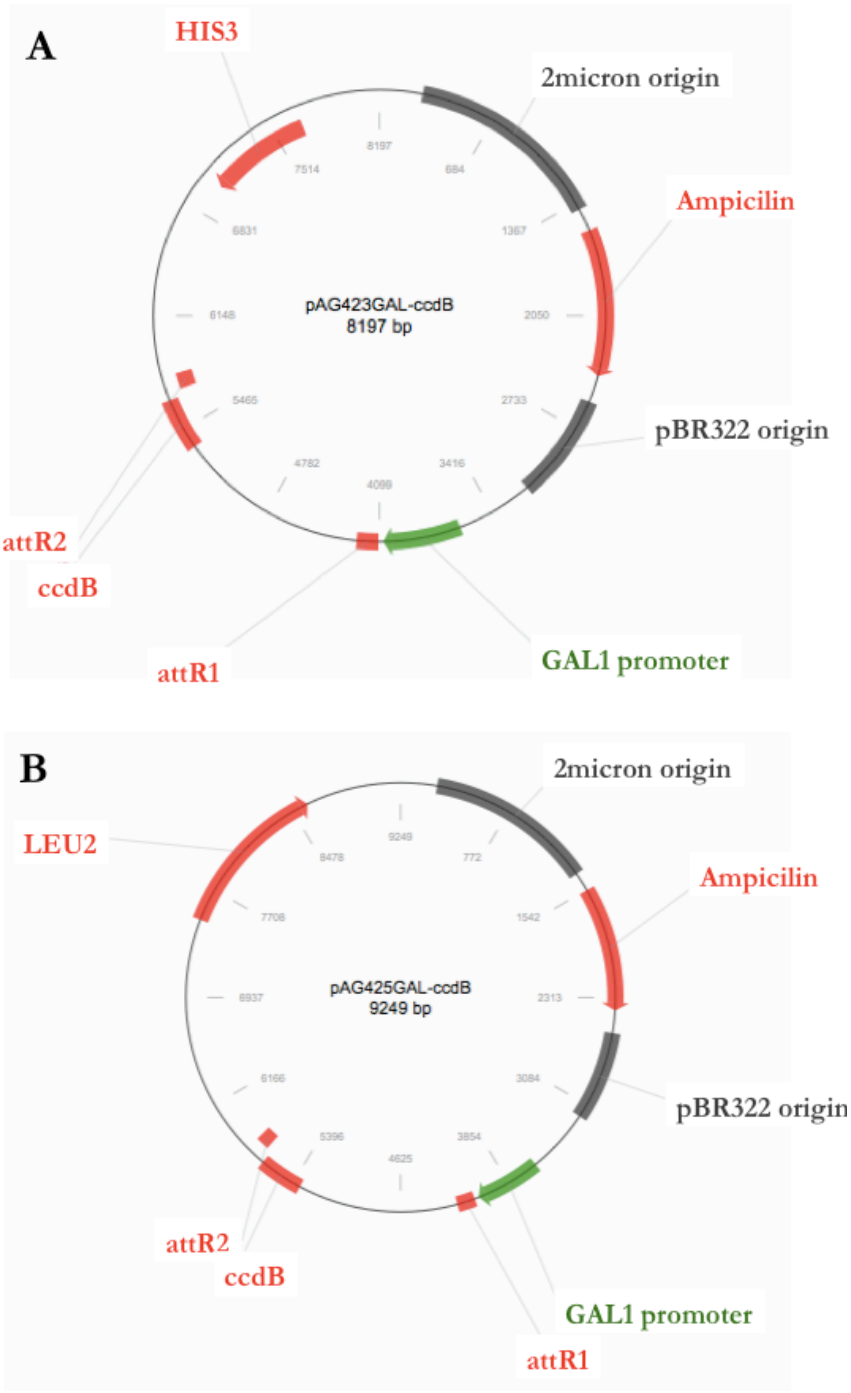


Figure 2.2 Plasmid map of addgene vector pAG423/pAG425GAL-ccdB. A) pAG 423GAL-ccdB is yeast expression; gateway destination vector. It contains GAL1 promoter to allow inducible expression of gene into vector; attR1 and attR2 recombination site for cloning of the gene of interest from an entry vector; *HIS3* gene for selection of yeast transformants in histidine-deficient medium and 2-micron origin for high copy replication in yeast. B) Yeast expression vector, pAG423425GAL-ccdB contains all the features of pAG 423GAL-ccdB vector except the selectable marker *LEU3* which allows the plasmid grow in the absence of leucine –deficient medium.

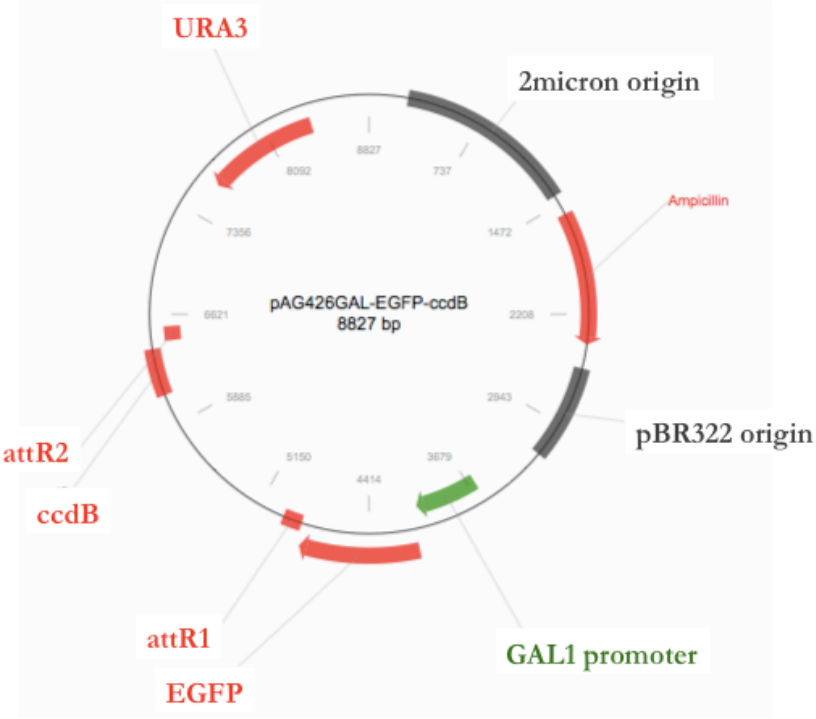


Figure 2.3 Plasmid map of addgene vector pAG426GAL-ccdB. Yeast expression, gateway destination vector pAG426GAL-ccdB is 2-micron origin type of plasmid for high copy replication in yeast. It contains GAL1 promoter to allow inducible expression of gene into vector; attR1 and attR2 recombination site for cloning of the gene of interest from an entry vector; *URA3* gene for selection of yeast transformants in uracil-deficient medium and EGFP fusion protein on c-terminal backbone.

Media

i) For *E.coli*: The 2xyeast extract and tryptone (2×YT) rich media and plates were used to propagate bacterial strains. Media was prepared by adding the 16g bacto-tryptone, 10g bacto-yeast extract and 5g NaCl in 1L of distilled water and sterilized by autoclaving for 20 min at 15 psi. Once the media was cooled to 50-60°C, ampicillin/kanamycin was added to a final concentration of 100 or 50µg/mL respectively, followed by agar @ 15g/L to make 2×YT rich plates.

ii) For yeast: Yeast strains were grown using either rich media or minimal media. The rich medium was formulated using yeast extract, peptone and dextrose (YPD). Initially yeast extract (10 g) and peptone (20 g) were dissolved in 900 ml of water and autoclaved for 20 min. To the mixture of yeast extract and peptone, 100 ml of 20% dextrose (sterilized by disposable

flask filters (Millipore, 1000ml, SCGPU10RE) was added to complete the YPD medium. Alternatively, galactose and raffinose were used in place of glucose as a carbon source to induce expression from a galactose promoter vector. The minimal media or synthetic defined medium was used as a basal media to which one or two amino acids were omitted to maintain the selection of a particular plasmid. Minimal medium was comprised of yeast nitrogen base (6.7 g), CSM minus selective amino acid (Complete synthetic medium containing amino acid additives but lacking marker amino acid, Table 2.3 and 2% carbon source (glucose or galactose+ raffinose). The co-expressed genes were expressed on the plasmids bearing different selective markers and the transformed yeast cells were grown on the double selective media as per the requirements mentioned in Table 2.4.

2.3 Molecular biology

2.3.1 Plasmid constructions

2.3.1.1 Sub-cloning of the *STE3* gene into pUC19 vector

The yeast mating receptor gene *STE3* was sub-cloned into the *BamH*1 restriction site of a small size vector pUC19 (2.6 kb) to facilitate site directed mutagenesis of these two receptors. Genes were amplified using primers (with *BamH*1 flanking sequences; Table 2.5), Expand High Fidelity ^{PLUS} PCR Kit (Roche applied science, USA) and PRL307-Ste3 (Shi *et al.*, 2007) construct as template. PCR reaction was run on thermocycler with following thermocycle conditions; initial denaturation at 94°C for 2 min, denaturation at 94°C for 30 sec, annealing at 58°C for 30 sec, extension at 72°C for 1 min 30 sec (27 repeat cycles of denaturation, annealing and extension) and final extension at 72°C for 7 min.

PCR products thus obtained were electrophoresed on 1% agarose gel containing ethidium bromide (0.5 µg/mL final concentration) using a gel-electrophoresis unit (BioRad, Canada) for approximate 45 min at 100V. The gel was then transilluminated to visualize the PCR products. A band corresponding to 1.4 Kbp (*STE3*) was excised and purified using the Quick gel extraction kit (Qiagen, Ontario, Canada). In the following step, empty pUC19 vector and purified *STE3* DNA (obtained from the above steps) were digested with *BamH*1 restriction enzyme (New England Biolabs, Ontario, Canada). Following digestion, linearized pUC19

vector was dephosphorylated using Calf intestinal phosphatase (New England Biolabs, Ontario, Canada) to prevent its religation. Both digested fragments were then ligated using the T4 DNA ligase enzyme (New England biolabs, Ontario, Canada) and the ligated products were subsequently transformed into the DH5α cells.

Table 2.5 List of cloning primers used in different experiments.

Primers	Primers sequences
Ste3 BamH1 F	5'-CGCGGATCCATGTCATACAAGTCAGCAATAATAGGGCTT TG-3'
Ste3 BamH1 R	5'-CGCGGATCCTTAAGGGCC TGCAGTATTTTCTGAACTATGTTC-3'
pENTR Ste2 F	5'-CACCATGTCTGATGCGGCTCCTTC-3'
pENTR Ste2 R	5'-TAAATTATTATTATCTTCAGTCCAGAACTTTCTGGCTTCCTC-3'
pENTR Ste3 F	5'-CACCATGTCATACAAGTCAGCAATAATAGGGCTTTG-3'
pENTR D Ste3 R	5'-AGGGCCTGCAGTATTTTCTGAACTATGTTC-3'
attB Art 1 F	5'-GCCGACAAGTTTGTACAAAAAAGCAGGCTATGGCATTTTCACGTCTTA-3'
attB Art 1 R	5'-GCCGACCACTTTGTACAAGAAAGCTGGGTCCTGGGTTATTCTATTGG AATC-3'
attB Art 3 F	5'-GCCGACAAGTTTGTACAAAAAAGCAGGCTATGCCCATGGACCAATC-3'
attB Art 3 R	5'-GCCGACCACTTTGTACAAGAAAGCTGGGTCAAGGGTACTCTCATTTA TAC-3'
attB Art 6 F	5-GCCGACAAGTTTGTACAAAAAAGCAGGCTATGCTCCAATTCAATACAG AAAATG-3'
attB Art 6 R	5'-GCCGACCACTTTGTACAAGAAAGCTGGGTCTTTTCTCTTTTCGCGAAA TGC-3'

*Underscored sequence in the Ste3 BamH1F and R represents the BamH1 restriction site.
* Underscored sequence in the attB preimers represents attB sequence with four terminal GCs.

Transformation of competent E.coli cells: Chemically competent DH5α cells were thawed on ice and 2 µL of DNA to be transfered was added into the cells. After mixing the contents gently by flicking, the tubes were incubated on ice for 30 min. The cells were then heat shocked by incubating them in a water bath at 42 °C for 30 sec. Following this, the tubes were immediately placed on ice for 5 min. To each of the tubes, 400 µL of sterile 2YT media was added and tubes were incubated at 37°C for 60 min under constant shaking. After the incubation, the transformed cells were plated onto separate 2YT-Ampicillin agar plates using a

sterile spreader and incubated at 37°C for 16 h to allow the transformed cells to form colonies. The colonies obtained from transformation were grown and purified using plasmid miniprep Kit (Qiagen, Ontario, Canada) and further sent for DNA sequencing. The DNA sequencing facility at the National Research Council (NRC), Saskatoon, Canada, confirmed the successful cloning of *STE3* gene into pUC19 vector. The pUC19 Ste2p construct was available in our lab as used in a previous study (Shi *et al.*, 2007).

2.3.1.2 Molecular cloning of WT and mutant *STE2, STE3* and arrestin genes into Yeast Gateway expression vectors

Entry clone: The genes of interest (*STE2/STE3*) were first cloned into an entry clone using the Gateway system technology through one of two methods. The targeted gene was cloned first into pENTR/D-TOPO vector for which, blunt end PCR products were produced by using pfu turbo DNA polymerase, a forward primer that contains, CACC to ensure the directional cloning of PCR products, at the 5`end and a reverse primer (Table 2.5). In the next step, the blunt end PCR products were cloned into pENTR/D-TOPO vector by mixing 7-8 ng of PCR mixture with the TOPO vector (Invitrogen/Life technologies, California, USA) and incubated for 30 min at room temperature and transformed into One Shot Top10 competent cells (Invitrogen/Life technologies, California, USA) and plated onto 2YT kanamycin plates for 24 h at 37°C. Next day in morning colonies were purified by Qiagen Miniprep Kit and further sent for DNA sequencing at NRC, Saskatoon, Canada.

Alternatively, a terminal attB sequence containing the PCR product for arrestin genes was created using primers that had 20-25 bp long attB sequence and four terminal GC's. The attB PCR product was recombined with the pDONR vector to create the entry clone containing the gene of interest. The primers used in the generation of different entry clones are given in Table 2.5.

Destination clone: The genes of interest were subsequently transferred from entry vector to destination vectors through recombination reactions that were facilitated between attL sites present in the entry vector and attR sites of the destination vector. The recombination reaction mix is prepared by adding the entry clone (100 ng), destination vector (150 ng) with Gateway LR clonase enzyme mix (Invitrogen). After recombination, the reaction mix was transformed into Top 10 competent cells (Invitrogen) and plated onto 2YT (plus ampicillin) plates.

Colonies were cultured into 2YT ampicillin solutions for 24 h at 37°C and purified using Qiagen Miniprep Kit. Constructs were confirmed by sequencing DNA sequencing laboratory, NRC, Saskatoon. Sequencing data was processed using the DNASTAR Lasergene 8 – SeqMan Pro software package.

2.3.2 Site directed mutagenesis

The single and double mutations in *STE3* and *STE2* genes were introduced using the Quick-change site directed mutagenesis (Agilent technologies, Ontario, Canada) kit. All mutagenic primers for the reactions were designed according to the site directed mutagenesis kit guidelines (Table 2.6). The melting temperatures for all the mutagenic primers were calculated using the equation given in the guidelines and set for ≥78°C. Both forward and mutagenic primers were designed to contain the desired mutation. The mutagenic reaction consisted of 1×reaction buffer, 0.1 mM dNTPs, 125 ng each of forward and reverse primers, 50-70 ng of pUC19 based plasmid constructs and 2.5U of P*fu Turbo* DNA polymerase enzyme in a total 50 µL of reaction volume. HPLC purified water was added in place of the plasmid construct as a negative control. The mutagenesis thermal cycling conditions were: (1) initial denaturation step at 95°C for 30 sec; (Blahos *et al.*, 1998) denaturation step at 95°C for 30 sec; (Bao *et al.*, 2004) annealing step at 55°C for 1 min; (4) extension step at 68°C for 1 min/kb of plasmid length; (5) repeat 17 cycles from step (Blahos *et al.*, 1998) to step (4). After PCR directed mutation, the construct was digested by Dpn1 restriction enzyme (New England Biolabs, Ontario, Canada) and the digested sample was transformed into One-Shot Top10 competent cells. The transformants were selected on 2YT Ampicillin agar plates incubating for 18 h at 37°C. The colonies obtained from transformation were cultured and purified by Qiagen Miniprep Kit and further sent for DNA sequencing facility at NRC, Saskatoon. The final receptor mutant arising from the mutagenic reactions are given in Table 2.7. Once the mutants were constructed in pUC19 vector, they were transferred in yeast Gateway expression vectors using the Gateway cloning method.

Table 2.6 List of mutagenic primers used in different experiments.

Mutagenic primers	Primer sequences
Ste3 P222L (F)	5'-TTCTGTTTCATTATTATTTTAGTCATGTTCCTGTTTTCTGTTTACACCTTTGTTCAAGATTTAC-3'
Ste3 P222L (R)	5'-GTAAATCTTGAACAAAGGTGTAAACAGAAAACAGGAACATGACTAAAATAATAATGAAACAGAA-3'
Ste3 L194Q (F)	5'-CGCAAGGACGTTAGGGATATTGAACACTGTACCAATTCAGGTTTAAAC-3'
Ste3 L194Q (R)	5'-GTTTAAACCTGAATTGGTACAGTGTTCAATATCCCTAACGTCCTTGCG-3'
Ste2p S254L (F)	5'-CATATTTTACTCATAATGTCATGTCAACTTTTGTTGGTTCCATCGATAATATTCATC-3'
ScSte2p S254L (R)	5'-GATGAATATTATCGATGGAACCAACAAAAGTTGACATGACATTATGAGTAAAATATG-3'
Ste2p P258L (F)	5'-CATGTCAATCTTTGTTGGTTCTATCGATAATATTCATCCTCGC-3'
Ste2p P258L (R)	5'-GCGAGGATGAATATTATCGATAGAACCAACAAAGATTGACATG-3'
Ste2p S254LP258L (F)	5'-CATGTCAACTTTTGTTGGTTCTATCGATAATATTCATCCTCGC-3'
Ste2p S254L P258L (R)	5'-GCGAGGATGAATATTATCGATAGAACCAACAAAAGTTGACATG-3'
Ste2p S254L P258L S259L (F)	5'-CATGTCAACTTTTGTTGGTTCTACTGATAATATTCATCCTCGCATACAG-3'
Ste2p S254L P258L S259L (R)	5'-CTGTATGCGAGGATGAATATTATCAGTAGAACCAACAAAAGTTGACATG-3'
Ste2p S251L (F)	5'-CATATTTTACTCATAATGTTATGTCAATCTTTGTTGGTTCC
Ste2p S251L (R)	5'-GGAACCAACAAAGATTGACATAACATTATGAGTAAAATATG-3'
Ste2p S251L S254L (F)	5'-CATATTTTACTCATAATGTTATGTCAACTTTTGTTGGTTCC-3'
Ste2p S251L S254L (R)	5'-GGAACCAACAAAAGTTGACATAACATTATGAGTAAAATATGTCA-3'

Table 2.7 List of receptor mutant produce in pUC19 plasmids used in experiments.

Receptor	Plasmids
Ste3	P222L
	L194Q
	L194Q P222L
Ste2	S254L
	P258L
	S254L P258L
	P258L S259L
	S254L P258L S259L
	S254L P258L S259L
	S251L
	S251L S254L

2.3.3 Yeast transformation protocol

The cloned wild type (Taylor *et al.*, 1996) and mutant genes of *STE2* and *STE3* were transformed into the receptor null yeast strains for expression of the receptors. Among the different available means for yeast transformation, the LiAc/PEG mediated transformation was used as it has been reported as a simple, highly reproducible and efficient method for introduction of gene of interest into yeast cells (Gietz *et al.*, 1992).

A single colony of the yeast strain to be used in the transformation was inoculated in 5 mL YPD media and incubated overnight at 30°C in a shaking incubator at 200 rpm. Next day in the morning, the yeast culture was diluted to an OD_{600} of 0.4 in 50 mL of YPD media and was grown for an additional 3-4 h. After sufficient growth when OD_{600} reaches 1.0 (or close), the culture was pelleted and re-suspended first in 40 mL TE buffer (10 mM Tris pH 7.5, 1mM EDTA) and then 2 mL of 1X LiAc solution. LiAc solution was prepared by mixing 100 mM LiAc (pH 7.5), 5 mM Tris-HCL (pH 7.5) and 0.5 mM EDTA. The suspensions of yeast cells in LiAc solution were incubated for 10 min at room temperature. Transformation mix was prepared by adding 1µg plasmid DNA and 100 µg denatured sheared salmon sperm DNA in

100 µL of yeast suspension. For co-transformation, two plasmids were transformed in single yeast cell by using 1µg DNA for each plasmid. The solution of 1×LiAc/ 40 % PEG-3350/ 1×TE {100 mM LiAc (pH 7.5), 40% polyethylene glycol-3350 (PEG), 10 mM Tris-HCl, (pH 7.5) and 1mM EDTA) was prepared freshly and added to the above transformation mix and incubated for 30 min at 30°C. The denatured salmon sperm DNA (single stranded carrier) was used to increase the transformation efficiency up to 10^5 transformants/µg of vector DNA (Schiestl and Gietz, 1989) and prepared as per the method mentioned previously (Schiestl and Gietz, 1989). After incubation, 150 µL DMSO was added to enhance the transformation efficiency (Hill *et al.*, 1991) and cells were heat shocked for 15 min at 42°C. In the following steps, cells were centrifuged and re-suspended in sterile TE buffer twice and plated on minimal media plates. The minimal media plates were selected based on the specific selection marker of a particular plasmid Table 2.3.

2.4 Western blots

Ste3p: For western immune-blots of WT and mutants Ste3p receptors, approximately 2.8×10^6 mid logarithmic phase YKL178CBY4742 cells were harvested and lysed with freshly prepared solution of 7.4 % β-merkapto-ethanol in 1.85 N NaOH. Proteins were precipitated by adding150 µl of concentrated TCA to the tubes. Proteins were separated by electrophoresis on an SDS/10% polyacrylamide gel. After SDS PAGE, proteins were electrophoretically transferred to nitrocellulose membrane and then probed with anti-V5 HRP antibody (Invitrogen) in 1:5000 dilutions in TBST solution. The immuno-reactive proteins were detected by enhanced chemi-luminescence kit (Amersham).

Ste2p: JKY78 yeast cells containing WT and mutant Ste2p receptors were used to harvest the cells. Cells were lysed using the same procedure as described for Ste3p receptor. Proteins were fractioned by SDS-polyacrylamide gel and immunoblotted. Blots were probed with anti STE2 antibody (Abcam) as primary antibody and rabbit anti mouse IgG+IgM as secondary antibody. Blots were detected using the enhanced chemi-luminescence kit (Amersham).

2.5 Characterization of WT and mutant *STE2, STE3* and arrestin genes

2.5.1 *FUS1-lacZ* β galactosidase assay

The gene encoding galactosidase (*lacZ*) has been widely used as a reporter gene to monitor the gene expression in prokaryotic and eukaryotic organisms (Silhavy and Beckwith, 1985). In the case of yeast, the promoter of Fus1 protein of mating pathway has been linked to the β-galactosidase gene to assess the activity of β-galactosidase that indirectly indicates transcription of the mating pathway (Kippert, 1995). In the β-galactosidase enzyme assay, 2-nitrophenyl β-D-galactopyranoside (ONPG) was added as a substrate to detect the enzyme activity through yellow color development. The intensity of yellow color was measured by spectrophotometric detection as a direct indicator of *FUS1* gene induction. The Fus1p is involved in fusion and is transcribed early once the MAPK pathway is activated in response of pheromone binding to its receptor. The JKY78/JKY79 and JKY127-36-1 strains had *FUS1-lacZ* gene integrated into chromosomal DNA while other yeast strains were made to express the *FUS1-lacZ* gene from the uracil selectable vector pSB234 (Table 2.4).

The induction of the *FUS1-lacZ* gene was monitored in different WT and receptor null yeast strains expressing WT or mutant mating receptor genes. Also, arrestin knockout and arrestin knock-in strains were assessed for *FUS1-lacZ* induction. The details of all transformed yeast strains and their selection media are described in Table 2.8. Yeast strains, listed in Table 2.8, were grown overnight on CSM minimal culture media to maintain the plasmid genes. Next day morning, the genes were overexpressed in the yeast cells by transferring cells into RI media (YPD 2% galactose + 1% raffinose). After 4-5 h growth period, 1.5 mL yeast cultures were treated with 1 μM α-factor (Zymoresearch, California, USA**)**. The α-factor (500 nM) was again added to the cultures 45 min later to replenish the degraded peptide. Negative controls for the experiment were produced similarly, but without the addition of any pheromone.

For Ste3p assays, due to the lack of available purified a-factor, yeast strains expressing the mutant receptor were mixed in equal amounts with Mat a cells (source of a-factor ligand) and incubated for 2 h at 30°C. YPD media was added instead of Mat a cells in another set of experiments to measure the signaling activity in absence of the a-factor ligand.

All experiments were conducted in triplicate. After two hours of incubation with pheromones, yeast cells were centrifuged and re-suspended in the 300 μL Z-buffer solutions

(60mM Na_2HPO4, 40mM NaH_2PO4, 10mM KCL, 1m Mg_2SO4) and the OD was measured at 600 nM. All samples were then lysed in liquid nitrogen and stored at -20°C until processed. The samples were thawed, incubated at 30°C for 30 min and subsequently 700 µL Z-buffer containing β mercaptoethanol + 0.2% sarcosyl solution was added to each sample to permeabilize the samples. In the following step, ONPG (4 mg/mL) was added and the mixture was incubated at 30^0C until a yellow color developed. After 3-4 h of incubation, 400 µL of 1.5 M Na_2CO_3 was added to stop the reaction. The samples were then centrifuged (10 min, 16873×g and OD was taken at 420 nM. The β-galactosidase activity was calculated as per the following formula and arbitrary units were used to highlight the relative values.

β galactosidase units = OD (420nm)×1000/t×v×OD (600 nM) where: t= total time of incubation; v= 0.3X concentration factor (Concentration factor is 5 fold, because 1.5 mL yeast culture was first lysed in 300 µL Z-buffer)
OD_{600} and OD_{420}=$Absorbance_{600}$ and $Absorbance_{420}$ of 1 mL of culture, respectively.

Table 2.8 List of yeast strains for *FUS1-lacZ* signal transduction assay

Ste3p strains	**Selection Media**
YKL/1987 pAG423 STE3pP222L pSB234	-His-Ura
YKL/1987 pAG423 STE3p pSB234	
YKL/1987 pAG423 pSB234	
SCYO6O pSB234	-Ura
Ste2p strains	
JKY78/79/127 STE2p	-Ura
JKY78/79/127 pDEST STE2p	
JKY78/79/127 pDEST	
JKY78/79/127 pDEST STE2p S254L	
JKY78/79/127 pDEST STE2p P258L	
JKY78/79/127 pDEST STE2p S254L/P258L	
JKY78/79/127 pDEST STE2p P258L/S259L	
JKY78/79/127 pDEST STE2p S254L/P258L/S259L	
Arrestin knockouts strains	
EN06 pSB234	-Ura
EN07 pSB234	
EN08 pSB234	
EN09 pSB234	
201388 pSB234	
EN59 pSB234	
EN60 pSB234	
201389 pSB234	
Arrestin knock-ins strains	
EN60 pEN311 pSB234	-Leu-Ura
EN60 pEN340 pSB234	
EN60 pEN341 pSB234	
EN60 pEN342 pSB234	
EN60 pEN343 pSB234	
EN60 pEN344 pSB234	
EN60 pEN345 pSB234	
EN60 pEN346 pSB234	
EN60 pEN347 pSB234	

2.5.2 Qualitative mating efficiency assay for WT and mutant Ste2p/Ste3p

In qualitative mating efficiency assays, Mat a and Mat α yeast cells were mixed with each other and plated on double minimal media plates for the selection of diploid zygotes. The WT Mat a and Mat α strains were mated with a counterpart strain consisting of a ΔSte3p/ΔSte2p strain that was transformed with WT or other mutant receptor genes in pYES DEST52 constructs and selected for mated diploid zygote on minimal media plates. During the mating experiments, all strains were cultured overnight in CSM minimal media containing 2% glucose. Next day in the morning, cells were washed with TE buffer and diluted to 0.4 OD_{600} in rich YPD culture media and grown for another 4-5 hrs. If the gene of interest is under the galactose promoter, the RI media is used to express the gene. To initiate mating, tester cells (2×10^7) were mixed with an excess of opposite strain cells (8×10^7) to facilitate the pairing with the opposite cell type. The mating mixture was incubated for 5 h at 30°C. At the end of the incubation period, cells were collected, re-suspended in 1 ml sterile water and were spotted on double minimal media plates for diploid zygote selection by incubating the plates for 2-4 days at 30°C.

2.5.3 Quantitative mating efficiency assay

2.5.3.1 Ste2p/Ste3p mating assay

In the quantitative yeast mating efficiency test, the yeast cells were cultured overnight in minimal media containing 2% glucose and next day transferred and cultured in YPD media for 4-5 h. The tester strain (2×10^7) was mixed with excess of opposite mating type strain (8×10^7) to facilitate mating (Table 2.9 and Table 2.10) and filtered onto 0.22 μm filter paper. In addition, each yeast strain alone was collected on the filter paper to serve as a negative control for the mating reaction. The filter paper with cells were placed on the YPD plates and incubated for 5 h at 30°C. At the end of incubation, the cells were collected in 1xPBS buffer (Gibco, 10010-031) and sonicated in a water bath (Elmasonic S30H) for 30 seconds to disrupt the cell clumps. From the cell suspension, 10 μL neat suspension (~5×10^4 cells), as well as its three and nine fold dilutions were plated on double minimal media selection plates. Two days later, single zygote colonies formed as a result of the mating reaction were quantified and the

mating efficiency was calculated according to the method described previously (Sprague, 1991a).

Table 2.9 List of Mat a and Mat α yeast strains in Ste2p related mating reaction.

Mat a	Mat α	Diploid selection
SCYO61	SCYO6O	-His-Leu
JKY78/79 pDEST STE2p	*SCYO6O or *T24D	-Ura-Leu
JKY78/79 pDEST		
JKY78/79 pDEST STE2p S254L		
JKY78/79 pDEST STE2p P258L		
JKY78/79 pDEST STE2p S254L/P258L		
JKY78/79 pDEST STE2p P258L/S259L		
JKY78/79 pDEST STE2p S254L/P258L/S259L		

*SCYO6O or SM1229 Mat a was used in three different mating reactions against Ste2p Mat α strain. SCYO6O is WT Mat α, whereas SM1793 is *MFα1* and *MFα2* gene deletion strain. *MFα1* and *MFα2* are responsible for the α-factor production in Mat α cells.

Table 2.10 List of Mat a and Mat α yeast strains in Ste3p related mating reaction.

Mat α	Mat a	Diploid selection
SCYO60	SCYO61	-His-Leu
YKL/1987 pDEST STE3p	*SCYO61 or *YDR461W or *SM1458	-Ura-His/ -Ura-Lys/ -Ura-Leu
YKL/1987 pDEST		
YKL/1987 pDEST STE3p P222L		
YKL/1987 pDEST STE3p L194Q		
YKL/1987 pDEST STE3p L194QP222L		

* SCYO61 or YDR461W or SM1458 Mat α strain was used in three different mating reactions against Ste3p Mat a strain. SCYO61 is WT Mat α whereas YDR461W and SM1458 are *MFA1* and *MFA1* and *MFA2* gene deletion strains, respectively. *MFA1* and *MFA2* are responsible for the a-factor production in Mat a cells and *MFA1* accounts for the major production of a-factor.

2.5.3.2 Arrestins mating assay

All arrestin knockouts/ knockins were mated against Mat-a or Mat-α yeast strains (201388/201389). The mating was allowed to occur for 2 h and mating products were grown on minimal media plates (SCMM) lacking histidine and uracil or leucine and uracil. Different

'Mat a' and 'Mat α' mating mixtures used in the arrestin mating assay are described in Table 2.11.

Table 2.11 List of Mat a and Mat α yeast strains in arrestins related mating reaction.

Mat α	**Mat a**	**Diploid selection**
Arrestin knock outs		
EN06	201388	-Lys-Met
EN07		
EN08		
EN09		
Mat a	**Mat α**	**Diploid selection**
EN59	201389	-His-Ura
EN60		
Arrestin knock-ins		
EN60 pEN311		-Leu-Ura
EN60 pEN340		
EN60 pEN341		
EN60 pEN342		
EN60 pEN343		
EN60 pEN344		
EN60 pEN345		
EN60 pEN346		
EN60 pEN347		

2.5.4 Quantification of nuclear fusion in DAPI labeled cells

The cells in the mating mixture were labeled with the 4'-6-diamidino-2-phenylindole (DAPI dilactate) 1mg/mL (Invitrogen, catalogue# D3571) solution to stain nuclear material and the fused nuclei were quantified by fluorescence microscope (LEICA DMR 40x objective, and filters with excitation/emission maxima of 360 nM/400 nM). Before labeling, cells were fixed in 3.7% final concentration of formaldehyde solution (F-79, Fisher Scientific) for 1h at room temperature. Following fixation, 10 µL of cell suspension was added to the DAPI solution (2.5 µg/mL) and incubated for 10 min at room temperature. After labeling, cells were harvested by centrifugation (8609×g) and washed with 1×PBS solution three times. Finally cells were resuspended in 50 µL of 1×PBS solution and observed under an epifluorescence microscope (40×objective, and filters with excitation/emission maxima of 360 nM/400 nM). The

proportion of cells with fused nuclear compartments to the total number of cells was calculated and compared to the values for WT 'MAT a' cells.

2.5.5 Cell cycle arrest (CCA) and Mating projection formation MPF) assays

Yeast strain (LM102) expressing WT and mutant *STE2* genes were grown overnight on CSM-Leu minimal media. Next day in the morning, the culture was inoculated into RI media for 4-5 h to induce the expression of genes. The logarithmic phase cells were treated with different doses (ranging from 10^{-10} nM to 10^{-5} nM concentration) of 'α factor' for either 4 or 7.5 h. At the end of treatment with α factor, cell clumps were dispersed using sonication in a bath sonicator (Elmasonic S30H) for 3 min and cells were subsequently fixed as mentioned in the previous section. Cells were washed thrice with 1×PBS solutions and stored at 4^0C until inspected further. The total numbers of cells in a 10 µL solution were counted using a Z1 threshold coulter counter (Beckman Coulter). Samples for the coulter counter were prepared by mixing the 10µl yeast samples with 20 ml isoton II solution (Beckman coulter). The mutant yeast cells treated with 'α factor' for 4 h were evaluated for percent-unbudded cells under 60X LEICA DMR contrast phase microscope as an indicator of CCA. Whereas, the mutant yeast cells treated with ligand for 7.5 h were examined for the proportion of cells with MPF and then compared with the wild type cells. The MPF scoring in WT and different Ste2p mutants are shown in Figure 2.4, Figure 2.5, Figure 2.6, Figure 2.7, Figure 2.8 and Figure 2.9. The yeast culture was diluted 20 times and 10 µL of sample was used for observation under the microscope. C-chip disposable hemo-cytometer chambers (Incyto, catalogue #DHC-N01) were used for counting the unbudded cells and projection containing yeast cells. Approximately 200 yeast cells were observed in the central square of hemo-cytometer (that is further divided into 25 small squares) at 40x magnification.

The data obtained in the CCA and MPF assays were plotted as dose response curves using Graph pad prism software 6.0. The amount of α-factor was transformed as log concentration and data values were normalized to a common scale. A Non-linear regression curve fit was used to fit the sigmoidal curve shape and further to obtain EC_{50} values.

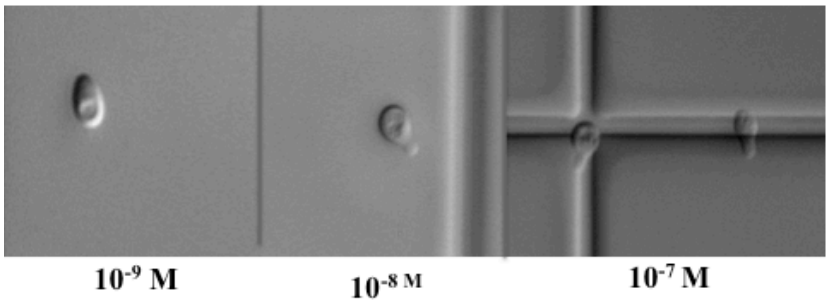


Figure 2.4 WT mating projection formation at 7 h. WT Ste2p starts forming mating projection at only at 10^{-8} M concentration of α-factor ligand.

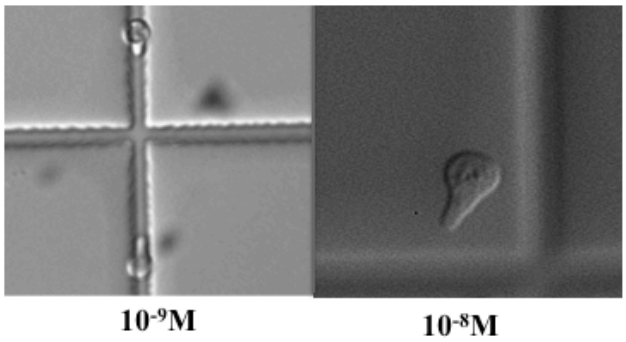


Figure 2.5 S254L mutant allele mating projection formation at 7 h. S254L mutant allele showed mating projection formation at lower concentration (10^{-9} M) of α-factor ligand compared to the WT.

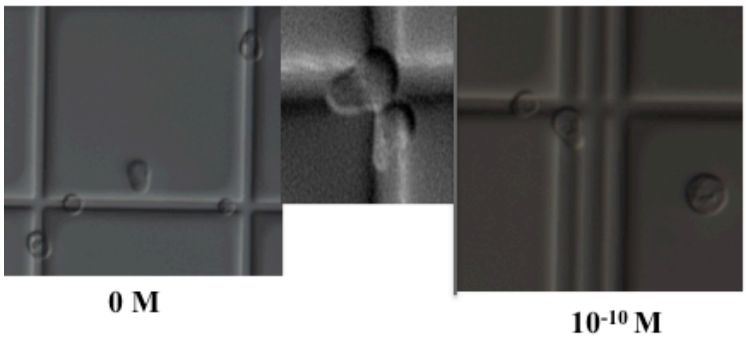


Figure 2.6 P258L mutant allele mating projection formation at 7 h. P258L mutant allele showed mating projection fromation in the absence of α-factor ligand (0 M). The projections formed by P258L are blunt shaped in the absence and in lower concentration (10^{-8}) of α-factor ligand compared to the pointed shaped projections formed at high concentration of ligand.

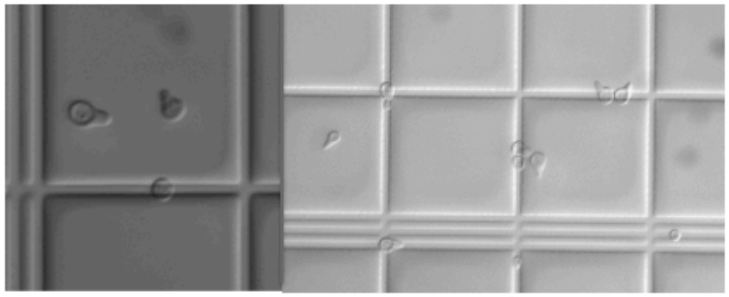


Figure 2.7 S254L/P258L mutant allele mating projection formation at 7 h. S254L mutant allele showed mating projection formation at lower concentration (10^{-8} M) of α-factor ligand compared to the WT and even in the absence of α-factor ligand (0 M).

Figure 2.8 P258L/S259L mutant allele mating projection formation at 7 h. P258L mutant allele showed mating projection fromation in the absence of α-factor ligand (0 M). The projections formed by P258L/S259L are blunt shaped in the absence and in lower concentration (10^{-8}) of α-factor ligand compared to the pointed shaped projections formed at high concentration of ligand. The number of projection fromation by P258L/S259L never reached to the level of WT mating projection formation.

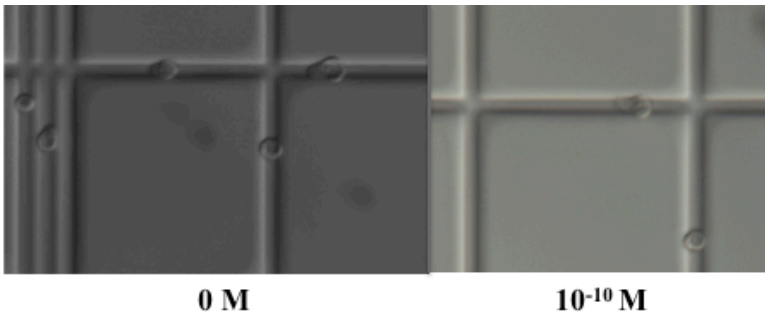


Figure 2.9 S254L/P258L/S259L mutant allele mating projection formation at 7 h. S254L/P258L mutant allele showed mating projection fromation in the absence of α-factor ligand (0 M). The projections formed by S254L/P258L/S259L are blunt shaped in the absence and in lower concentration (10^{-8}) α-factor ligand compared to the pointed shaped projections formed at high concentration of ligand. The number of projection fromation by P258L/S259L never reached to the level of WT mating projection formation

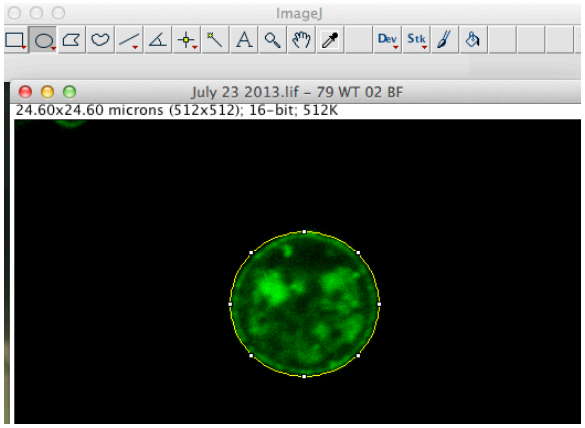
2.5.6 Localization of receptor through confocal microscopy

In order to determine the localization and endocytic trafficking of the Ste2p and mutant receptors, GFP was tagged to the wild type and mutant Ste2p receptors using GFP expression plasmids. Yeast strains with GFP tagged receptors were cultured overnight in the CSM-Ura minimal media to maintain the vector. Next day, yeast cells were washed with the TE buffer and sterile water and re-suspended in the RI media. After 4-5 h of growth in the RI media, yeast cells were treated with α-factor ligand for different time periods (10 min, 30 min, 1 h and 2 h), or not to determine receptor trafficking. The treated and untreated yeast cells were mounted on the microscopic slides (VWR, cat# 89049-670) using the hard set mounting media. Cells were fixed in 3.7% final concentration of formaldehyde solution (F-79, Fisher Scientific) for 1h at room temperature before mounting in hard-set media. Following fixation, one drop of cell suspension was mixed with one drop of mounting medium (approximately 25 µL) on a slide to mount cells and covered with the coverslip (VWR, cat# 48382-041). The slides were left undisturbed overnight at room temperature and observed under confocal microscope (Leica SP5, 63x, oil immersion lens and filters with excitation/emission maxima of 488nm/493-582 nm). For each mutant, at least 3 cells were imaged using the following technique. Initially the top and bottom surfaces of the yeast cell in focus were observed to determine the z-thickness of the yeast cell. Subsequently, 2D images (16 bit, 512x512, 24.6x24.6 micron, both brightfield and epifluorescent) were taken at the center of the yeast cell in focus with the same zoom factor (10), power and gain settings (750) across all images.

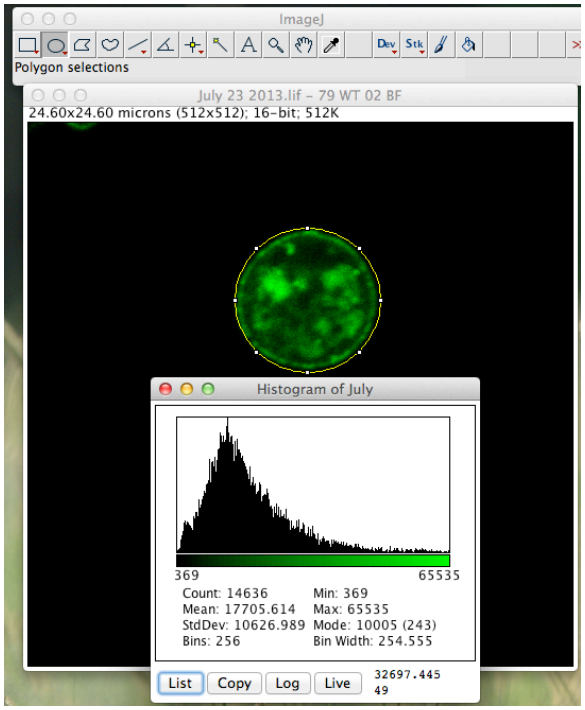
The amount of GFP signal (representing receptor localization) in different mutants at different time points was assessed using Image J software. In order to determine whether the receptor localized peripherally, the 0.5 micron distance from the yeast plasma membrane to the inside of cytoplasm was taken as the peripheral region and the remaining area as the central region of the yeast cytoplasm. After importing an image into Image J software, the total intensities of GFP signal in the peripheral region, central region and total cell were calculated for the region of interest using built in software settings.

2.5.6.1 Work flow for Image analysis in Image J

- Import the lif sequence and select image file to open in Image J.
- Select oval/elliptical ROI, fit it to the image**.**

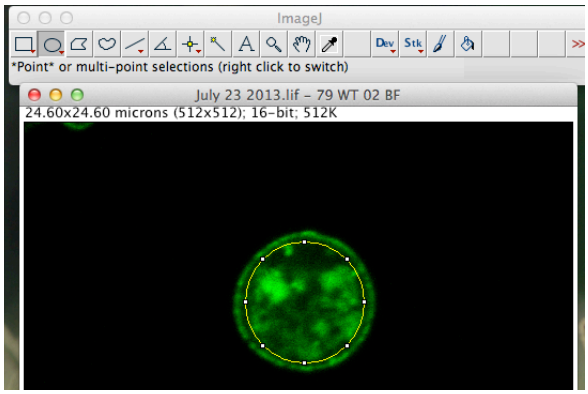


- After drawing the oval/ellipsoid, the values for total pixel count were multiplied by mean intensity of pixels to calculate the total GFP intensity for individual images.

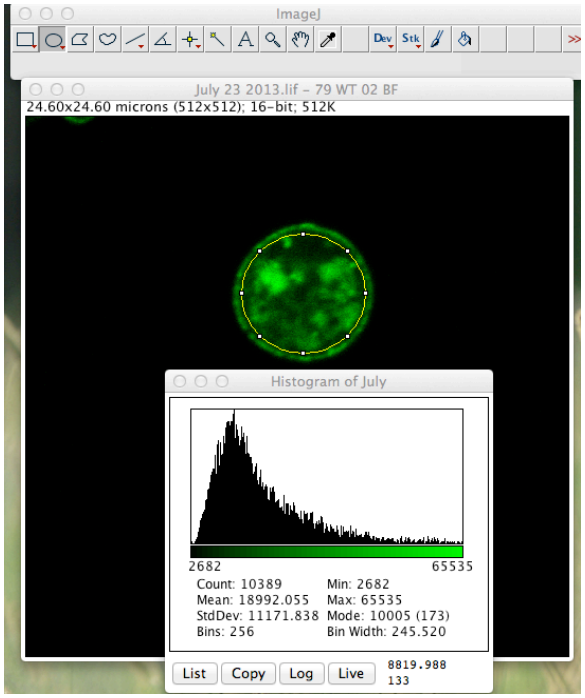


- **Selecting a peripheral vs. central region:** After drawing the ovoid/ellipsoid, the image was shrunk by 0.5 micron from the plasma membrane to inside so as to designate the peripheral and central regions of cytoplasm. For shrinking, go to "Edit" and then to "Selection". Under Selection go to "enlarge". Shrink the ROI by putting the value of

distance from plasma membrane to inside and put "-" in front of the value to indicate its a negative value



- After selecting the central region, histogram of selected image was generated using the inbuilt function within Image J.



- For the central region, the total GFP intensity for individual images was calculated as mentioned above for the entire image.

2.5.7 Characterization of mutation-linked functional bias factors for Ste2p receptor

Various doses of α-factor ligand (ranging from 10^{-10} nM to 10^{-5} nM) were used to assess the dose response curve of WT and mutated receptors in *FUS1-lacZ* induction, cell cycle arrest and mating projection formation assays, which are described in previous sections. The EC_{50} values thus obtained for mutant receptors were compared to the WT EC_{50} to get relative EC_{50} ratio of mutant receptors for each assay. The relative EC_{50} ratios were compared to get the bias factor between different assays related to a specific mutant receptor.

Realtive EC_{50} (*FUS1-lacZ* induction) =WT EC_{50}/ Mutant EC_{50}

Realtive EC_{50} (Mating projection formation) =WT EC_{50}/ Mutant EC_{50}

Bias factor= Realtive EC_{50} (*FUS1-lacZ* induction)/ Realtive EC_{50} (Mating projection formation)

2.6 Prediction and modeling

All 3-D models of Ste2p were viewed and labeled with the PyMOL pdb viewer software using the coordinates from Eilers *et al.*, 2005.

2.7 Statistical analysis

All statistical analyses were performed using Graph Pad Prism 6 (Graph pad, California, USA) software. All data are reported as mean ± SEM. Groups were compared using either t-test or analysis of variance after assumptions of normality and equal variance were met for all data analyzed. In the case of proportionate data, arcsine transformation was done to normalize the data before applying statistical tests. Differences between multiple groups for different endpoints were tested using Fischer's least significant difference and probabilities ≤ 0.05 were considered significant.

CHAPTER 3

3 RESULTS

3.1 Characterization of the ligand-induced and constitutive MAPK signaling, as well as mating activities of select Ste2p and Ste3p mutants

Constitutive activity is defined as an active state of a receptor that is capable of inducing signaling events even in the absence of ligand/agonist. Spontaneous or constitutive activity has been reported for many WT receptors, such as DOR, cannabinoid receptor (CB1), growth hormone secretagogue receptor (GHSR), and melancortin-1 receptor (MC1R) (Costa and Herz, 1989; Cohen *et al.*, 1997; Seifert and Wenzel-Seifert, 2002). Apart from naturally occurring constitutive activity in WT GPCRs, mutations can also increase the basal signaling levels in receptors compared to their WT counterparts. Several mutations has been reported in the past that cause the generation of constitutive activity in the receptor and are termed constitutively active mutants (CAMs; Horn *et al.*, 2001). CAMs serve as important tools to understand the mechanisms involved in the activation of the receptor upon ligand binding as they mimic the active conformation of the receptor to a certain extent. The activated conformation of the receptor is capable of activating the G protein and downstream signaling pathway (Cohen *et al.*, 1997; Gether, 2000; Spalding and Burstein, 2001). Mutations in the TM6 domains of GPCRs have been shown to be important to make the receptor active in the absence of ligand (Dube and Konopka, 1998; Han *et al.*, 1998; Geva *et al.*, 2000; Greasley *et al.*, 2001). A proline residue in the TM6 domain is of special interest as it is present in 90% of GPCRs and it keeps the receptor in an inactive conformation by producing a kink in the TM6. Mutational analysis of the yeast mating receptor Ste2p also demonstrates the importance of the proline residues in TM6 for constitutive activity, despite their low sequence similarity with other GPCRs. Further analysis shows that polar amino acids of Ste2p TM6 and TM7 domains are also likely involved in keeping the receptor in an inactive conformation (Konopka *et al.*, 1996; Dube and Konopka, 1998). Interestingly, mutations in the TM6 domain of Ste2p resulted in constitutive MAPK signaling activity but without the formation of mating projections (Konopka *et al.*, 1996). In order to investigate mutation-derived functional bias in Ste2p, the basal, ligand-induced

signaling and CA, as well as the mating ability of a selection of TM6 mutants, targeting residues Ser 254, Pro 258 and Ser 259 were initially characterized (Figure 3.1). The CA of mutant receptors was obtained by taking the ratio of basal to maximum (Emax) signaling activity levels of the receptors. In addition to the previously reported S254L, P258L and double P258L/S259L mutants (Konopka *et al.*, 1996; Dube and Konopka, 1998), the double S254L/P258L mutant and the triple S254L/P258L/S259L mutants were also evaluated. This allowed consideration of the effect of incorporating a 'strong mating' mutation into weak-mating constitutive mutants (Figure 3.1).

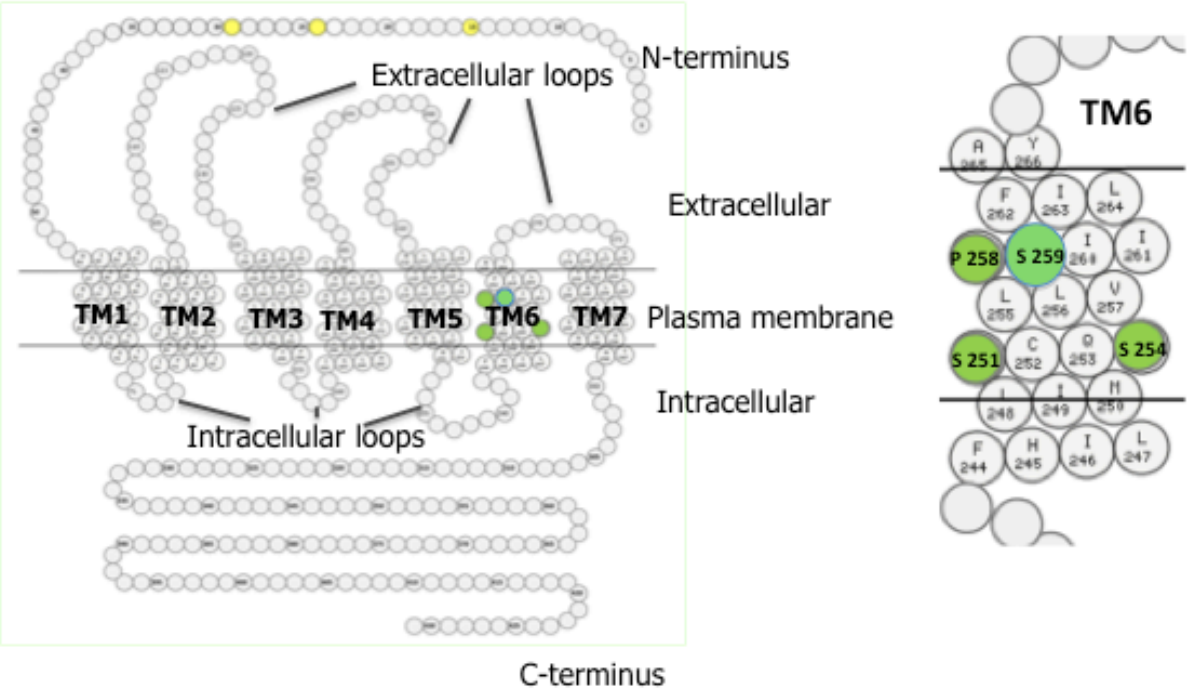


Figure 3.1 Residues of TM 6 domain in the α-factor receptor that were targeted for mutagenesis. The snake plot of Ste2p was made using the GPCR-SSFE database software.

3.1.1 Ste2p mutants

The MAPK signaling activity of Ste2p mutants was measured by the *FUS1-lacZ* β galactosidase reporter assay. WT *STE2* and mutant *STE2* genes expressed from the uracil selectable vector pYES-DEST52 were transformed into the *ste2* null JKY78, JKY79 and

JKY127-36-1 strains. All these yeast strains contained pheromone responsive *FUS1-lacZ* reporter gene integrated into the chromosome. With the endogenous *STE2* gene deleted from JKY78/79/127-36-1 strains, WT and mutant genes can be uniquely expressed through a yeast expression plasmid (pYES-DEST52). The JKY79 strain carries a temperature sensitive mutation in the post receptor component (Ste5ts) of the pheromone-signaling pathway. The temperature sensitive mutant (Ste5ts) allows the yeast cells to grow normally at a restrictive temperature (34°C) but it doesn't activate the pheromone-signaling pathway at that temperature. Shifting the yeast cells from 34°C to 23°C activates the signaling pathway (Dube and Konopka, 1998). The advantage of using this strain is that the yeast strain can be grown overnight without activating the signaling pathway. JKY127-36-1 strain (*SST2* knockout strain) was used to increase the sensitivity for basal signaling activity for WT and mutant receptors. This Sst2 knockout strain is more sensitive to α-factor because Sst2p acts as a regulator of G protein signaling (Mathew *et al.*, 2011) and shortens the life span of the active G protein (Gα-GTP) by increasing the GTP hydrolysis rate (Chan and Otte, 1982; Apanovitch *et al.*, 1998).

3.1.1.1 Ligand dependent MAPK signal transduction activity of mutant receptors

To examine the ability of Ste2p mutant receptors to respond to ligand, the β-galactosidase activity of yeast cells expressing mutant receptors was examined in the presence of 1.5 μM α-factor. The results showed (Figure 3.2) that S254L/P258L, P258L/S259L and S254L/P258L/S259L mutants were induced by α-factor to produce similar or even higher levels of signaling compared to the WT cells induced with α-factor ligand, in both JKY78/79 strains (Figure 3.2A and B). All the double and triple mutants showed very high levels of MAPK signaling in the *Δsst2* JKY172-36-1 strain (Figure 3.2C). The exact mechanism of hypersensitivity observed with these mutants in JKY127-36-1 strain is not clear. The combination of CA conformation and high-activated Gα concentration could be one possibility that might explain the hypersensitivity of these mutant receptors. The ability of all Ste2p mutants to respond to the α-factor ligand stimulation indicates that mutations don't severely impair their structure or function. Moreover, all mutant receptors were observed to express at similar levels, albeit at slightly lower levels than those observed for WT receptor level (**Figure 3.3**).

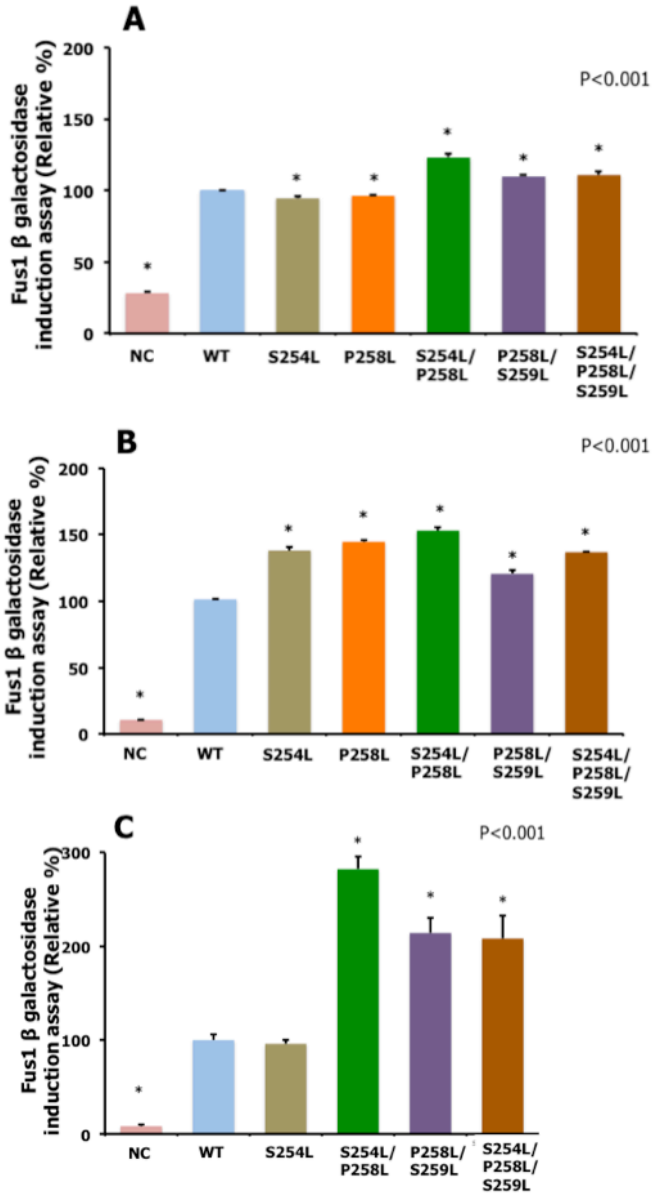


Figure 3.2 Characterization of ligand-dependent MAPK signaling activity of TM6 mutants, overexpressed in receptor null strains. A) Yeast strain (JKY78) carrying the vector only (pYES-DEST 52) or WT or mutant receptors was incubated with the 1.5 µM α factor for 2 h and then induction of *FUS- lacZ* reporter gene was assayed. B) *ste5ts* mutant JKY79 strain carrying the WT or mutants were grown overnight in minimal media at 34°C and then shifted to the 23°C for next 5-6 h. Ligand dependent induction of *FUS1-lacZ* reporter gene was measured in the presence of 1.5 µM α factor. C) The ligand dependent activity of *FUS1-lacZ* reporter gene was measured in the supersensitive sst2-1strain JKY127-36-1 in the presence of 1.5µM α-factor ligand. The β galactosidase activity for WT *STE2* was normalized to 100%. The results represent three independent assays, each done in triplicate. Comparisons between mutant values with those of WT were made using one-way ANOVA test. * $P<0.05$ was used as the cutoff value to define significant difference between mutant values compared to the WT.

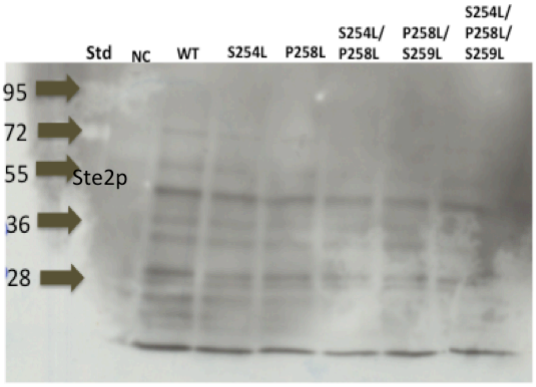


Figure 3.3 Immunoblot analysis of WT and mutant Ste2p receptor. Yeast strain JKY78 carrying the indicated WT or mutant receptors were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti Ste2p antibody. Immunoblots were detected using the enhanced chemi-luminescence kit (Amersham). The relative molecular weights in

3.1.1.2 Basal MAPK signaling activity of receptor

The relative level of basal MAPK signal transduction activity of WT and mutant Ste2p in the JKY78 strains is shown in Figure 3.4A. The double (S254L/P258L combination of Ste2p P258L mutant with an exceptionally efficient mating mutant Ste2p S254L), and triple mutants of Ste2p receptor (S254/LP258L/S259L) show 53 and 46% higher basal signaling activity respectively compared to the WT Ste2p. Another double mutant Ste2p P258L/P259L exhibited 37 % higher basal signaling activity.

In addition, ligand-independent activity of WT and mutant receptors was measured in the JKY79 strain as well. The relative induction levels of the *FUS1-lacZ* reporter gene are reported in Figure 3.4B. The maximum induction level (90%) was found in the S254L/P258L mutant followed by the triple mutant S254L/P258L/S259L (81%), another double mutant P258L/S259L (58%) and single mutant P258L (39%) compared to the WT levels (Figure 3.4B).

The analysis of the double mutant S254L/P258L in the JKY127-36-1 strain showed a very high increase (25 fold) in basal signaling activity while other mutants (double P258L/S259L and triple mutant S254L/P258L/S259L) caused a greater than 15 fold increase in the basal signaling activity (Figure 3.4C). These results indicate that substitutions at residues 254, 258 and 259 of Ste2p can contribute to activation of the G protein dependent MAPK signaling in the absence of ligand stimulation.

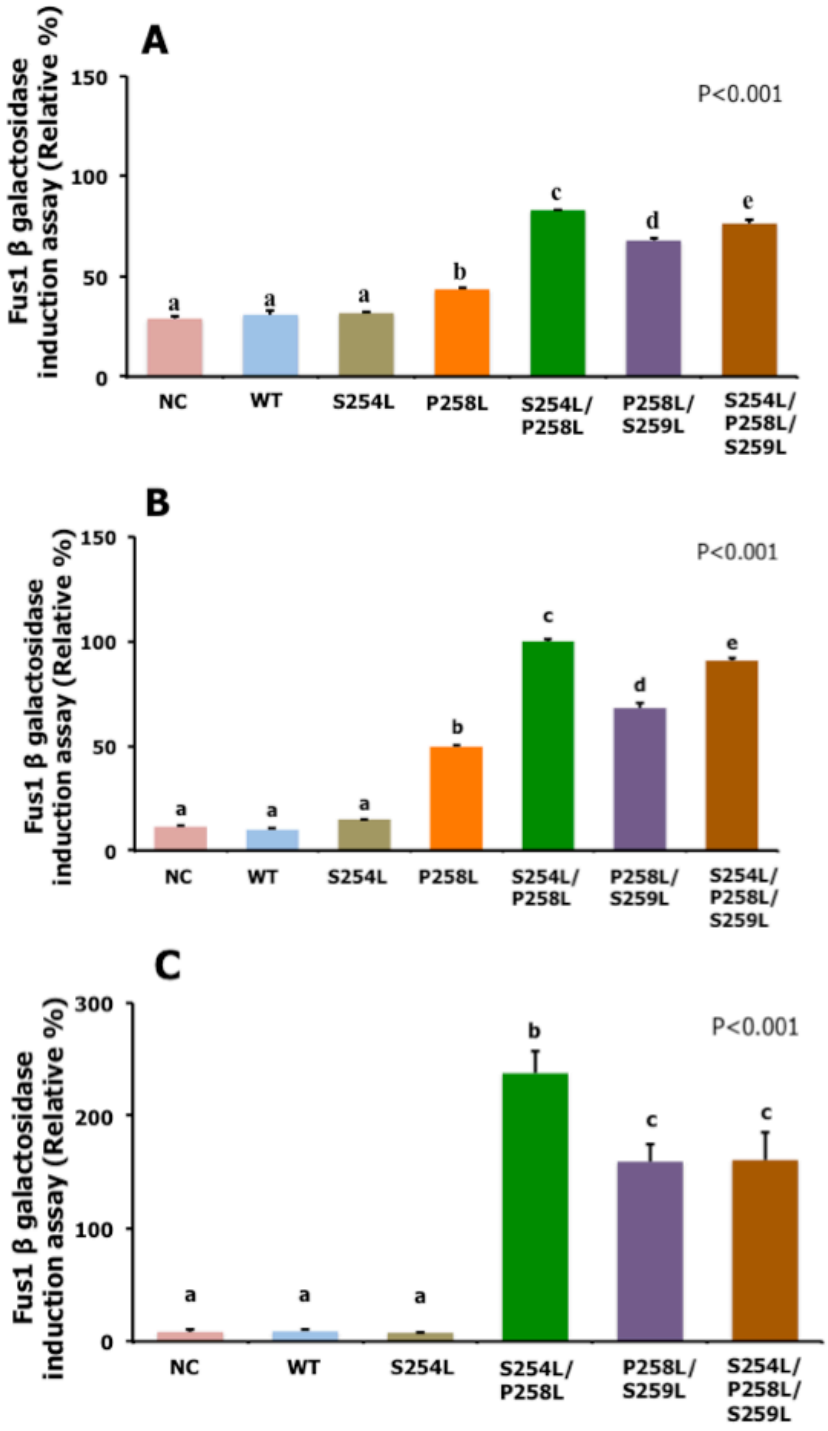


Figure 3.4 A, B and C: Basal MAPK signaling activity of TM6 Ste2p mutants, overexpressed in receptor null strains. JKY78/79/127-36-1 strain carrying the Empty vector or WT *STE2* receptor gene or the mutant *STE2* S254L or *STE2* P258L or *STE2* S254L/P258L or *STE2* P258L/S259L or *STE2* S254L/P258L/S259L was tested for the basal activity of *FUS1-lacZ* reporter gene in the absence of added α-factor ligand (Figure 3.3 A, 3.3 B and 3.3 C, respectively). The β galactosidase activity for ligand induced WT *STE2* was normalized to 100%. The results represent three independent assays, each done in triplicate. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was set as cut off value to indicate significant difference between groups. Values with no common superscript are different.

3.1.1.3 Constitutive activity of mutant receptors

The constitutive activities of different mutant receptors are shown in Figure 3.5. The constitutive activity was significantly elevated by 16, 37, 31 and 38 % for P258L, S254L/P258L and S254L/P258L/S259L mutants, respectively, compared to the WT in JKY78 strain (Figure 3.5A). However, S254L mutant showed constitutive activity similar to the WT receptor. In *STE 5ts* mutant strain (JKY79), the constitutive activities of both S254L/P258L and S254L/P258L/S259L were increased by 56% of the WT activity. While other mutants, P258L/S259L and P258L had a 46 and 24% increase in constitutive activity, respectively, compared to the WT (Figure 3.5B). Analysis of mutants in the supersensitive strain JKY127-36-1 revealed nine fold, nine fold and eight fold increase in constitutive activity of S254L/P258L, S254L/P258L/S259L and P258L/S259L mutants, respectively, compared to WT. However, the constitutive activity level of the S254L mutant didn't differ from the WT receptor in the Sst2- strain (Figure 3.5C). Similar to the case of basal activity, substitutions at residues 254, 258 and 259 contribute to making the Ste2p receptor constitutively active for the G protein dependent MAPK signaling, with the level of activity varying among depending on the combination of two or more of the mutations.

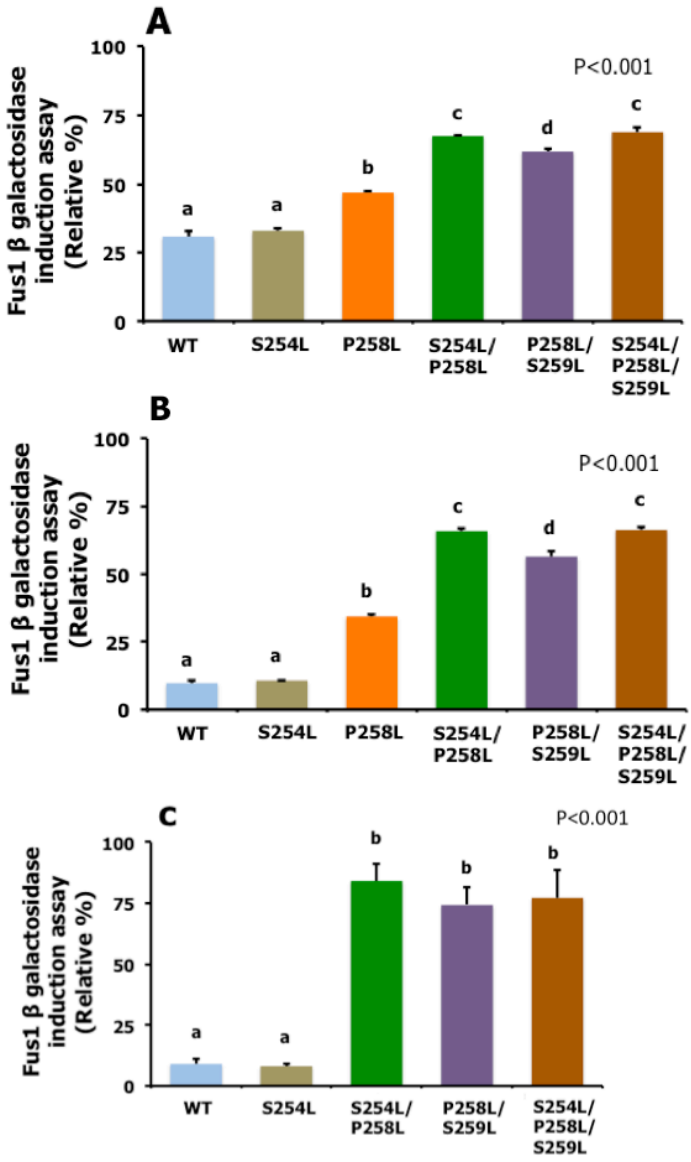


Figure 3.5 Constitutive MAPK signaling activity of TM6 Ste2p mutants overexpressed in receptor null strains. Constitutive activity of *FUS1-lacZ* reporter gene for WT *STE2* and *STE2* mutants were analyzed in JKY78 (A), JKY79 (B) and JKY127-36-1 (C) strains. The ratio of basal activity and maximum ligand induced activity (basal activity/Emax*100) for WT or mutant was used to calculate the constitutive activity. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.1.1.4 Mating activity of constitutively active in MAPK signaling Ste2p mutants

The overall functional ability of the mutant receptors was further assessed by their mating efficiency in qualitative and quantitative mating assays. The JKY78/ JKY79 Mat a strain carrying the WT and mutant *STE2* receptor were mated with Mat α cells and diploid zygotes were grown on selective minimal media plates.

On the one hand, Ste2p P258L/S259L and Ste2p S254L/P258L/S259L, respectively, showed very little mating activity even in the presence of α-factor. Interestingly, when tested for basal mating activity, which might be expected for mutants with high basal MAPK signaling, by mating against a Mat α strain (T24D) that is mat α null (i.e. in the absence of pheromone ligand), no evidence of basal mating was detected (Figure 3.6A). In contrast, the newly identified CA Ste2p S254L/P258L receptor was able to mate in the presence of α-factor ligand, although in the absence of ligand, mating efficiency is also completely blocked (Figure 3.6B).

In the quantitative assay, mating efficiency of the double mutant (Ste2p S254L/P258L) was 76% and 72% (in the presence of α-factor) of the WT receptor activity in JKY78 and JKY79 strains, respectively (Figure 3.7, upper and lower panels). On the other hand, very low mating activity was observed for Ste2p P258L/S259L and Ste2p S254L/P258L/S259L mutants even in the presence of α-factor, highlighting a negative contribution of the S259L mutation towards mating activity. Together these results, in particular the reduced mating ability of MAPK signaling CAM's and the complete lack of mating in the absence of ligand even for very potent MAPK signaling CAM's, support the possibility of an alternate function for not just Ste2p, but very specifically the α-factor-Ste2p complex, in mating events downstream of the classical Ste2p mediated MAPK signal transduction and cell cycle arrest.

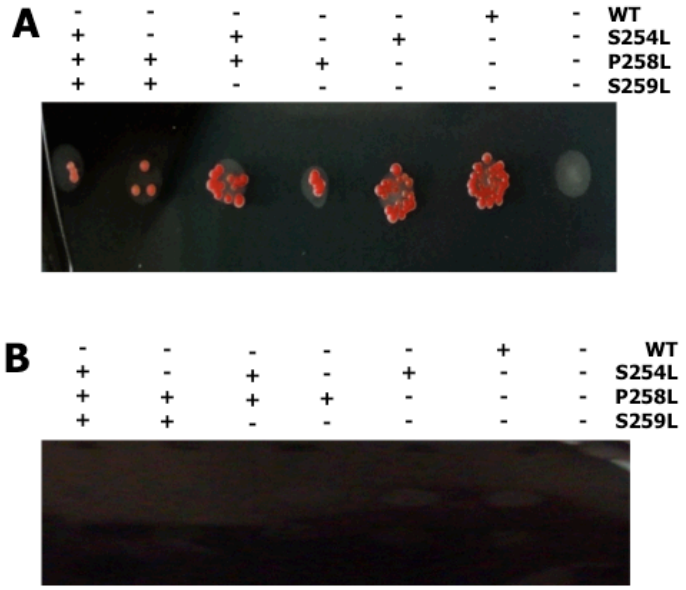


Figure 3.6 Qualitative yeast mating assay for Ste2p mutants. A) Ligand-stimulated mating. Lane 1 represents the mating between JKY78 strain transformed with empty pYES DEST52 vector and WT Mat a SCYO6O. Lane 2,3,4,5,6 and 7 shows the mating activity of JKY78 strains transformed with pYES DEST vector expressing the WT *STE2*, S254L, P258L, S254L/P258L, P258L/S259L and S254L/P258L/S259L respectively and WT Mat α SCYO6O. SCMM (-Leu-Ura) plates were used for selection of diploid zygotes. B) Basal mating in the absence of ligand. Lane 1 represents the mating between JKY78 strain transformed with empty pYES DEST52 vector and Δmfα1Δmfα2 T24D strain. Lane 2,3,4,5,6 and 7 shows the mating activity of JKY78 strains transformed with pYES DEST52 vector expressing the WT *STE2*, S254L, P258L, S254L/P258L, P258L/S259L and S254L/P258L/S259L genes respectively and Δmfα1Δmfα2 T24D strain. SCMM (-Leu-Ura) plates were used for selection of diploid zygotes.

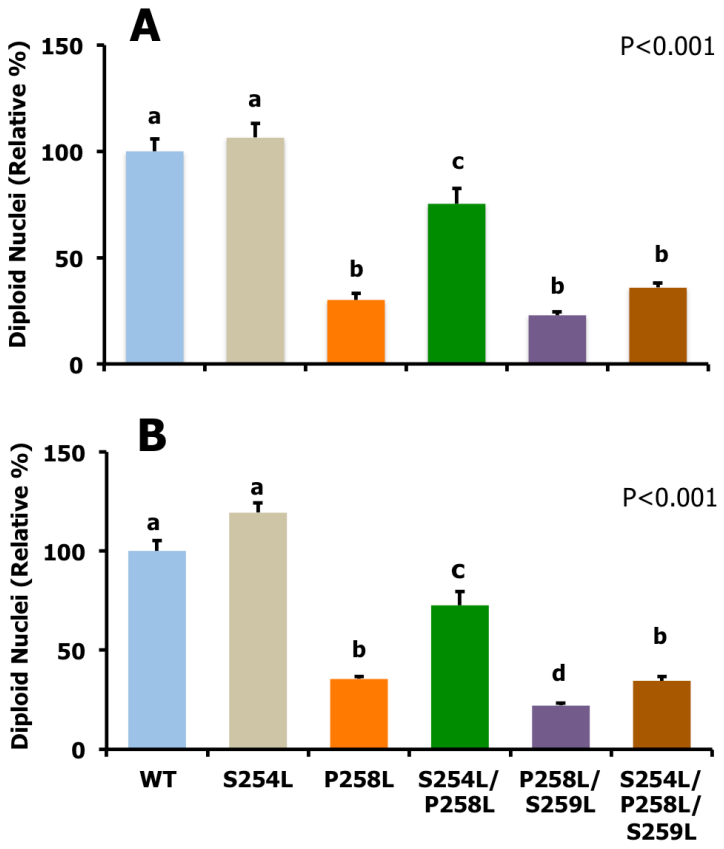


Figure 3.7 Percent ligand-induced mating activity (relative value) of Ste2p mutants in comparison to the WT Ste2p against WT SCYO6O. Mating efficiency of mutant receptors in JKY78 (upper panel) and JKY79 (lower panel). For mating efficiency, number of diploid zygote nuclei was quantified using the Sprague method (Sprague, 1991a) . Mating ability of WT Ste3p is taken as 100%. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.1.1.5 Characterization of MAPK signaling and mating efficiency of other Ste2p weak–mating mutants

As discussed in previous sections, the N terminal mutants (P15C, I24C and I29C) and TM6 mutant (S251L) have a differential effect on G protein mediated MAPK signal transduction (normal compared to WT) vs. diploid zygote formation (defective compared to

WT) (Konopka *et al.*, 1996; Dube and Konopka, 1998; Shi *et al.*, 2009a). In addition, the strong mating mutant S254L, when combined with the constitutive non-mating mutant (P258L) turned S254L/P258L mutant into a CAM with stronger mating mutant (this study). Whether the mating phenotype elicited by the S254L mutant can overcome the non-mating effect of other non-CAM, reduced-mating mutations (e.g. I24C and S251L) remains to be tested.

Toward this the MAPK signal transduction activity of different Ste2p mutants was determined and is shown in Figure 3.8. Consistent with the previous reports, non-mating mutants, I24C and S251L, showed 77 and 85% *FUS1-lacZ* β-galactosidase gene induction, respectively, compared to the WT in the presence of α-factor ligand. When combined with the S254L mutant, there was very little change in the induction of *FUS1-lacZ* β-galactosidase gene when compared to these non-mating mutants (Figure 3.8A). Moreover, none of these mutants showed basal MAPK signaling activity (Figure 3.8B).

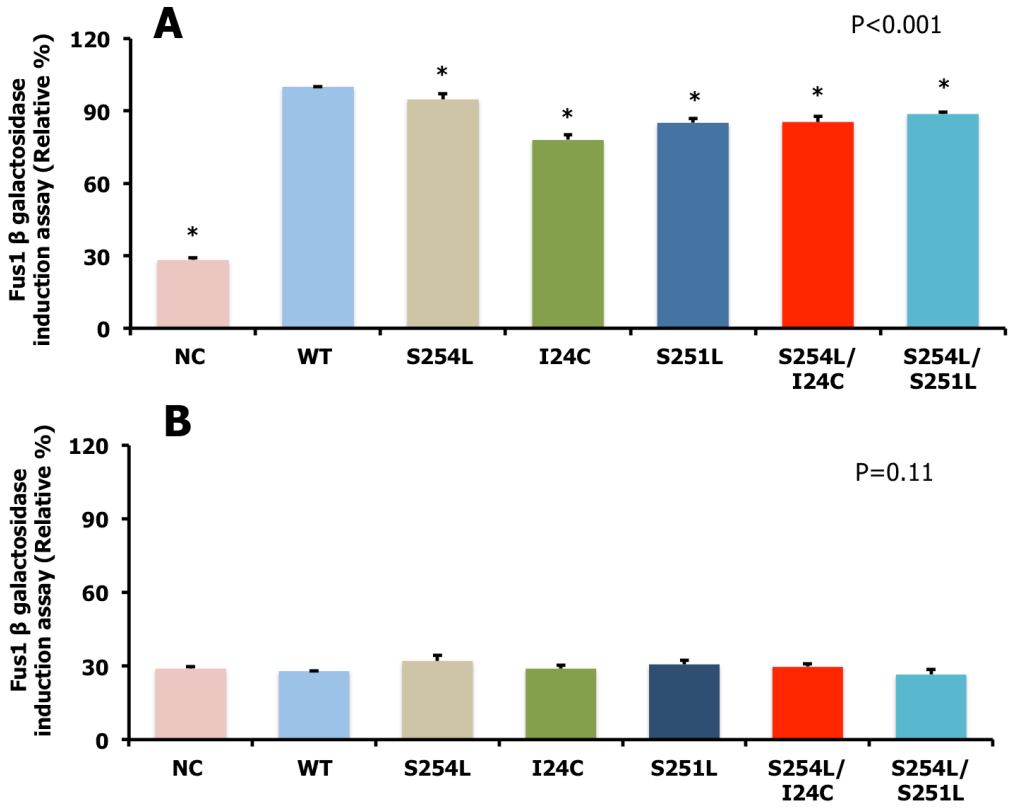


Figure 3.8 Ligand dependent and basal MAPK Signal transduction activity of Ste2p mutants in JKY78 cell line. A) Ligand dependent activation of a *FUS-lacZ* reporter gene was measured in JKY78 strain. B) Basal activity of *FUS1-lacZ* reporter gene in the absence of added α-factor ligand was measured in JKY78 strain. Data are expressed as a percent of the β-galactosidase activity of WT Ste2p treated with α-factor ligand. Comparisons between mutant values with those of WT were made using one-way ANOVA test. *P<0.05 was set as the cutoff value to define significant difference in mutant receptor compared to the WT.

The quantitative mating assay of these Ste2p mutants in JKY78 strain is shown in Figure 3.9. The N-terminal mutant containing lines in this study showed 37 % and 34 % mating efficiency compared to the WT Ste2p. These values are higher than the values mentioned in the literature; the use of a different cell line in the present study might be one of the reasons for differences in the values of mating efficiency. Interestingly, stacking the S254L mutant with N-terminal I24C or the TM6 mutation S251L did not increase the mating efficiency in both weak-mating mutants. These results indicate that the S254L mutation doesn't change the conformation of the I24C and the S251L mutants into a mating favorable conformation. This suggests that the weak-mating observed in these mutants is mediated through a different mechanism/conformational effect that the weak mating observed in the CAMs, which were rescued by the S254L mutation. Thus the strong mating mechanism of S254L is somehow specifically related to the mechanism of the CAMs.

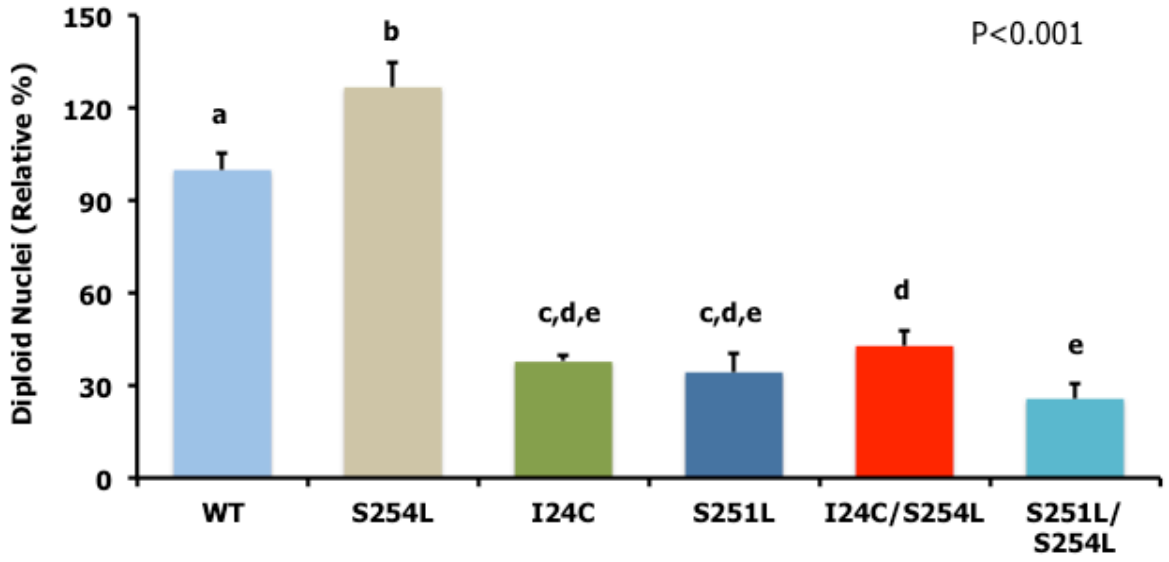


Figure 3.9 Percent ligand-stimulated mating activity (relative value) of Ste2p mutants TM6 and N-terminal mutants) in comparison to the WT Ste2p. JKY78 strains transformed with pYES DEST vector expressing the WT Ste2p, S254L, I24C, S251L, I24C/S254L and S251L/S254L gene were mated with WT Mat a SCYO6O. SCMM (-Leu-Ura) plates were used for selection of diploid zygotes. For mating efficiency, number of diploid zygote nuclei was quantified using the Sprague method (Sprague, 1991a). Mating ability of WT Ste2p is taken as 100%. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.1.2 Ste3p mutants

Like other GPCRs, the Pro residue (P222 in Ste3p) is conserved in TM6 of the Ste3p receptor and is very important in keeping the receptor in an inactive conformation. Expression levels of the Ste3p P222L mutant were comparable to the WT in western blot analysis and displayed a heterogeneous banding pattern also similar to the WT expression pattern, indicating that they entered the secretary pathway efficiently (Figure 3.10). However, the relative ligand-dependent MAPK signal transduction activity of WT Ste3p and mutant Ste3p P222L in the YKL178C BY4742 strain shown in Figure 3.11, indicate only very low signaling. This is in contrast to a previous report where a 2-fold increase in constitutive signaling activity was reported for the Ste3p-P222L mutant when transformed into an *SST2* deletion *STE3* null yeast strain (Stefan *et al.*, 1998). As discussed earlier, Sst2p is a negative regulator of the signaling pathway as it increases the GTP hydrolysis rate of the active Gα subunit, thus one would expect overall high signal activity levels in this cell line.

Interestingly, the third cytoplasmic loop (3CL) of Ste3p acts as a dominant negative regulator of signaling activity, as substitution of Glu for Leu at residue 194 (L194Q) makes the receptor hypersensitive and constitutively active (Bone et al, 1993). From this work it was hypothesized that the combination of the TM6 mutation (P222L) with the 3CL (L194Q) would yield better constitutive signaling activity for Ste3p.

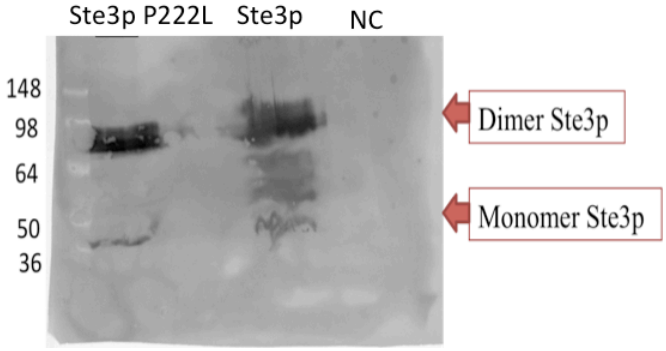


Figure 3.10 Immunoblot analysis of WT and mutant Ste3p receptor. Yeast strain YKL178CBY4742 carrying the indicated WT or mutant receptor were separated by SDS-PAGE, transferred to nitrocellulose membrane and probed with anti V5 HRP antibody. Immunoblots were detected using the enhanced chemi-luminescence kit (Amersham). The relative molecular weights (kDa) of prestained molecular weight markers (Bio-Rad) are given in left.

3.1.2.1 Ligand-dependent MAPK signal transduction activity of Ste3p WT and mutants:

The relative ligand dependent signal transduction activity of WT Ste3p and Ste3p P222L/L194Q in YKL178C By4742 and the Δ*sst2* 1987 yeast strains are compared in Figure 3.11A and Figure 3.11B. Both single (L194Q) and double mutant (P222L/L194Q) had WT signal transduction activity in YKL178C BY4742 strain. However, in Δ*sst2* 1987 strain, L194Q mutant showed only 65% signaling activity compared to WT. Another single mutant, P222L, had very weak signaling in YKL178C BY4742 strain while in Δ*sst2* 1987 strain it showed 40 % signal transduction activity as compared to the WT Ste3p.

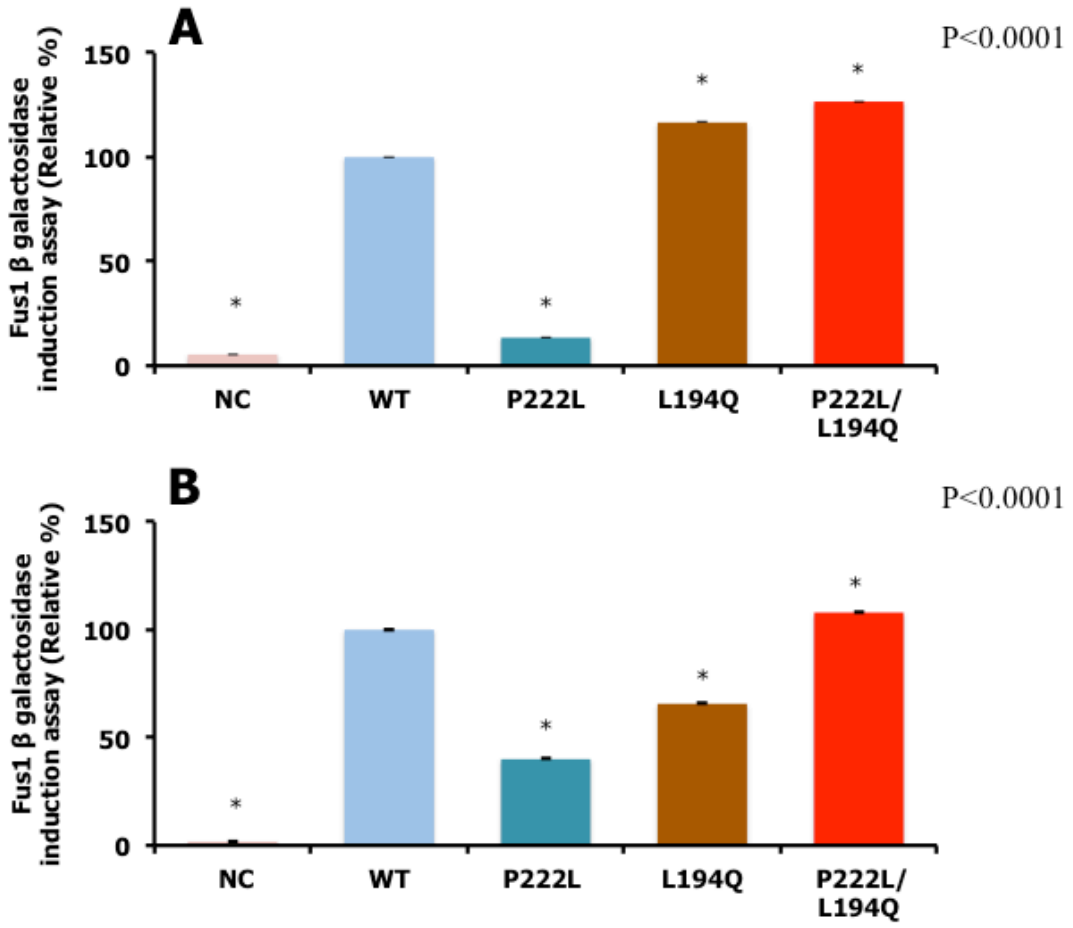


Figure 3.11 Ligand-dependent MAPK signal transduction activity of Ste3p mutants in YKL and 1987 Δ*sst2* deletion strains. Ligand (a-factor) dependent activation of a *FUS1-lacZ* reporter gene in was measured in the A) YKL178C BY4742 and B) Δ*sst2* 1987 strain. Data are expressed as a percent of the *FUS1-lacZ* β-galactosidase activity of WT Ste3p treated with a-factor ligand. YKL178C BY4742 or 1987 strain was co-transformed with pAG 423 vector carrying WT *STE3* or mutant *STE3* gene (P222L or L194Q or P222L/L194Q) and pSB234 vector to measure the induction of *FUS1-lacZ* reporter gene. Empty pAG423 vector transformed into YKL178C BY4742/1987 strain represents the negative control. Comparisons between mutant values with those of WT were made using one-way ANOVA test. * P<0.05 was used as the cutoff value to define significant difference between mutant receptor compared to the WT.

3.1.2.2 Basal MAPK signal transduction activity of Ste3p and mutants:

The relative basal signal transduction activity of WT Ste3p and Ste3p mutants in the absence of a-factor ligand are shown in Figure 3.12. WT as well as the single mutants P222L and L194Q didn't have any basal activity in YKL178C BY4742 or 1987 strains. However, the double mutant Ste3p P222L/L194Q could achieve 56 and 53 % basal signaling in the YKL178C BY4742 and Δ*sst2* 1987 strain, respectively. Thus in Ste3p, substitutions at both the third ICL residue and the TM6 residue, changes the receptor's conformation into an active one such that it is able to show basal signaling activity.

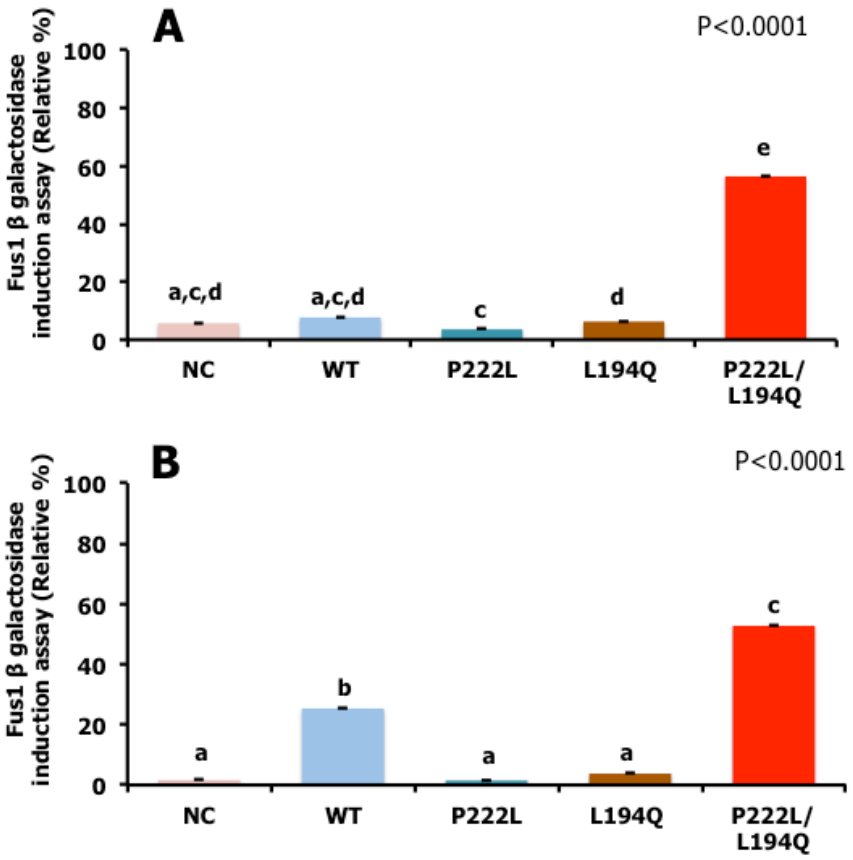


Figure 3.12 Basal MAPK signaling activity of TM6 Ste3p mutants, overexpressed in receptor null strains. A) Yeast strain YKL178C BY4742 or B) Δ*sst2* 1987 strain carrying the WT *STE3* or mutant *STE* receptor gene carrying in pAG 423 vector were co-transformed with pSB234 vector, was grown overnight in minimal media (-His-Ura) at 34°C and then shifted to the 23°C for next 5-6 h. The induction of *FUS1-lacZ* reporter gene was measured in the absence of a-factor ligand. Data are expressed as a percent of the *FUS1-lacZ* β-galactosidase activity of WT Ste3p treated with a-factor ligand. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.1.2.3 Mating activity of a constitutive active Ste3p mutant

The mating efficiencies of Ste3p mutant receptors were assessed in YKL and 1987 strains. The WT Mat a SCYO61 strain or Δ*mfa1*Δ*mfa2* Mat-a strain SM1458 was mated with either Mat-α YKL178C BY4742 or Mat α *Δsst2* strain 1987 transformed with various pYES DEST 52 Ste3p expression constructs and selected for mated diploid zygote on minimal media plates.

Qualitative mating assay: The double mutant Ste3p P222L/L194Q in YKL strain showed mating efficiency similar to WT Ste3p in the presence of a-factor ligand (Figure 3.13A). While in the absence of a-factor ligand, (mated with SM1458 strain), it did not show any mating activity (Figure 3.13B).

Quantitative mating assay: Similarly, the Ste3p mutants were compared against WT Ste3p for their mating abilities and the results are shown in Figure 3.14. The Ste3p L194Q and Ste3p P222L/L194Q mutants exhibited 1.5 and 1.7 fold increase in mating efficiency compared to WT. The double Ste3p mutants P222L/L194Q demonstrated only $3/4^{th}$ of the WT Ste3p mating efficiency when the1987 yeast strain, was mated with SCYO61 strain Figure 3.14. A probable reason for the lower mating ability could be that the supersensitive Δ*SST2* strain used in the present study has been observed to exhibit decreased directional accuracy in mating projection formation towards the mating partner (Moore *et al.*, 2008).

Overall, as concluded for Ste2p, these Ste3p results highlight a complex relationship between CA and mating, likely related to competition between unique receptor conformations that mediate MAPK signaling versus mating events.

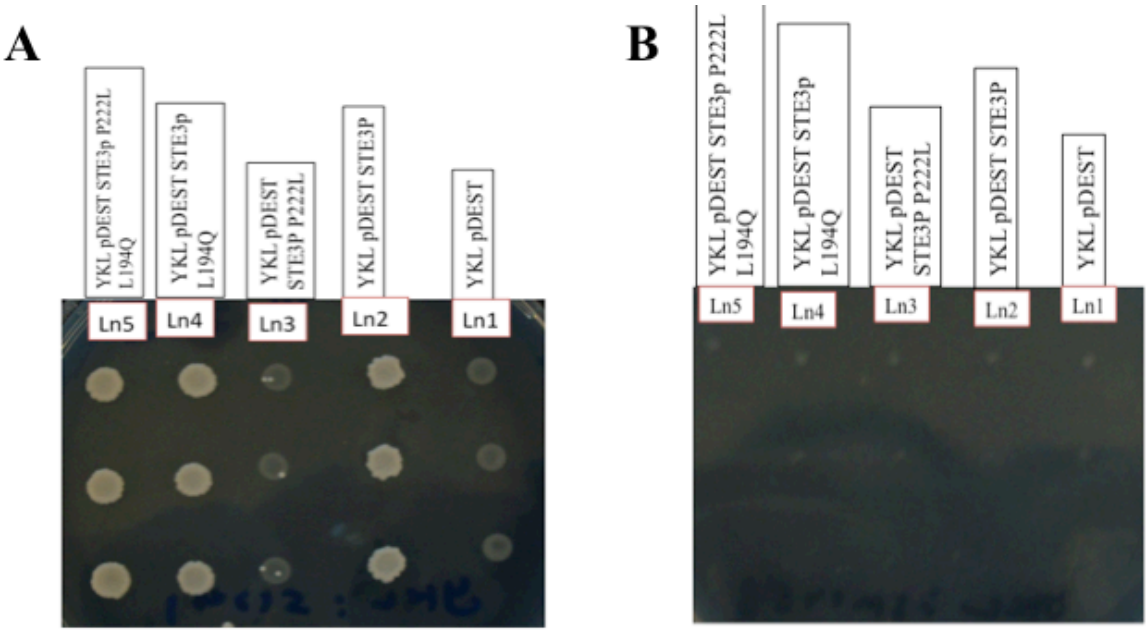


Figure 3.13 A: Qualitative yeast mating assay for Ste3p mutants against WT Mat a SCYO61 yeast strain. Lane 1- Mating between YKL strains transformed with empty pYES DEST 52 vector and WT Mat a SCYO61. Lane 2-Mating between YKL178C BY4742 strain transformed with Ste3p and Lane 3 –Mating between Ste3p P222L mutant transformed into YKL178C BY4742 strain with WT Mat a strain. Lane 4- Mating between Ste3p L194Q mutant and Lane 5 indicates the mating between Ste3p P222l L194Q and SCYO61.SCMM (-Leu-Ura) plates were used for selection of diploid zygotes. **Fig 3.13 B: Qualitative yeast mating assays of Ste3p mutants against Mat a Sm1458 yeast strain.** Lane 1- Mating between YKL strains transformed with empty pYES DEST vector and Mat a Sm1458 strain. Lane2-Mating between YKL strain transformed with Ste3p and Lane 3 –Mating between Ste3pP222L mutant transformed into YKL strain with Mat a Sm1458 strain. Lane 4–Mating between Ste3pL194Q mutant transformed into YKL strain with Mat a Sm1458 strain, Lane 5 –Mating between Ste3p P222L L194Q transformed into YKL strain with Mat a Sm1458 strain. SCMM (-His-Ura) plates were used for selection of diploid zygotes.

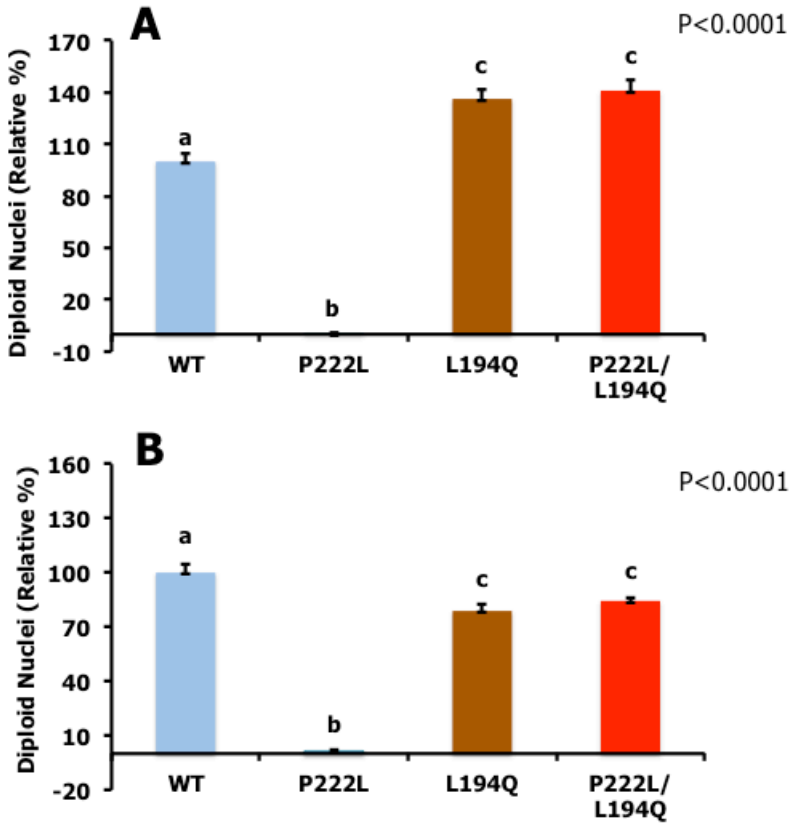


Figure 3.14 Quantitative mating efficiency of Ste3p mutant in YKL (A) and 1987 (B) yeast strains. Mating efficiency of Ste3p mutant receptors in YKL178C BY4742 (upper panel) and 1987 (lower panel). For mating efficiency, number of diploid zygote nuclei was quantified using the Sprague method (Sprague, 1991a). Mating ability of WT Ste3p is taken as 100%. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.2 Evidence for mutation derived biased functionality in Ste2p mutants

As discussed earlier, mating in yeast is initiated by the binding of pheromones secreted by the opposite cell type (Bender and Sprague, 1989; Slessareva and Dohlman, 2006). After pheromone binding, the yeast cell undergoes a series of events that prepare the yeast cell for mating. The events include significant changes in the expression of about 200 genes (almost 3% of the yeast genome) associated with arrest in the G1 stage of the cell cycle and formation of a projection towards the opposite mating type cell (Sprague, 1991b; Roberts *et al.*, 2000)

(Wittenberg and Reed, 1996; Madden and Snyder, 1998; Oehlen *et al.*, 1998) .

Many of these changes can also be induced when the yeast cells are exposed only to the purified pheromones of the opposite cell type. Previously, a quantitative kinetic approach was used in order to study the different steps of yeast mating by comparing dose response curves for various events of yeast mating stimulated by α factor ligand under an identical set of conditions (Moore, 1983). This study proposed the possibility of two different α-factor binding sites (high and low affinities) on Ste2p, such that the high affinity-binding site is saturated during the cell cycle arrest and agglutination step, whereas the low affinity-binding site is filled up in the mating projection formation step as pheromone concentrations increase. Interestingly, based on this earlier work, it was proposed that Ste2p might play an alternate role in mating events, mediated by two distinct (high affinity versus lower affinity) pheromone-binding sites (Moore, 1983; Konopka *et al.*, 1996). However, precedent for alternate functionalities for GPCRs has now been set in the literature, and standards for demonstrating alternate functionality based on biased-ligand signaling and mutational-biased signaling have been reported (Reiter *et al.*, 2012; Kenakin and Christopoulos, 2013). Essentially, evaluation of dose responses of an array of ligands or receptor mutants relative to a single standard enables comparison of two assays by accounting for systemic and observational biases that might otherwise mislead comparative interpretations. On the other side, the commercial unavailability of a-factor ligand makes it almost impossible to develop dose response assays for the Ste3p receptor or to further assess the differential activities of Ste3p mutants.

3.2.1 Comparison of the dose response curve of signal transduction activity, cell cycle arrest and mating projection formation for different Ste2p mutants

Initial dose response experiments carried out on WT Ste2p, towards reproducing the findings of the previous kinetic studies, yielded EC_{50} values of 1.7 nM for cell cycle arrest and 45 nM for mating projection formation (Figure 3.15). These EC_{50} values, while not identical, are both in the same range and relative affinity as those reported previously for the same experiment (Moore, 1983). Differences between the original values and those reported here are likely attributable to variations in cell lines and other experimental conditions. To further

calibrate and investigate this possibility, the dose response for WT Ste2p in the *FUS1-lacZ* β galactosidase reporter assay was also determined. Although numerically higher, the *FUS1-lacZ* β galactosidase EC_{50} value of 6 nM concentration of α-factor ligand was within the same range as the EC_{50} values obtained for cell cycle arrest. The EC_{50} value for MPF was 8 times higher than β galactosidase induction and 27 times higher than the EC_{50} for cell cycle arrest (CCA). While, the EC_{50} for β- galactosidase induction was only 4 times higher than the EC_{50} of CCA (Figure 3.15). These results emphasize that direct comparison of Ste2p dose responses between assays can't be used to evaluate receptor signaling or functional bias due to the interference of observational/systemic bias. The presence of observational/systemic bias in different assays emphasizes a need for a mutational derived approach to validate alternate functionality for the Ste2p receptor.

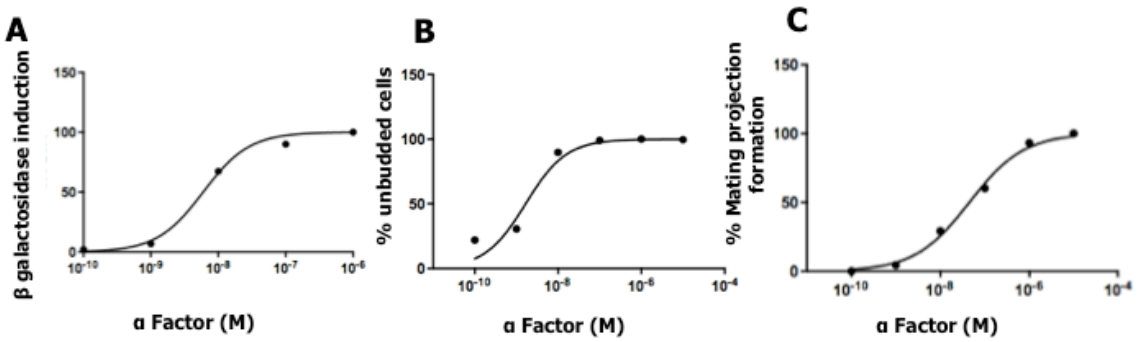


Figure 3.15 Concentration-dependent functional bias of WT Ste2p receptor. The EC_{50} for *FUS1-lacZ* β galactosidase activity, cell cycle arrest and mating projection formation was 6 nM, 1.7 nM and 45 nM, respectively. Previous reported EC_{50} values for cell cycle arrest and mating projection formation 0.25 nM and 14 nM (Moore, 1983). The EC_{50} for mating projection formation was 27 times and 8 times higher than the EC_{50} for cell cycle arrest and β- galactosidase activity, respectively.

The comparisons of dose responses between WT and mutant receptors for β galactosidase, cell cycle arrest and mating projection formation are given in Figure 3.16A, B and Figure 3.17 respectively. The mutants showed MAPK signaling dose responses approximately comparable to WT (EC_{50} in the range of 3.5 - 4.3 nM) with the exception of those containing the S259L mutations, which showed a 5 fold reduction in response (EC_{50} of 27 nM and 28 nM respectively (Table 3.1). The cell cycle arrest dose response curves showed a left side shift only for the S254L mutant to that of the WT Ste2p (Figure 3.16B).

Both S254L containing mutants (S254L and S254L/P258L) showed higher potency in mating projection formation as half maximal response was obtained at lower concentrations of α-factor (Figure 3.17; Table 3.1). Surprisingly, the P258L allele showed projection formation at a lower concentration of α-factor when compared to the WT Ste2p, but this mutant was less efficient in diploid zygote formation as only 30% of P258L mutants showed mating compared to WT Ste2p (Figure 3.7A and B). Together these results further indicate the differential effect of mutants on MAPK signaling (*FUS1-lacZ* β galactosidase induction, cell cycle arrest) and mating activity (mating projection formation).

Table 3.1 EC_{50}values for WT *STE2* and its mutant alleles in different assays.

	Signaling Activity: Fus1:β-Gal Induction		**Signaling Activity: Percent unbudded cells**		**Mating Activity: Mating Projection Formation**	
	Log EC_{50} **+/- S.E.(nM) (n=3)**	EC_{50} **(nm)**	**Log** EC_{50} **+/- S.E.(nM) (n=3)**	EC_{50} **(nm)**	**Log** EC_{50} **+/- S.E. (nM) (n=3)**	EC_{50} **(nm)**
WT	-8.2±0.3	6	-8.8±0.1	1.6	-7.3±0.1	45
S254L	-8.4±0.4	4.3	-9.3±0.17	0.41	-8.8±0.2	1.4
P258L	-8.5±0.5	3.6	-9.9±0.9	0.12	-7.6±0.3	25
S254L/P258L	-8.4±0.7	3.5	-8.9±0.3	1.3	-8.5±0.2	3.1
P258L/S259L	-7.5±0.8	27	-8.2±0.4	5.7	-6.8±0.3	142
S254L/P258L/ S259L	-7.5±0.8	28	-8.6±0.3	2.4	-7.0±0.2	91

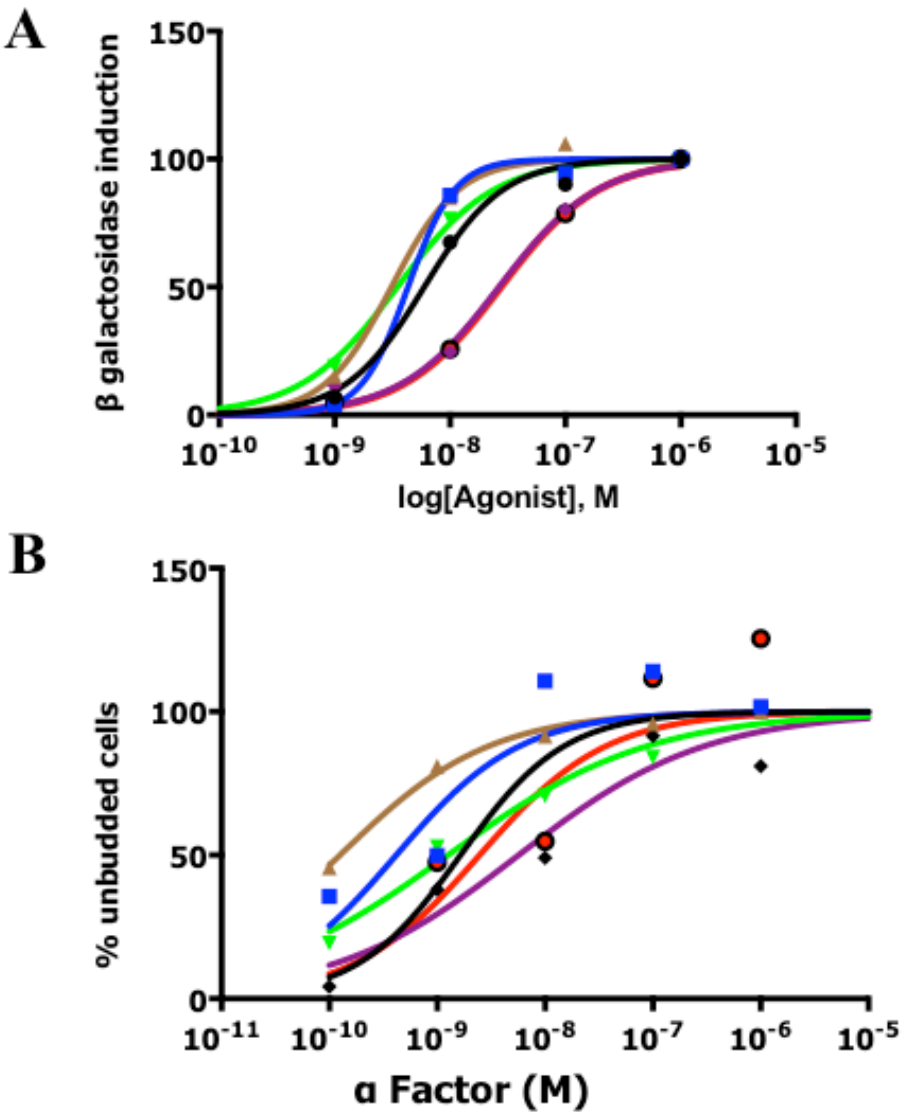


Figure 3.16 A, B: Dose response curve for α-factor induced MAPK-siganling responses (β-galactosidase and cell cycle arrest) at different concentration of α-factor ligand. The JKY78 strain carrying WT or mutant *STE2* alleles on plasmid vector pYESDEST were incubated with the α-factor for 2 h and then assayed for β-galactosidase activity to measure the induction of the pheromone responsive *FUS1-lacZ* reporter gene for WT and *STE2* mutant alleles shown in Figures 3.14 A. The LM102 strain carrying WT or mutant *STE2* alleles on plasmid vector pAG425 were incubated with the α-factor for 4 h and 7.5 h and collected in 37% formaldehyde. Cells were examined microscopically to determine the percent of cells that were arrested at the unbudded stage of cell division (B).

● WT (Black), ■ S254L (Blue), ▲ P258L (Brown), ▼ S254L P258L (Green), ◆ P258L S259L (Purple), ○ S254L P258L S259L (Red)

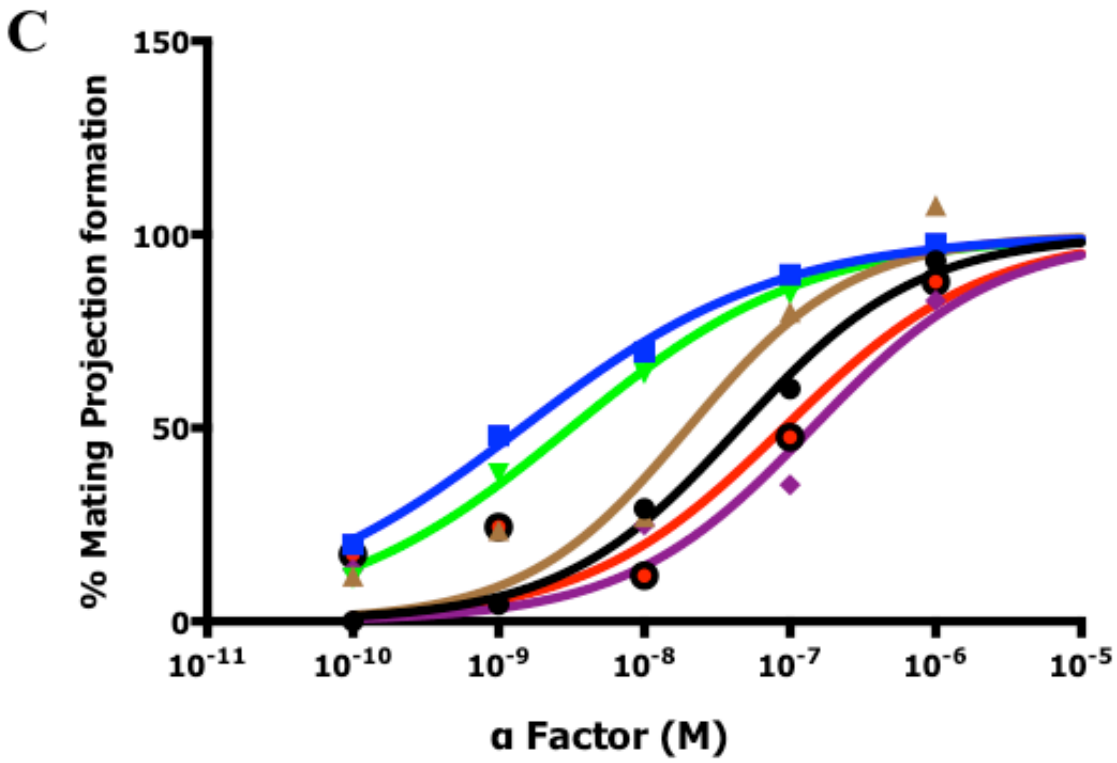


Figure 3.17 Dose response curve for α-factor induced mating response (mating projection formation) at different concentration of α factor ligand. The LM102 strain carrying WT or mutant *STE2* alleles on plasmid vector pAG425 were incubated with the α-factor for 7.5 h and collected in 37% formaldehyde. Cells were examined microscopically to determine the percent of cells that has pointed projection towards one end for WT and *STE2* mutant alleles.

● WT (Black), ■ S254L (Blue), ▲ P258L (Brown), ▼ S254L P258L (Green), ◆ P258L S259L (Purple), ○ S254L P258L S259L (Red)

3.2.2 Evaluation of mutational-derived mating bias for Ste2p mutants

The differential effect on the dose response curves or EC_{50} values of Ste2p mutant receptors suggests the presence of mutational bias for mutant receptors. In order to remove the systemic and observational bias to demonstrate the mutational bias, the relative EC_{50} was measured by comparing the EC_{50} value of mutants to the WT for each particular assay (Table 3.2). Then, the bias for the pathway was calculated by taking the ratio of the relative activities of mutants in mating projection efficiency to the relative activity in signaling events (β

galactosidase induction or CCA) (Table 3.3). The relative EC_{50} of mutants in β-galactosidase induction was comparable to WT with the exception of those containing the S259L mutations, which showed a 5-fold reduction in values. However, in the cell cycle arrest assays, the P258L mutant showed an aberrantly high value of relative EC_{50} that needs to be interpreted with caution as the values for this mutant had high variation between the replicates indicated by a large standard error (Table 3.1 EC_{50}values for WT *STE2* and its mutant alleles in different assays.) Therefore, the *FUS1-lacZ* β-galactosidase reporter assay was selected over cell cycle arrest as the representative assay for classical MAPK signal transduction, based on it being more reliable and more efficient for ease of reproducibility to calculate the bias factor. In contrast, mating projection responses were generally more variable, with the strong mating mutant, S254L, yielding the most potent response representing a 32-fold relative decrease in EC_{50} (EC_{50} 1.4 nM; Figure 3.17; Table 3.1) consistent with the observed trend in diploid zygote formation (Figure 3.7). Overall, these results provide strong evidence in support of α-factor-dependent alternate-functionalities for Ste2p in downstream mating functionalities.

Table 3.2 The relative EC_{50} values of mutant receptors

Receptor	**Relative EC_{50}**		
	BGI	CCA	MPF
WT	1	1	1
S254L	1.4	3.9	32
P258L	1.6	13.3	1.8
S254L/P258L	1.7	1.2	14.5
P258L/S259L	0.2	0.3	0.31
S254L/P258L/S259L	0.21	0.6	0.5

Table 3.3 Mating bias of different mutants in comparison to WT Ste2p

Receptor	**Relative activity ratios**
	MPF: BGI
WT	1
S254L	23.2
P258L	1.1
S254L/P258L	8.5
P258L/259L	1.5
S254L/P258L/S259L	2.1

MPF: Mating projection formation
BGI: β galactosidase induction

3.3 Expression and localization of Ste2p mutants in response to α-factor ligand

3.3.1 Expressed Ste2p-GFP localizes to the periphery and endocytic compartments

To characterize the localization and expression of WT Ste2p receptor, green fluorescent protein (GFP) was tagged onto the C-terminus of the receptor. In addition, carboxy-terminal Ste2p mutant (CT-345 Ste2p) was attached to the GFP as well. The CT-345 Ste2p is an internalization defective mutant, in which potential phosphorylation sites within the C-terminus of Ste2p are mutated (Kim *et al.*, 2012). The localization of WT Ste2p-GFP and CT345-GFP was examined in the presence and absence of α factor ligand as described in section 2.5 of the Materials and Methods.

Consistent with previous reports (Stefan and Blumer, 1999; Kim *et al.*, 2012), Ste2p was localized to the periphery as well as in the intracellular vesicle compartments in the absence of ligand (Figure 3.18, Figure 3.19). When quantified, almost 50% of the total Ste2p was present on the periphery (Figure 3.20). Upon ligand treatment, Ste2p receptor was rapidly removed from the periphery in as little as 10 min after α factor exposure (Figure 3.21). Approximately 90% of the total receptor was observed to move from the periphery into the cytoplasmic organelles (vesicle possibly) when quantified using Image J software; Figure 3.21, bar plots). The levels of Ste2p remained low for at least 30 min after the α-factor administration (Figure 3.22). In contrast, the CT-345 Ste2p mutant was predominantly present on the periphery even after 10 min of ligand exposure, in agreement with the previous study (Figure 3.18).

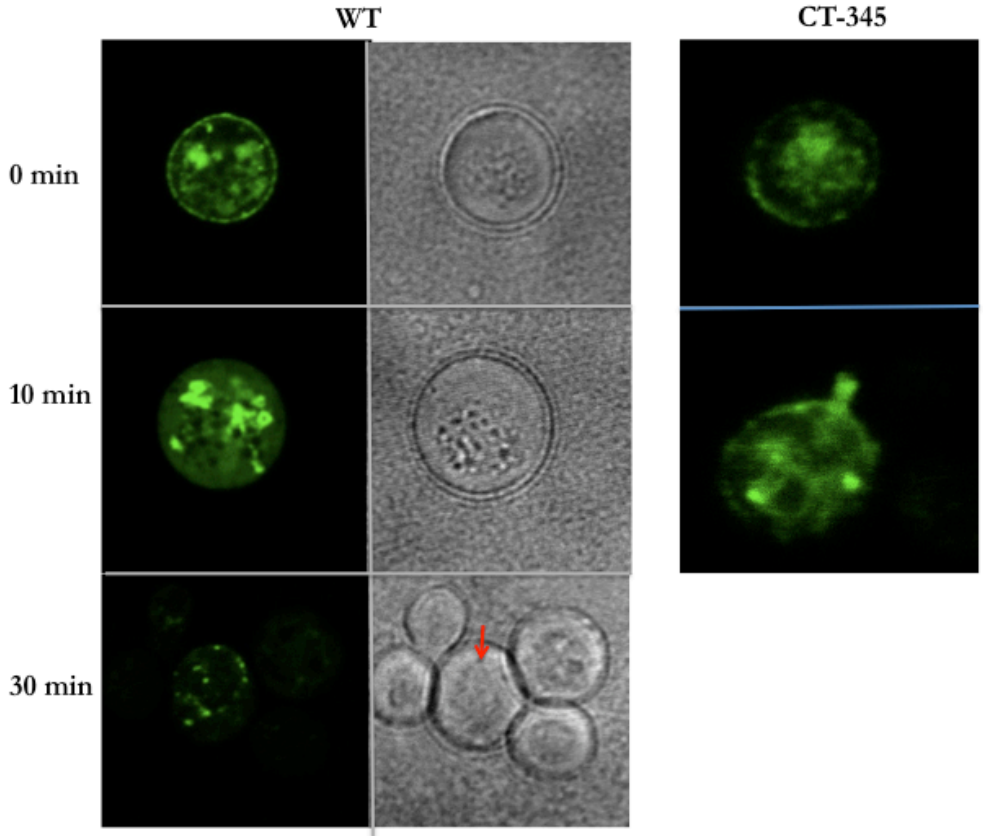


Figure 3.18 Localization of GFP tagged α-factor stimulated Ste2p receptor: The JKY78 strain cells containing Ste2p-GFP/ CT-345 Ste2p-GFP receptor in pAG425 vector were observed in the absence of α-factor ligand (0 min) and following treatment with α factor at 10 and 30 min at 30^0C prior imaging. At 0 min time point, Ste2p is distributed on periphery and endcocytic compartments. Upon ligand exposure, WT Ste2p was internalized and present in the intracellular vesicles. CT-345 Ste2p-GFP mutant failed to internalize following ligand stimulation. For WT both confocal and bright field images are given. Red arrow indicates the cell chosen for confocal imaging.

3.3.2 Expression of TM6 Ste2p mutants in the absence of α-factor ligand

TM6 Ste2p mutants (expressed in JKY78 strain) were tagged with the green fluorescent protein at the C-terminal to examine their localization and trafficking in the absence of α- factor ligand. The total expression levels of mutants never achieved levels comparable to WT, showing 50% or less total expression (Figure 3.19). In agreement with the lower total expression levels, peripheral distribution of mutant receptor was also lower than the WT levels at 0 time point (Figure 3.20) bar plot).

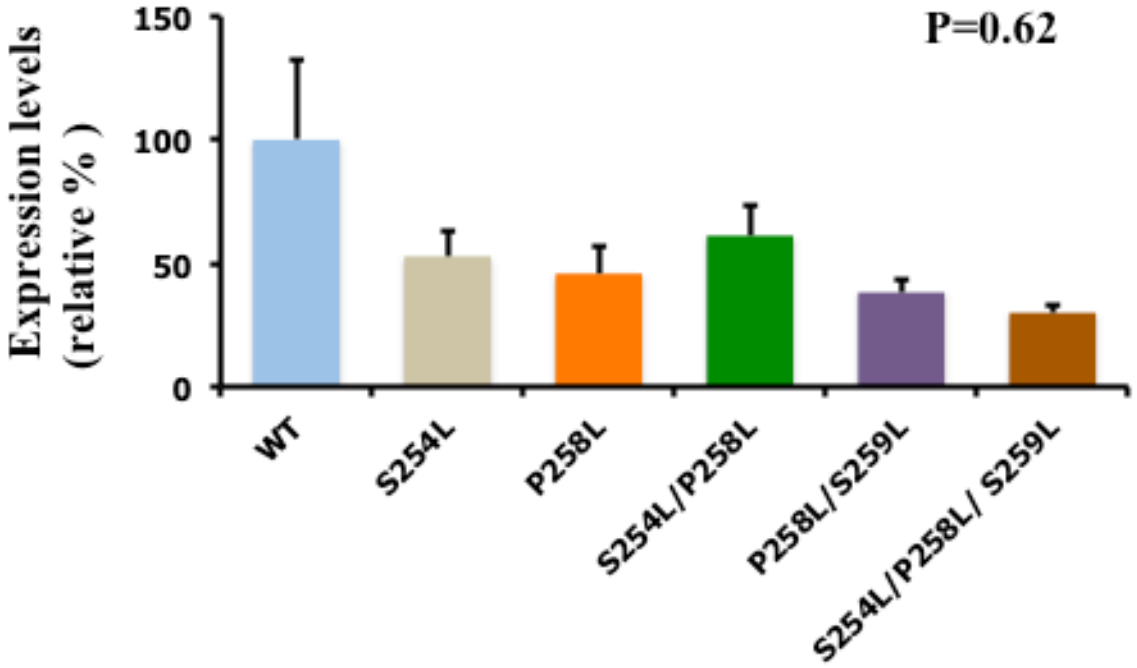


Figure 3.19 Total expression levels of WT and mutant Ste2p. WT and mutant STE2 genes were tagged with GFP and total cellular fluorescence measured by confocal microscopy. Data are expressed as a percent of the total expression levels of WT Ste2p. Comparisons between mutant values with those of WT were made using one-way ANOVA test. $P>0.05$ was considered as the non-significant difference between mutant receptor compared to the WT.

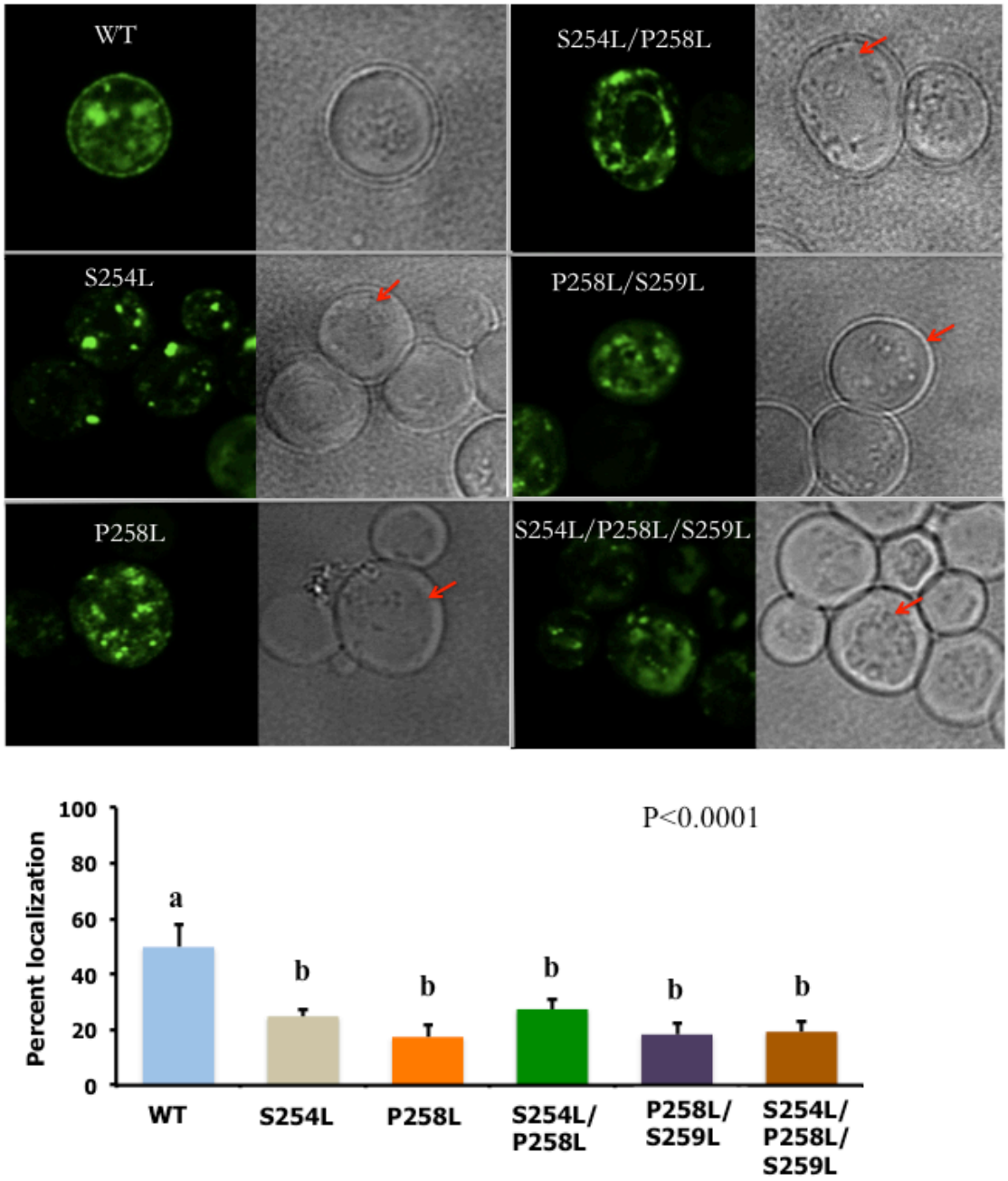


Figure 3.20 Expression of GFP tagged WT and Ste2p mutants at periphery in the absence of α-factor. 2-D images showing distribution of Ste2p mutants in the JKY78 strain taken in the absence of α-factor. The adjacent graph shows peripheral distribution (percent) of these Ste2p mutants that were quantified using Image J software. Percent data shown here represent the proportionate values within each receptor type. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different. Red arrows indicate the cell chosen for confocal imaging.

3.3.3 Expression of TM6 Ste2p mutants in the presence of α-factor ligand

All mutations containing the P258L variation followed the kinetics of WT receptor localization after ligand stimulation with decreased localization of the receptor on the periphery at 10 min time (Figure 3.21). However, unlike WT, by 30 min most of these P258L containing mutants began to recover some of receptor content back into the periphery by 30 min (Figure 3.22 bar plot). In contrast, the S254L mutant showed only a very slight decrease in localization to the periphery 10 min after application of ligand, and these levels were maintained through at least 30 min (Figure 3.21, Figure 3.22 bar plot).

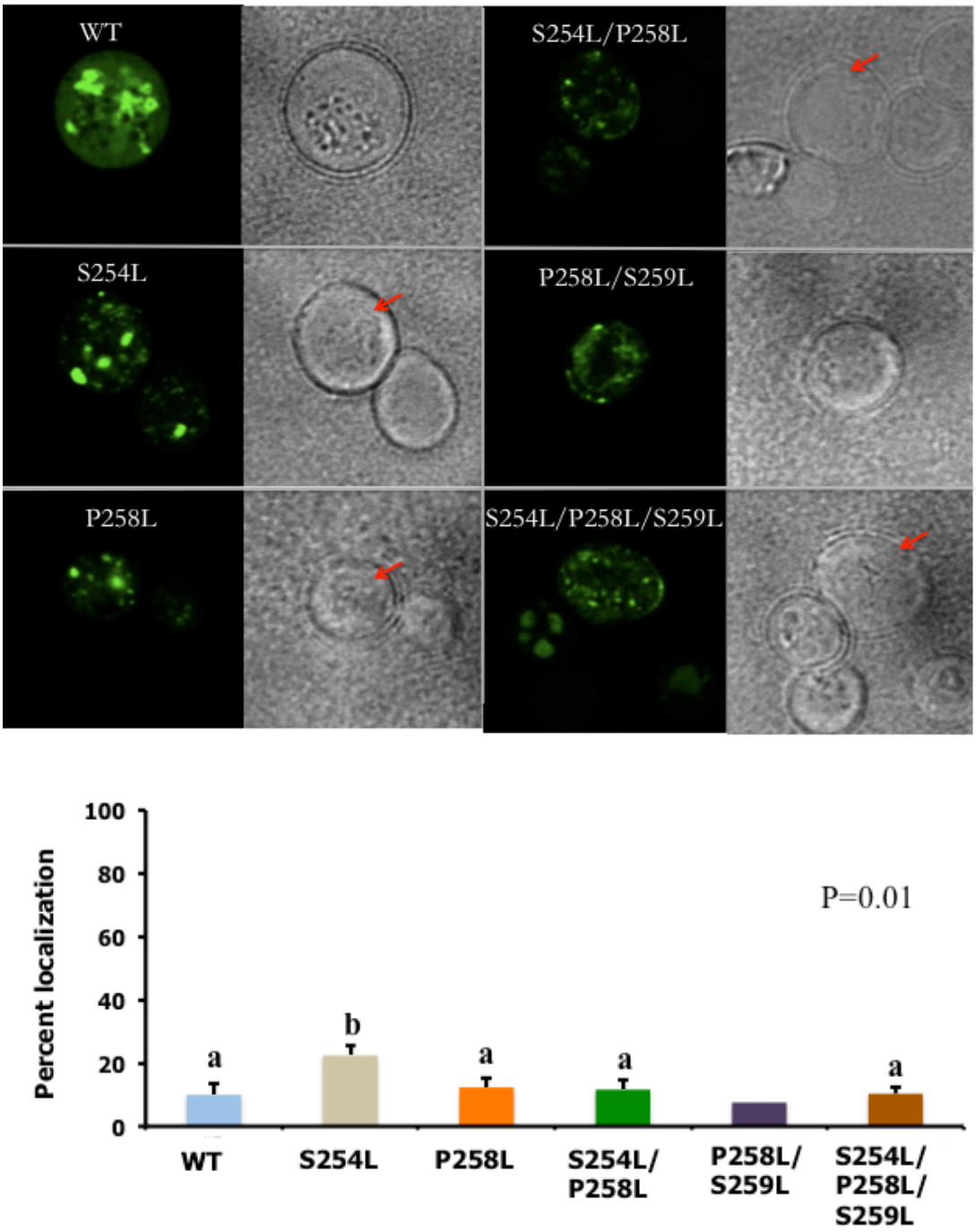


Figure 3.21 Expression of GFP tagged WT and Ste2p mutants on periphery at 10 min time point after exposure to 1µM α-factor. 2-D images showing distribution of Ste2p mutants in the JKY78 strain taken at 10 min post α-factor addition. The adjacent graph shows peripheral distribution (percent) of theseSte2p mutants that were quantified using Image J software. Percent data shown here represent the proportionate values within each receptor type. Comparisons between groups were made using one-way ANOVA test. *P<0.05 indicates significant difference between groups. Values with no common superscript are different. Red arrows indicate the cell chosen for confocal imaging.

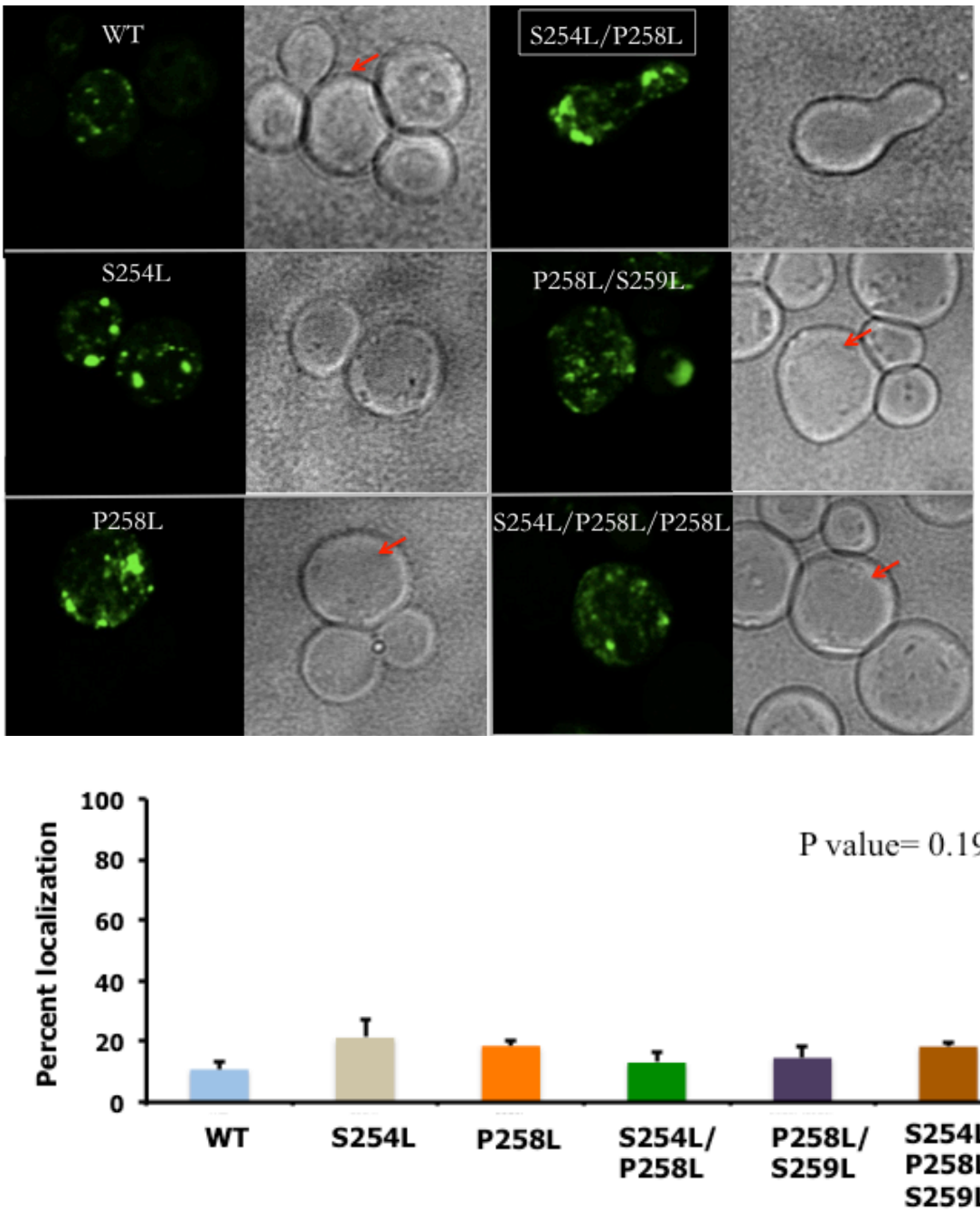


Figure 3.22 Localization and distribution of GFP tagged WT and Ste2p mutants on cell surface at 30 min time point after addition of 1μM of α-factor. 2-D images showing distribution of Ste2p mutants in the JKY78 strain taken at 30 min post α-factor addition. The adjacent graph shows peripheral distribution (percent) of these Ste2p mutants that were quantified using image J software. Percent data shown here represent the proportionate values within each receptor type. Comparisons between groups were made using one-way ANOVA test. P>0.05 is considered non- significant. Red arrows indicate the cell chosen for confocal imaging.

3.4 Role of arrestins in the mating associated functionality of Ste2p receptor

Members of the arrestin family have been reported to regulate GPCR functioning in mammalian systems at several levels. Apart from their desensitizing effect (DeWire *et al.*, 2007), arrestins have been shown to mediate the endocytosis of GPCR through ubiquitination (Goodman *et al.*, 1996) and mediate signaling pathways by themselves (Luttrell and Gesty-Palmer, 2010). Previously, it was believed that yeast do not have arrestin proteins, a concept that has been challenged recently, as arrestin-like proteins (α-arrestins) were shown to be involved in the endocytosis of various other membrane proteins in yeast (Table 3.4) (Nikko and Pelham, 2009). As well, a very recent report has indicated the contribution of three different α-arrestin family members in desensitization and internalization of Ste2p ((Alvaro *et al.*, 2014)). This recent study demonstrated the presence of sustained and even increased MAPK signaling in cells lacking Ldb19/Art1, Rod1/Art4 and Rog3/Art7 arrestins. However, in contrast to Rod1 and Rog3, the overexpression of Ldb19 didn't rescue the cells from pheromone induced cell cycle arrest. It might be possible that Ldb19 acts via a different mechanism or may be involved in more functions than Rod1/Rog3.

In order to determine whether or not α-arrestins modulate alternate Ste2p mating functionalities, a series of yeast arrestin knockout strains were tested for both MAPK signal transduction and mating activities.

Table 3.4: Name of yeast α-arrestins (Nikko and Pelham, 2009)

Arrestin	Gene Name
Art1	Ldb19
Art2	Ecm21
Art3	Aly2
Art4	Rod1
Art5	Ygr068c
Art6	Aly1
Art7	Rog3
Art8	Csr2
Art9	Rim8
Art10	Ylr392c

3.4.1 The involvement of arrestins in pheromone regulated MAPK signal transduction pathway of Ste2p receptor:

The effect of arrestins on MAPK signal transduction was evaluated in six knockout strains, including four double 'Mat α' knockouts and two 'Mat a' complete knockout strains (Table 2.2 in material and methods). These strains were all transformed with the *FUS-lacZ* receptor vector and tested for α-factor stimulated signal transduction. The relative levels of signal transduction activity of WT Ste2p and arrestin knockout strains are shown in Figure 3.23. The signal transduction activity of EN59 and EN60 strains (10 and 9 arrestins knockouts, respectively) was 94% and 84%, respectively, of that observed for WT Ste2p (Figure 3.23A). All arrestin double knockouts strains, except EN07, showed signal transduction activity comparable to the WT (Figure 3.23A). A significant, but weak effect for the EN07 line, which included knockdown of Art3 (gene *ALY*2) and Art6 (gene *ALY*1) arrestins was observed. These results are further reflected in the complementation experiments, where each arrestin was overexpressed one at a time in the arrestin null strain (EN60) and all show some increase possibly due to the improved general health of the yeast cells. It is interesting to note that Art6 overexpression had the maximum effect on signaling, 45% increase in signaling over WT, while the Art3 lines does not (Figure 3.23B). Knockdown and complementation results in combination suggest a possible link between Art6 and MAPK signal transduction, however the effect is relatively weak.

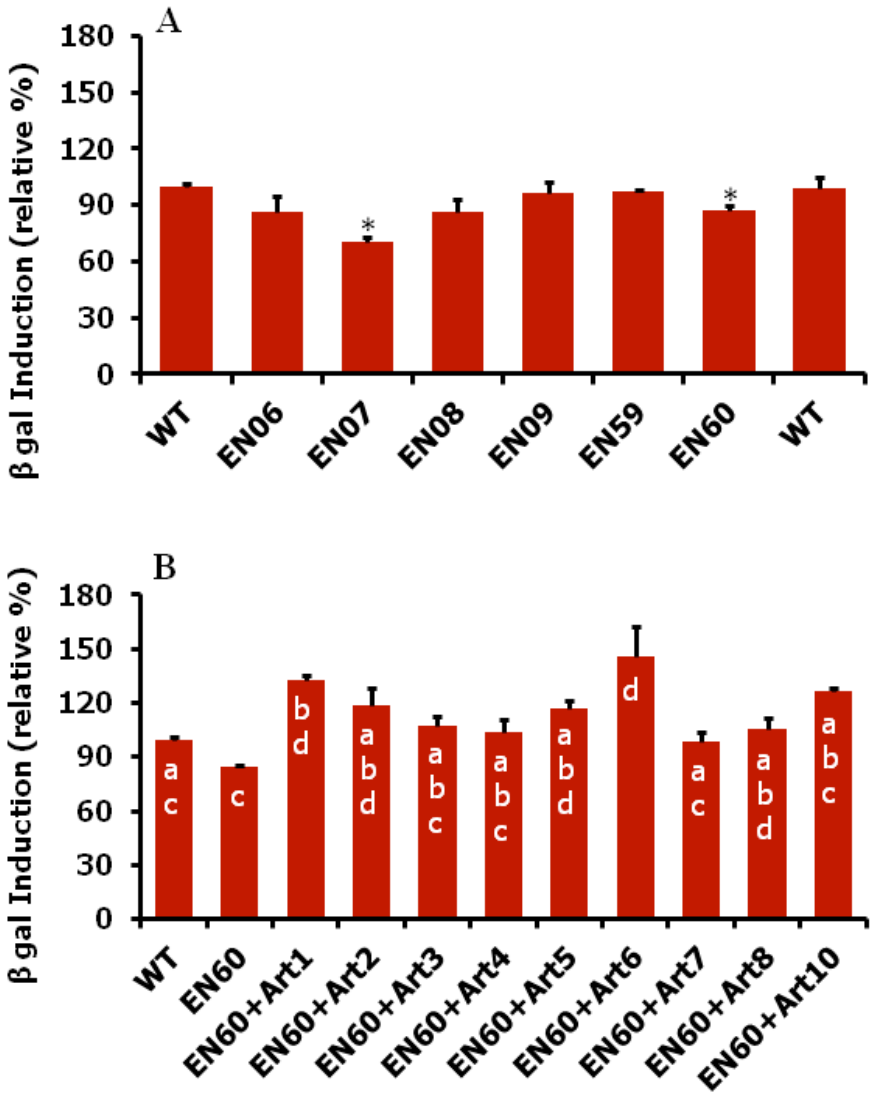


Figure 3.23 Characterization of MAPK signaling of arrestin knockouts A: Signal transduction activity (relative value) of arrestin knockouts in the presence of α-factor ligand in comparison to the WT yeast strain. Arrestin double knockouts (EN06, EN07, EN08 and EN09) were incubated with 1μM α-actor ligand for 2 h and then assayed for β galactosidase activity. Complete knockout strains EN59 and EN60 were treated with equal amounts of Mat α cells (201389) as a source of a-factor and incubated for 2 h at 30^0C. Values are representative of two independent experiments (triplicate each time). Comparisons between mutant values with those of WT were made using one-way ANOVA test. *P<0.05 was used as the cutoff to define significant difference between mutant values compared to the WT. B: Signal transduction activity of complete knockout strain (EN60) following transformation of different arrestin proteins. Arrestin null yeast stain EN60 carrying different arrestin genes in a plasmid was tested for the induction of the *FUS1-lacZ* reporter gene in the presence of α- factor ligand. Values (mean±SE) are representative of two independent experiments (triplicate each time). Comparisons between groups were made using one-way ANOVA test. P<0.05 was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.4.2 Modulation of pheromone based mating events by yeast arrestins

To evaluate the effects of different arrestins on pheromone based mating activities of yeast, double knockouts and complete knockout strains were used. Mat a and Mat α knockout strains were mated against WT Mat α (201389) and Mat a (201388) strains respectively. The quantitative mating efficiencies of different arrestin knockouts are given in Figure 3.24A. Complete knockout strains, EN59 and EN60, showed a 56 and 66% decline (down to 44 to 33% of WT values), respectively, in the mating efficiency compared to the WT strain (Figure 3.24A).

The EN06, EN07 and EN09 strains showed decreased mating effeciencies down to 62, 55 and 52 % respectively in comarison to the WT strain (Figure 3.24A). However, the knockout strain EN08 showed mating efficiency comparable to the WT (Figure 3.24A). Expression of arrestin proteins in the complete knockout strain (EN60) partially reversed the drop in mating efficiency. Specifically, Art1 (Ldb19) overexpression yielded a significant recovery of mating activity, showing a 35% increase over the EN60 knockdown level, none of the other single arrestins yielded any significant effect, highlighting a possible role for Art1 as a key component in downstream mating events.

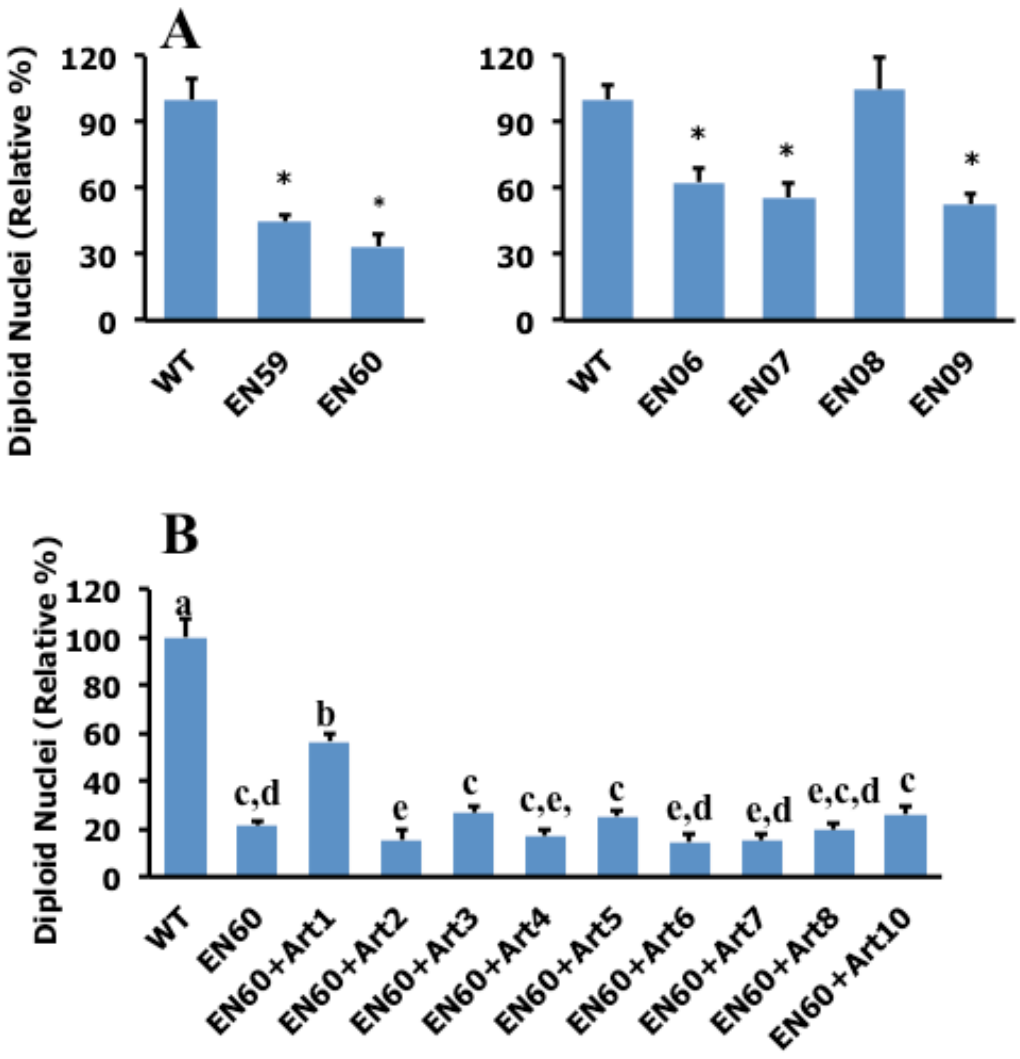


Figure 3.24 Characterization of role of arrestins in mating activities A: Percentage mating activity of arrestin knockouts as compared to the WT yeast strain. Arrestin knockouts were mated against 'Mat a' or 'Mat α' yeast strains (201388/201389). The mating was allowed to occur for 2 h and mating products were grown on minimal media (SCMM-Ura-His) plates. Values are representative of six to eight independent experiments (triplicate each time). Comparisons between mutant values with those of WT were made using one-way ANOVA test. * $P<0.05$ was used as the cutoff value to define significant difference between mutant receptor compared to the WT. B: Percent mating abilities of arrestin proteins: EN60 yeast strain transformed with plasmid carrying different arrestin proteins was mated against WT 201389 strains and diploid zygotes were selected on (SCMM-Lys-Met) minimal media plates. Comparisons between groups were made using one-way ANOVA test. $P<0.05$ was used as the cutoff value to define significant difference between groups. Values with no common superscript are different.

3.4.3 The localization of GFP tagged Art1 in response to α-factor ligand

Gene deletion and complementation studies indicate a possible role for Art1 in the mating activity of yeast α-factor receptor. Further, localization of arrestin-GFP expressed in the EN60 strain, was monitored by confocal microscopy following α-factor treatment. In the absence of any added ligand, Art1-GFP was distributed uniformly in the cytoplasm of yeast cells (Figure 3.25A). Ligand induced translocation of Art1-GFP was seen in endocytic vesicles at 30 and 120 min time, suggesting the involvement of Art1 in the later events of mating (Figure 3.25A). Interestingly, Art1-GFP was concentrated to the projection tip after treatment with α factor at the longer time point of 120 min. As a negative control, the pattern of GFP-vector (pAG426 GFP) was compared with that obtained for the Art1-GFP localization (Figure 3.25B).

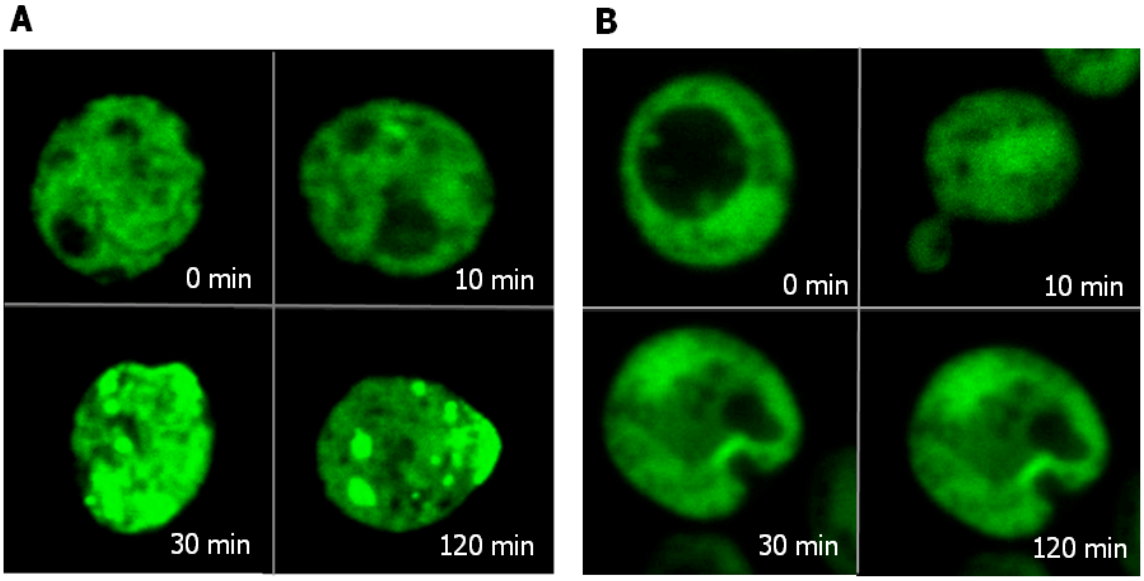


Figure 3.25 Trafficking of arrestin protein in response to α-factor ligand. GFP tagged Art1 (A) and vector (GFP only, B) was transformed into arrestin null EN60 yeast strain. The trafficking of arrestin was monitored in the absence (at 0 min) and presence (at 10 min, 30 min, 60 min and 120 min time points) of α-factor ligand under confocal microscopy.

To further evaluate involvement of Art3 and Art6 in MAPK signal transduction, Art3 and Art6 were tagged to GFP and observed under the microscope. Both Art3 and Art6 didn't show altered localization in response to α-factor ligand (Figure 3.26A and Figure 3.26B). Thus overall, both complementation and localization work indicate a possible role for Art1 in mating events that is downstream of G protein MAPK signaling events.

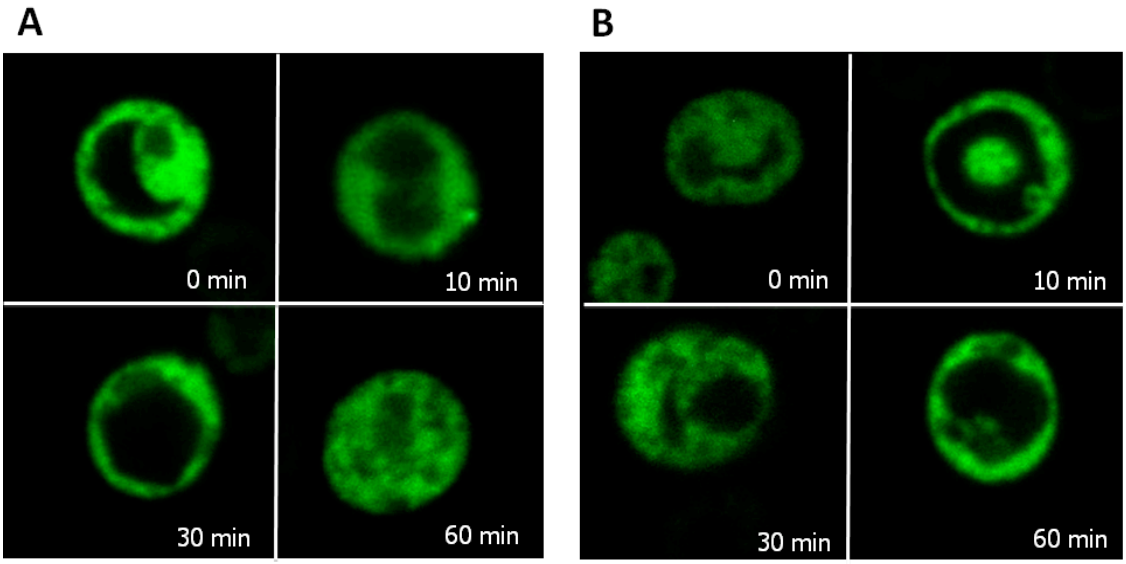


Figure 3.26 Trafficking of arrestin protein in response to α factor ligand. GFP tagged Arrestin 3 (A) and Arrestin 6 (B) were transformed into arrestin null EN60 yeast strain. The trafficking of arrestin was monitored in the absence (at 0 min) and presence (at 10 min, 30 min and 60 min time points) of α factor ligand under confocal microscopy.

Chapter 4

4. Discussion

4.1 MAPK dependent signaling and mating events are sequential yet distinct steps in yeast mating

4.1.1 CAM of Ste2p and Ste3p do not show constitutive mating activity

The TM6 domain of Ste2p has been reported to play an important role in the activation of receptor upon ligand stimulation (Konopka *et al.*, 1996; Dube and Konopka, 1998). Within the TM6 domain, the P258L mutation is proposed to stimulate CA activity through release of the P258 induced kink in TM6 (Figure 4.1A and B), where the straight helix stabilizes a conformation in the cytosolic domain similar to that achieved by ligand binding to WT and facilitates the constitutive activity of Ste2p receptor (Figure 3.5A, B and C). Kink predictions, using TM Kink prediction software (tmkinkpredictor.mbi.ucla.edu) revealed putative kinks at eight positions each, including two in the TM6 for the WT Ste2p and S254L mutant (Table 4.1). The P258L variation alone or in combination with S254L was predicted to relieve the kinks at two positions in TM6 (Table 4.1). The increase in constitutive activity of the Ste2p receptor after combining the S259L and P258L mutations compared to WT further substantiates the crucial role of intra-molecular contacts in the activation of receptor (Figure 3.5A, B and C). Similarly, another polar residue S254L in combination with the P258L and P258L/S259L mutation gave very high constitutive activities compared to WT (Figure 3.5A, B and C). On the contrary, both single mutants (S254L and S259L) failed to exhibit any changes in their constitutive activity compared to the WT in JKY78, JKY79 and supersensitive *ΔSST2* JKY127-36-1 strains (Figure 3.5A, B and C) (Dube and Konopka, 1998).

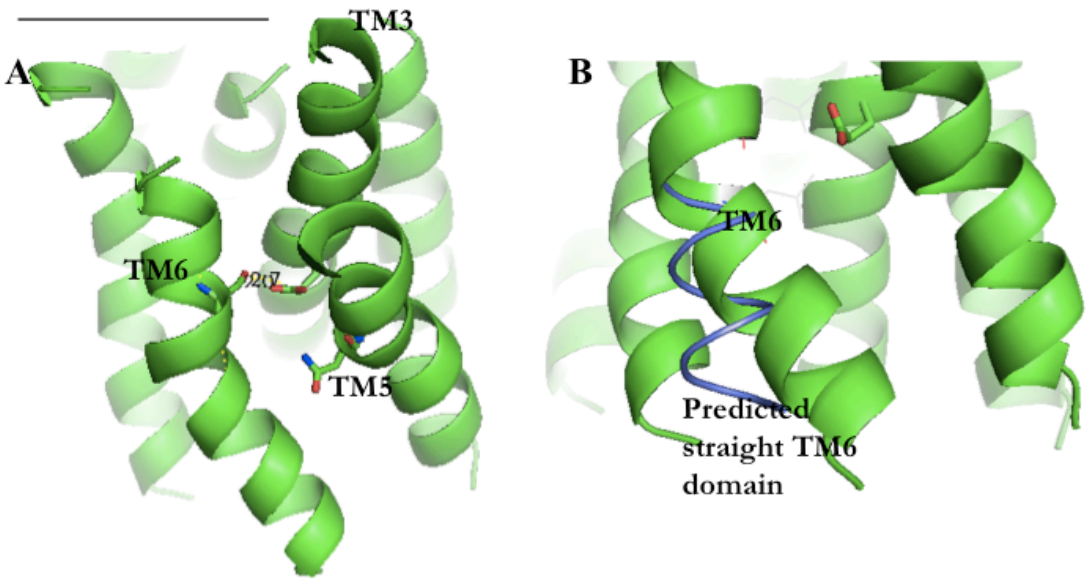


Figure 4.1 Proline 258 kink in TM6 domain of Ste2p. A: The Pro 258 residue in the middle of the Ste2p receptor produces a kink in the domain. B: Prediction of straightening of the TM6 domain after proline mutation. The Ste2p model was made using the Pymol software and model coordinates are based on those reported previously (Eilers *et al.*, 2005).

Table 4.1 Kink prediction by position in WT and mutants using tmkinkpredictor.mbi.ucla.edu

	WT	**S254L**	**P258L**	**S254L/P258L**	**P258L/S259L**	**S254L/P258L/S259L**
Predicted kinks by position	56 57 149 175 221 254 259 288	56 57 149 175 221 254 259 288	56 57 149 175 221 288	56 57 149 175 221 288	56 57 149 175 221 263 288	56 57 149 175 221 263 288

For the Ste3p receptor, the P222L variation in TM6 or L194Q mutation in the third cytoplasmic loop (L194Q) was reported to stimulate partial constitutive activity of the receptor. The constitutive activity was greatly enhanced in the *SST2* deletion strain for L194Q mutant, particularly (Boone *et al.*, 1993; Stefan *et al.*, 1998). These results were not reproducible in my

study; the reason might be the use of different yeast strains and differences in the experimental conditions of different assays. However, combining both mutations of the TM6 and the third ICL increased the spontaneous activation of MAPK-signaling that was further increased in the presence of a-factor ligand (Figure 3.11 and Figure 3.12). Together the removal of the TM kink, which is proposed to straighten the helix and intra-molecular interactions between the newly replaced negatively charged glutamic acid with the available positive charged side chain in the third ICL, thereby induced a new conformation that leads to agonist-independent G protein coupling and signaling of Ste3p receptor.

Interestingly, neither in the literature nor in the investigations reported here, has a Ste2p/Ste3p receptor mutant been identified that elicits CA signaling events/G1 arrest as well as undergoes mating in the absence of the ligand (Figure 3.5, Figure 3.6). From these findings I conclude, that either the mutations that lead to constitutive signaling and G1arrest must preclude constitutive mating (i.e. functional redundancy), or alternatively, the ligands themselves must play an essential 'structural/conformational' role in the downstream mating associated events which cannot be mimicked through mutagenesis.

4.1.2 Ligand-induced mating activity is blocked in some constitutively active Ste2p mutants

Despite showing increased basal and ligand-dependent signaling, CAMs P258L and P258L/S259L inhibited mating activity even in the presence of α-factor ligand (Figure 3.7). It is interesting to consider the loss of ligand-induced mating ability of P258L containing mutants with the stabilization/increase of basal signaling activity. While the primary effect of the P258L mutation appears to be stabilization of the cytosolic domain in an orientation that stimulates basal MAPK signaling, this mutation also inhibits mating. Taken together, these findings suggest that i) the conformation of the cytosolic domain of the receptor is critical to mediating its alternate mating functionality, and ii) that the MAPK signaling conformation is different than that of the mating conformation.

In contrast to the Ste2p results, the Ste3p receptor mutations that were assessed in the present study did not reveal a phenotype with CA and poor mating, however, polar (equivalent to S259 in Ste2p) residues in the TM6 domain of Ste3p were not studied.

4.1.3 Engineering of a constitutively signaling and ligand inducible mating receptor

When the strong mating mutant S254L was combined with the CAM P258L, the effect was to at least partially rescue the very weak mating phenotype of the CA mutant (Figure 3.7). However, unexpectedly, combining S254L also stimulated (doubled to almost WT-like ligand stimulated levels) the relative constitutive activity induced by P258L, despite the fact that S254L on its own did not induce any significant change in CA activity previously (Dube and Konopka, 1998), or in the experiments reported here (Figure 3.5). The effect of S254L on constitutive activity (in the presence of P258L) is likely similar to the effect of an alternate large space filling variation at S254F, as well as a variation at the adjacent residue Q253L, which showed 2.4 and 4.6 fold increases in basal activity respectively on their own (Dube and Konopka, 1998). That strong basal signaling can co-exist with at least moderately strong ligand-induced mating, suggests a more complex mechanism than a non-redundant cytosolic domain conformation mediating alternate signaling.

Looking at the structural model of Ste2p (Figure 4.2A), using the coordinates from (Eilers *et al.*, 2005) shows that the S254F variation clearly introduces sufficient bulk to cause a steric clash with residues L146, I142 and E143 in TM3. The steric clash between TMDs likely leads to a significant shift in the orientation of TM6 and/or TM3 and like the P258L mutation stabilizes a cytosolic domain conformation similar to the WT ligand induced conformation. Thus, while the S254L mutation does not introduce sufficient bulk in itself to elicit a CA effect, the structural impact of the P258L mutation may shift the 254-side chain closer to TM3 such that the added bulk in the S254L variation is in this instance sufficient to mediate additional conformational impact on the cytosolic domain conformation like S254F. That the effect of the two residues appears to be additive suggests their effects may be mediated through different conformational effects, perhaps with P258L primarily modulating TM6 and the S254L mutation modulating the orientation of the neighboring TM3. Also, the S254 and P258 residues of TM6 are involved in a H-bond cluster with Glu 143 of TM3 and Asn 216 of TM5 respectively (Figure 4.2B) (Eilers *et al.*, 2005). Thus, modifications to the relative motions of TM6 and TM3 that normally lead to the breaking of already existing intra-molecular contacts, may contribute to the traits observed in the TM6 mutants described here.

The strong mating ability of the S254L mutant in the presence of ligand might occur through one of the following possible mechanisms. Possibly S254L on its own, notably

eliminates H-bond interactions, but also introduces sufficient bulk to clash with residues L146, I142 and E143 in TM3, eliciting a small but significant conformational change in the cytosolic domain stabilizing the mating conformation, without impeding the MAPK signaling conformation. One might speculate that the S254L mutation enables an inducible (more redundant) cytosolic conformation that can interact with alternate signaling partners in their presence. This is proposed to occur with other GPCR, where unique conformational states of the GPCR bind to the β-arrestin and induce an alternate signaling pathway. Finally, while the possibility of S254L having an impact on the extracellular surface seems unlikely, it also cannot be strictly eliminated. Indeed, the impact of S254L on the extracellular domain would fit with reports in the literature, linking the N-terminal domain to mating functionalities (Shi *et al.*, 2007; Shi *et al.*, 2009a) as well as the possibility of there being two different α-factor ligand binding sites, a high-affinity site associated with MAPK signaling, and a lower affinity site associated with mating functionalities described here (Moore, 1983) . While also consistent with a previous report indicating a 5-fold increase for S254L α-factor affinity (Dube and Konopka, 1998), modulation of the extracellular domain by S254L remains to be demonstrated.

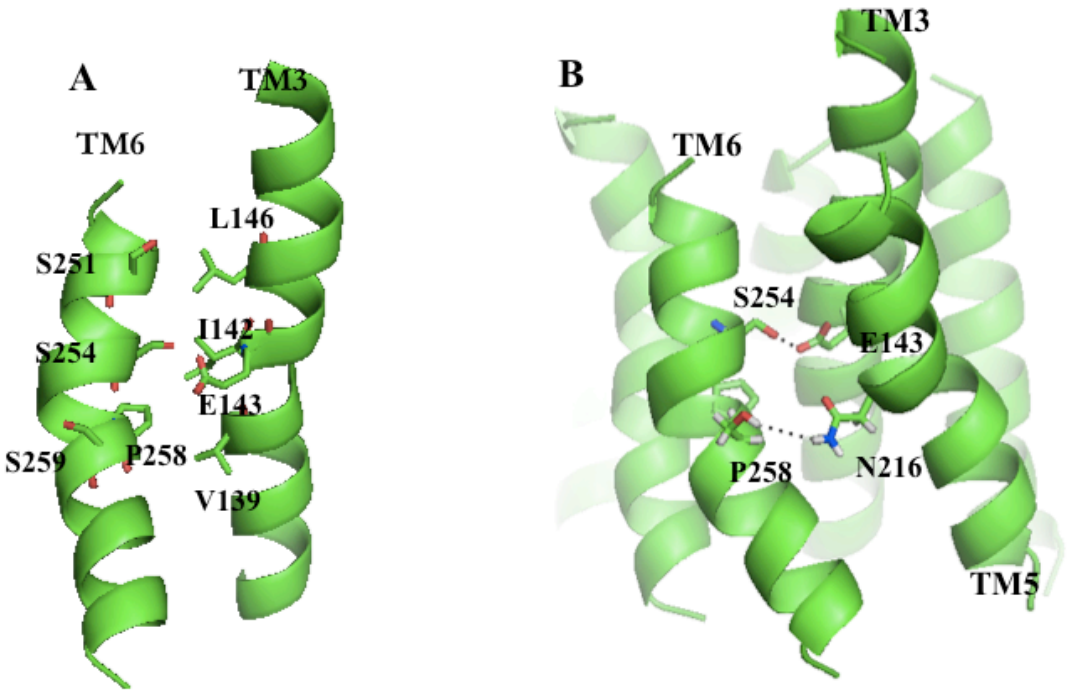


Figure 4.2 Interactions between TM6, TM3 and TM5 domains of Ste2 receptor. A) Residues facilitating the interactions between TM6 and TM3, including those targeted for mutagenesis in this report. B) H-bond cluster between TM6, TM3 and TM5 domains. The Ste2p model was made using the Pymol software and model coordinates are based on those reported previously (Eilers *et al.*, 2005).

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4.1.4 TM6 substitutions at residues 254 and 258 yield modified ligand-stimulated receptor internalization

Comparison of receptor localization and internalization within the yeast for WT and the mutant receptors highlights a number of interesting differences that possibly reflect in part the functional differences exhibited by different mutants. The total expression levels of mutant receptors were lower than the WT levels at 0 time points (Figure 3.19 bar plots). Interestingly, an enhanced basal signaling activity was found in some of the Ste2p mutant receptors (P258L, S254L/P258L, P258L/S259L and S254L/P258L/S259L) despite having lower expression than WT (Figure 3.5A, B and C). This indicates that the enhanced basal signaling is not an outcome of enhanced receptor expression.

In agreement with the total expression levels, the peripheral localization of mutant Ste2p receptors was overall consistently less than that of WT receptor (Figure 3.20). The lesser localization of mutant receptors on the cell membrane could be due to increased internalization and/or less efficient transport of receptors to the cell membrane. This would make sense in the context of CAMs that mimic the active conformation of the receptor, and showed enhanced

basal signaling, which should further lead to constitutive phosphorylation thereby inducing constitutive internalization and reduced cell surface localization of the receptor. Alteration in the surface localization of the receptor due to constitutive phosphorylation and subsequent internalization has been demonstrated for many CAM GPCRs including LH, α1 AR and β AR (Pei *et al.*, 1994; Min *et al.*, 1998; Mhaouty-Kodja *et al.*, 1999; Min and Ascoli, 2000). The possibility of increased internalization and intracellular recycling has been recently reported for some GPCR, i.e. AT1 receptor CAM (Miserey-Lenkei *et al.*, 2002), though recycling of Ste2p receptor has not to date been observed. Alternatively, increased internalization might result from unstable misfolded receptors. As for the P258L Ste2p mutant receptor, it has been speculated that the mutants are accumulated in the post ER compartment, thus preventing their trafficking towards the cell surface until their folding is complete (Stefan *et al.*, 1998). However this possibility is inconsistent with observed WT like ligand-induced signaling and high level of basal signaling observed for this mutant.

Indeed, in the present study, it was observed that the P258L containing mutant receptor relocalized to the periphery significantly faster after ligand-stimulation, than WT Ste2p (Figure 3.22). Whether this increased rate of relocation is linked to increased constitutive activity or enhanced release of the receptor from post-endocytic compartment remains to be determined. Furthermore, the constant localization of receptors on the cell membrane in the S254L mutant needs to be studied further so as to determine whether it is due to an increased rate of trafficking/turnover or decreased rate of internalization of receptor with this mutation. It also needs to be determined whether this sustained localization of the S254L receptor to the cell membrane following ligand stimulation actually contributes to the mating functionality bias of the S254L mutant.

4.2 Ago-allosteric modulators and mutational bias

4.2.1 Presence of low and high affinity binding site on WT Ste2p

In agreement with a previous report (Moore, 1983), the present study also reported the requirement of low (EC_{50} 1.6 nM) and high (EC_{50} 45 nM) α-factor ligand concentrations for CCA and MPF steps respectively (Figure 3.15). The requirement for different concentrations of ligand for sequential steps of yeast mating could be explained by the presence of low and

high affinity binding sites on the receptor as was proposed before (Moore, 1983). Possibly, the requirement for low and high concentrations of ligand for the different steps of mating might be representative of an ago-allosteric modulator concept, as explained for other GPCRs, where an agonist is capable of acting also as an allosteric modulator on its own promoting a distinct conformational state and signaling profile. However, ago-allosteric modulators are not classical allosteric modulators where they increase or decrease the action of an agonist by combining with a distinct binding site and binding at the same time as orthosteric ligand. Rather, an ago-allosteric modulator-binding site overlaps with the orthosteric-binding site. In the case of yeast mating receptor Ste2p, there is no evidence supporting the existence of agonist and ago-allosteric modulators so far. Instead, the α-factor ligand itself appears to work as an allosteric modulator in the later stages of mating, presenting a 'homotropic allosterism concept' for mating receptors. The different binding sites on the receptor might be overlapping each other and initial binding of available low (ligand) amount of α factor further exposes or creates the alternate low affinity-binding site (Figure 4.3). A high concentration of ligand in the later stages of mating is possible as the pheromone ligands are self-inducible and the mating projection tip on opposite partners facilitates the directional release of ligands (Strazdis and MacKay, 1983; Achstetter, 1989).

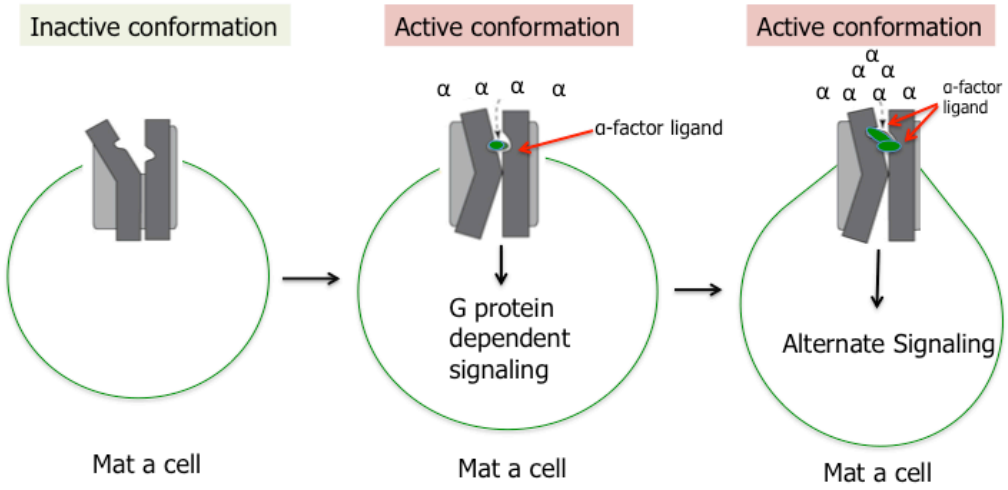

Figure 4.3 Time resolved allostery model for Ste2p. Initial binding of α-factor activates the Ste2p receptor and initiates G protein dependent signaling and further facilitates the exposure of a high affinity binding site and alternate signaling.

4.2.2 TM6 domain residue, Serine 254, shows a mutational bias in favor of mating vs. MAPK dependent signaling

In order to validate the possibility of alternate functionalities for Ste2p, the relative mutation derived functional bias method was applied. It revealed that the TM6 substitution S254L alone yields an α-factor-dependent mutational-bias ratio of 23 for mating (Table 3.3. This substitution was even able to counteract the mating inhibitory effect of the P258L CA variation, yielding a mating-bias ratio of over 8 relative to MAPK signaling for the double S254L/P258L mutant. Further to this, the lack of effect of S254L alone on MAPK signal transduction emphasizes that in WT, Leu at residue 254 forms part of a mechanism or ligand-binding site that selectively mediates one functionality (mating) and not the others tested herein. Interestingly, it appears that the slight increases in dose response sensitivity for the non-S259L containing mutants (Table 3.1) might account for the WT-like ligand induced signaling activity of these mutants (Figure 3.2), despite their relatively low level of membrane localization (Figure 3.20).

It is interesting also to note some of the subtle differences that are shown by the mutants on diploid zygote formation (Figure 3.7) compared to mating projection formation (Figure 3.17). Both functionalities are known to be downstream of classical G-protein mediated cell cycle arrest and are expected to respond similarly to mutations. However comparison of the data obtained here suggests otherwise. The P258L mutation shows a significant decrease in the proportion of diploid zygote (> 60 % decrease; Figure 3.7) and a significantly higher proportion of mating projection formation (2.1 times; extracted from dose response experiment (Figure 3.17; Table 3.1) than the WT receptor. It remains to be determined whether the differences are simply related to observational bias (a distinct possibility) or a role for some aspect of the mating partner cell (e.g. localization of a ligand concentration gradient) or represents mechanistic differences associated with roles for Ste2p early in mating projection formation and later in diploid zygote formation.

4.3 Linkage of yeast arrestins to the mating related functionality of the Ste2p receptor

For the first time I report here that arrestin may be a crucial component involved in Ste2p related mating functions in yeast. Only a very recent report has indicated that three different α-arrestin family members might facilitate the desensitization and internalization of Ste2p (Alvaro

et al., 2014). That study has demonstrated the presence of sustained MAPK signaling in cells lacking Ldb19 (Art1), Rod1 (Art4) and Rog3 (Art7) compared to control cells, as judged by the diameter of the halo of G1 arrested cells, phosphor *FUS3* and *FUS1*-GFP levels. The effect of sustained signaling was observed to be relatively modest when measured through phosphor *FUS3* and *FUS1* levels. However, in contrast to Rod1 and Rog3, the overexpressed Ldb19 didn't promote adaptation, as cells didn't recover from pheromone induced G1 arrest. It is quite possible that Ldb19 acts via a different mechanism or may be involved in more functions than Rod1/Rog3.

What could be the mechanism of arrestins in desensitization and internalization of Ste2p? It has been observed that Rsp5 (ubiquitin ligase) binds with the plasma membrane with its N-teminal lipid binding C2 domain (Cho and Stahelin, 2006) and to the PPxy motif target in the substrate via its three-tandem WW domain (Sullivan *et al.*, 2007; Lin *et al.*, 2008), in the ubiquitination step of membrane receptors. However, the absence of PPxY motif in Ste2p receptor made it difficult to understand how Rsp5 might bind to the Ste2p for ubiquitination to facilitate receptor internalization. Only recently, it has been demonstrated that arrestins act as negative regulators of the ubiquitination pathway as arrestins serve as an intermediary to recruit Rsp5 ubiquitin ligase to the Ste2p receptor (Alvaro *et al.*, 2014). Essentially, Ldb19 and Rog1 recruit Rsp5 to Ste2p via PPxy motif in its C-terminal region while Rog3 does it by the presence of an arrestin fold domain in the N-terminus.

Here a systematic evaluation of arrestin knockout lines has been described that demonstrates the existence of WT-like levels of MAPK signaling in arrestin knockout strains, similar to that observed in a recent report (Alvaro *et al.*, 2014). This is with the exception of a very weak down regulation of MAPK signaling activities in Art3 and Art6 knockouts (Figure 3.23A). In complementation experiments some recovery in the signaling was observed especially with Art6, which is possibly related to general cellular fitness, based on the lack of observed effect upon knockdown (Figure 3.23B). However, the lack of effect of overexpressing Art 4 and Art 7 on pheromone signaling is in contrast with the findings of the recent study (Alvaro et al, 2014), where significant (albeit weak) recovery of cells from pheromone signaling was observed for these. However, the effect of overexpressed Art1, Art4 and Art7 was measured only through the pheromone halo assay in this same previous study (Alvaro *et al.*, 2014).

Alternatively, arrestins seem to have a significantly stronger effect on mating that was linked primarily to Art1p (Ldb19) as indicated by Figure 3.24. This is further supported by the results of experiments on receptor localization that demonstrated a very distinctive association of the Art1p-GFP with the mating projection at 2 hours post-ligand stimulation (Figure 3.24). These results are consistent with a previous report that demonstrated higher densities of Ste2p-GFP localizing to the mating projection, subsequent to cell cycle arrest (Jackson *et al.*, 1991). Together these results support a model in which Art1p selectively plays a role in mating events subsequent to the classical Gpa1p mediated cell cycle arrest, and apart from its internalization of receptor and desensitization of G protein signaling functions. Whether, Art1p is involved in an alternate signal transduction pathway for mating events, remains to be determined. Based on mammalian examples, it is quite possible that Art1p acts as an adaptor for the alternate signaling pathway related to the yeast mating events.

4.4 Overall conclusion:

In conclusion, a systematic mutational-bias study probing the G-protein mediated MAPK signaling function of Ste2p versus the receptor role in downstream yeast mating events is reported. First, in functional assays, a mutation specific selective response was observed, where all the Ste2p mutants showed normal ligand dependent MAPK signaling and differences were in the level of constitutive MAPK signaling as well as in ligand dependent mating activities only (Figure 4.4). In particular, the S254L mutant can selectively induce ligand-dependent mating activity while having no effect on the CA in MAPK signaling of the receptor, whereas the P258L mutant had some effect (relatively weak) on constitutive activity in MAPK signaling with poor ligand-dependent mating phenotype. Interestingly, incorporation of S254L with the P258L mutant had both constitutive signaling and a good mating phenotype. While two other S259L containing mutants (P258L/S259L and S254L/P258L/S259L), had a strong effect on CA with negligible mating activity (Figure 4.4). Further, comparison of relative dose responses was made to account for the observational and systematic bias between functional assays. These relative dose response curves highlighted the mutation specific selective responses to pheromone stimulation. In accordance with the functional assays, strong bias toward mating was noted for the S254L containing mutants. These findings support a model of distinct α-factor-dependent MAPK signaling and mating biased functionalities for mutant pheromone receptors. In the end, demonstration of a possible role for arrestin in mediating the

alternate mating functionalities was discussed. As described, one of the α arrestins, Art1p, is involved in the later stages of mating events downstream of the MAPK signaling. These studies extend the recently identified role of the Art1p (LDB19), in the internalization of yeast Ste2p receptor to the putative alternate signaling pathway of receptor.

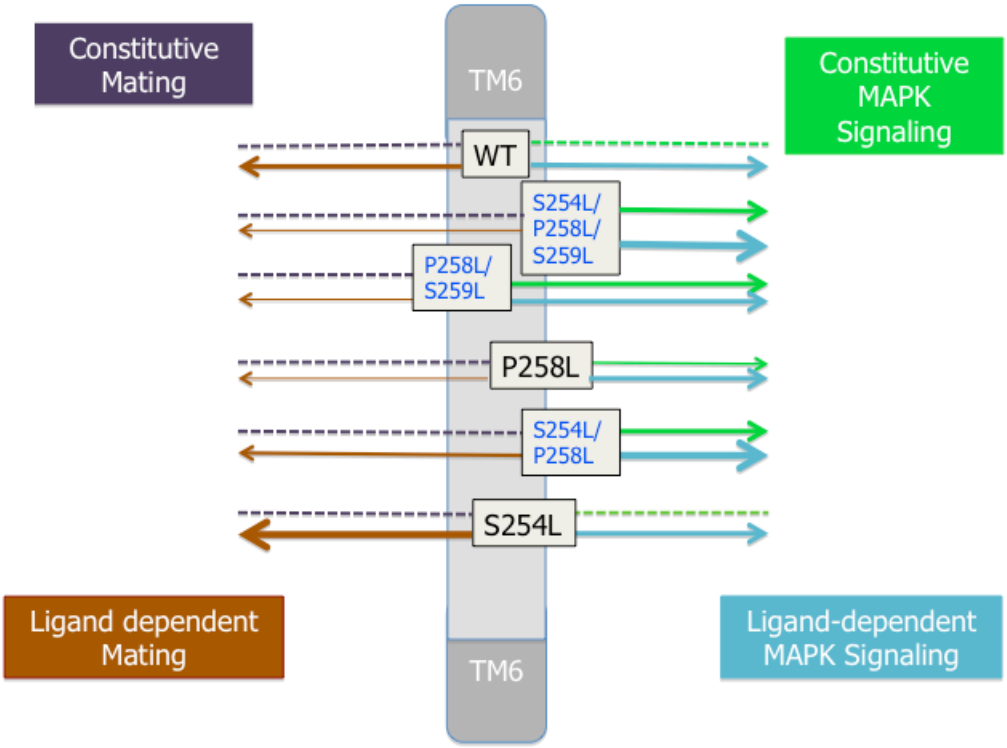

Figure 4.4 Summary of differential effects of TM6 Ste2p mutations on MAPK signaling and mating activities. The thickness of the arrows represents the degree of activation (dashed line, no activation; thin arrow, decrease in activity compared with WT; medium arrow, WT-like activation; thick arrow, increase in activity.

4.5 Proposed model:

Based on a systematic mutational study of yeast-mating receptor Ste2p described here in, a ligand-dependent alternate signaling model for mating functionalities of Ste2p receptor is proposed (Figure 4.5). Wherein, low concentration of ligand binds to the proposed high-affinity binding site on the receptor and stabilizes a 'conformation A' that is linked to G protein dependent MAPK signaling and results in cell cycle arrest. This initial binding of α-factor

ligand further exposes or create an alternate low affinity-binding site on the receptor, where binding of ligand, when present at high concentrations, promotes 'conformation B' of Ste2p receptor. This newly acquired conformation is able to bind to the α-arrestins (Ldb19, Rod1 and Rog3), which initiates the desensitization and internalization of receptor. Binding of Art1 (Ldb19) might then also act as an adaptor molecule for the components of alternate signaling for mediation of the mating functionality of Ste2p receptor.

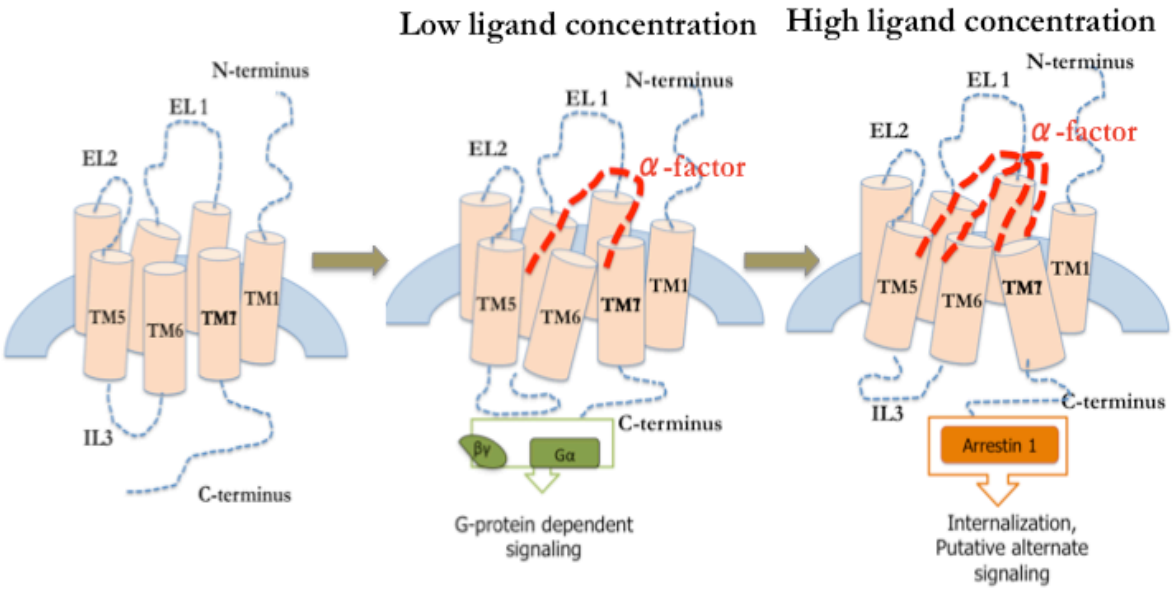

Figure 4.5 Proposed model of alternate signaling for mating events of Ste2p. Low ligand concentration activates the conformation A of Ste2p receptor and G protein dependent signaling and gives the cell cycle arrest of the receptor. While in high ligand concentration, receptor acquires another conformation that facilitates the binding of Art1 and further internalization, dampening of G protein signaling and also the alternate/biased signaling for mating projection formation or later mating events.

Another plausible model for Ste2p mating functionality involves the presence of high affinity binding sites on protomers, with a lower affinity binding site(s) formed at the interface of a dimeric ligand-stimulated complex (**Figure 4.6**). In the presence of lower ligand concentrations, high affinity binding sites present on the protomers are saturated thereby

activating the G protein dependent MAPK signaling and thus causing cell cycle arrest. Ligand binding to the protomer either stimulates dimer formation or affects the dimer interface of pre-dimerized receptors, exposing or creating a lower affinity binding site(s). Binding of ligand at the low affinity site of the stimulated dimeric-Ste2p induces the binding of Art1, internalization of the receptor and thereby facilitates the alternate signaling pathway for Ste2p receptor.

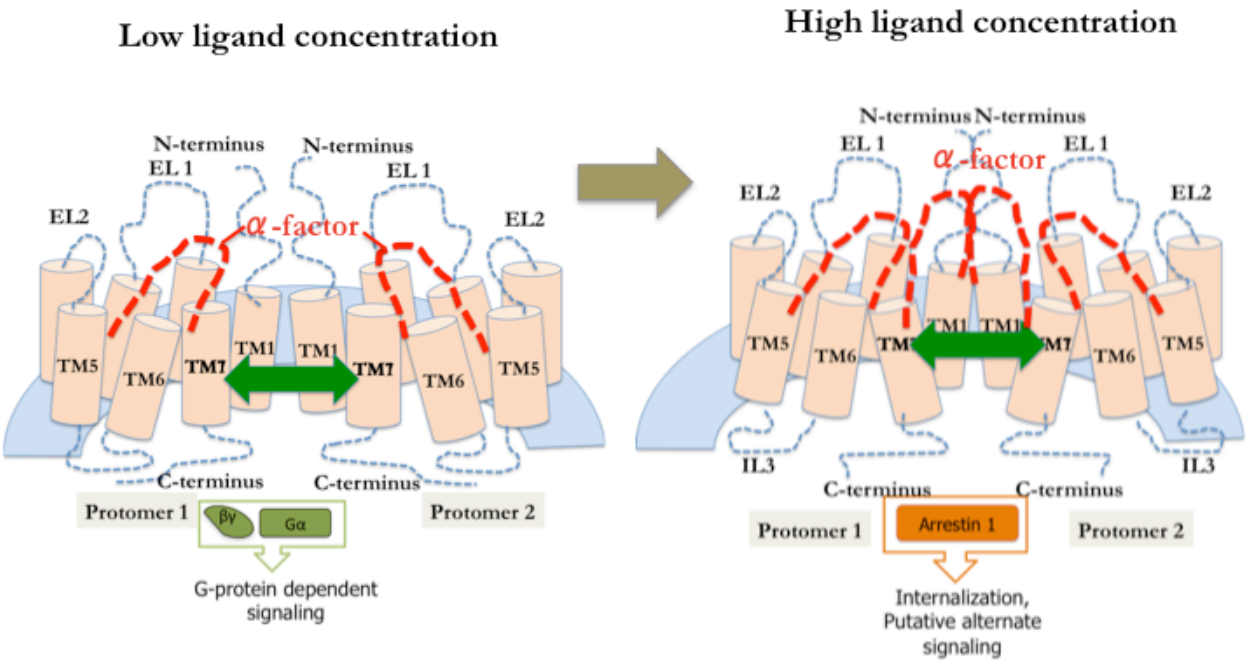

Figure 4.6 Proposed model of alternate signaling for mating events of Ste2p. Initially, in low ligand concentration, ligand binds to the protomer 1 of dimer-receptor unit. Receptor-ligand interaction activates the G-protein dependent signaling. It also brings the conformational change in another receptor of dimeric unit and exposes its binding site. Binding of ligand on both subunits facilitates the binding of Art1 and further internalization, dampening of G protein signaling and also the alternate/biased signaling for mating projection formation or later mating events.

Finally, the presence of two different pools of Ste2p receptor isomers that carry low or high affinity binding sites respectively due to alternative splicing and different posttranslational modifications of Ste2p receptor cannot be strictly eliminated. These different pools of Ste2p

receptor would regulate the G protein-dependent and Art1-dependent signaling of receptor in low and high concentration of ligand respectively.

4.6 Significance of work:

Yeast provides an exemplary model system to further improve our knowledge about human GPCRs and their complex signaling mechanism. Many important components and mechanisms of GPCRs and their signaling were first identified in yeast, and later discovered in humans. The present work is a first step toward justifying the continued use of yeast as a model system for mammalian GPCR screening/research related to alternate function/biased signaling mechanisms**.**

Moreover, the present work will be useful to understand mechanisms in fungal mating that might have potential applications in preventing or reducing fungal infections. In this context, many orthologs of Ste2p and Ste3p pheromone receptors have been identified in the ascomycetes group of the fungi kingdom and many of these receptors have been demonstrated as functioning in response to mating and pheromone sensing (Table 4.2). For example, in *Candida albicans*, an opportunistic human fungal pathogen, Ste2p/Ste3p-like pheromone receptors and a sexual life cycle have been identified in its opaque phase (Lockhart *et al.*, 2002). In *Candida albicans*, pathogen host interaction and virulence of fungi are related to phenotypic switching of an opaque-white transition, where white colonies are hemispherical colonies with typical attributes of budding yeast, whereas opaque phase shows flat grey colonies related to virulence (Slutsky *et al.*, 1985). The presence of mating components only in the opaque phase of *Candida albicans* further establishes a link between mating and white-opaque switching. Thus a thorough understanding of the mating pathway of *Candida albicans* should ultimately lead to the design of new anti fungal agents against *Candida albicans*. Moreover, in some other human fungal pathogens, where no sexual life cycle has been identified, the identification of pheromone receptor is surprising and warrants future investigations to examine whether mating receptors have evolved new functions independent of mating or functions in a novel way of cryptic sexual life cycle of these fungi. The examples of asexual fungi with pheromone receptors include, opportunistic the human fungal pathogen *Candida glabrata* and *Aspergillus fumigatus* (Srikantha *et al.*, 2003; Butler, 2007; Paoletti *et al.*, 2007).

Table 4.2 Fungal pheromone receptor homologs {adapted from (Xue *et al.*, 2008)}.

Species/references	Ste2 like pheromone receptor	Ste3 like pheromone receptor
Saccharomyces cerevisiae (Burkholder and Hartwell, 1985; Hagen *et al.*, 1986)	Ste2	Ste3
Schizosaccharomyces pombe (Kitamura and Shimoda, 1991; Tanaka *et al.*, 1993)	Mam 2	Map3
Candida albicans (Moore *et al.*, 2008)	Ste2	Ste3
Candida glabrata (Muller *et al.*, 2008b)	Ste2	Ste3
Aspergillus nidulans (Seo *et al.*, 2004)	GprA	GprB
Neurospora crassa (Kim and Borkovich, 2004)	Pre2	Pre1
Magnaporthe grisea (Kulkarni *et al.*, 2005)	Ste2	Ste3
Sordaria macrospora (Poggeler and Kuck, 2001; Mayrhofer *et al.*, 2006)	Pre2	Pre1
Penicillium chrysogenum (Hoff *et al.*, 2008)	Pcpre2	Pcpre1

4.7 Future work:

This final part of thesis outlines a few suggestions to elucidate the mating pathway of Ste2p receptor.

4.7.1 Identification of different conformational states of the mating biased mutants, S254L and S254L/P258L:

It will be interesting to identify the different conformational states of the mating biased mutants through fluorescence lifetime spectroscopy and ^{19}F-NMR spectroscopy. Site-specific incorporation of the environment sensitive fluorophore, tetramethylrhodamine (TMR) or

fluorine 19 labels will give specific insights into the confirmational equilibrium attached to the receptor.

4.7.2 Characterization of low and high affinity binding site on WT Ste2p and mutant receptors:

The presence of low and high affinity binding sites on Ste2p receptors could be assessed by *in vitro* SPR as have been done previously (Shi *et al.*, 2007). The His-tag of the recombinant purified receptor from yeast cells to bind to the Ni-NTA affinity Biacore chip and the binding of α-factor pheromone (Mw ~ 1kDa) detected over a pM to mM range of concentrations might be used to look for the multiple inflection points in kinetic plots as evidence of the low and high affinity binding sites and look for changes in theses parameters comparing associated signaling/mating mutants and WT.

4.7.3 Co-localization of Ste2p mutants and Art1 in response to pheromones:

Co-localization of Ste2p mating mutants (S254L/P258L) and Art1p will be interesting to study at in order to determine the role of Art1p in mating functionalities. The sustained/loose interaction between receptor and arrestins generally determines the fate of the receptor in terms of recycling vs degradation of receptor. Moreover, sustained interaction of receptor and arrestin gives the platform for alternate/biased signaling pathways in endosomes of the cell. The kinetic evidence of interaction between Ste2p and Art1 will give further insight into the involvement of arrestin in different steps of mating.

4.7.4 Identification of components of alternate signaling in WT Ste2p:

To identify the components of the alternate signaling pathway related to yeast mating functionalities will be a challenging task. One of the reasons might be due to the possibility of redundancy of the components of the MAPK pathway in alternate signaling as is observed to occur with other GPCRs (Wei *et al.*, 2003). Co-immuno-precipitation and pull down assay can be useful to identify the binding partner for Art1p following ligand stimulation. Further to decipher the arrestin mediated signaling, effect of down regulation of Art1p on putative signaling components will be preferred method.

CHAPTER 5

5. REFERENCES

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6. Appendix A

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6.1 Comparison of dose response curves of WT and Ste2p mutants in *FUS1-lacZ* β galactosidase induction, cell cycle arrest and mating projection formation.

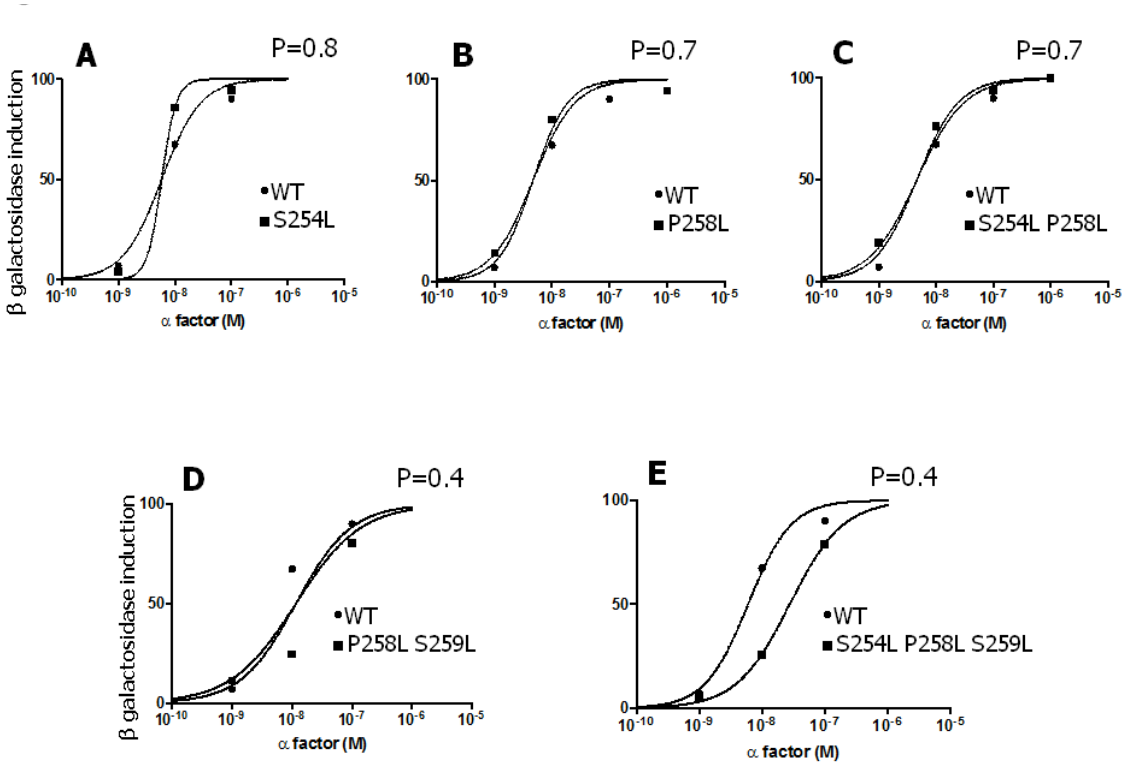

Figure 6.1 Comparison of log EC_{50} values of mutants with the WT Ste2p in *a FUS1-lacZ* β galactosidase assay. Log EC_{50} value of WT and mutant in *FUS1-lacZ* β galactosidase assay was compared using graphpad software. P>0.05 was was considered statistically significant.

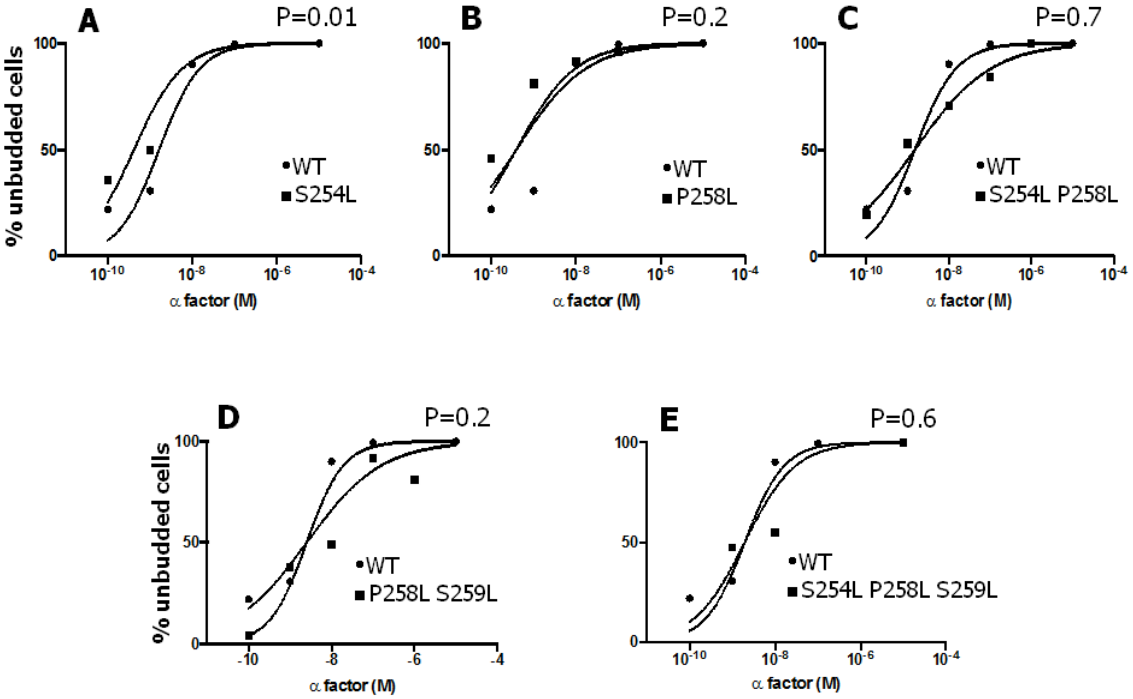

Figure 6.2 Comparison of log EC_{50} values of mutants with the WT Ste2p in cell cycle arrest assay. Log EC_{50} value of WT and mutant in cell cycle arrest was compared using graph pad software. P>0.05 was considered statistically significant.

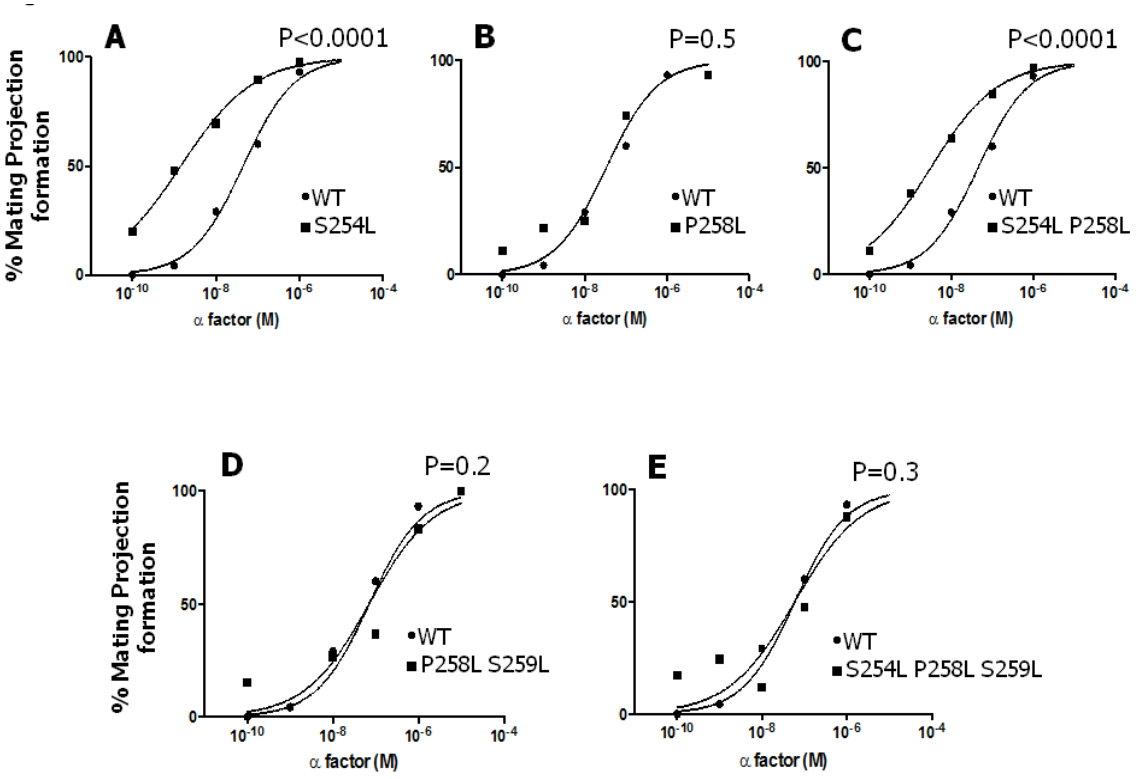

Figure 6.3 Comparison of log EC_{50} values of mutants with the WT Ste2p in mating projection formation assay. Log EC_{50} value of WT and mutant in mating projection formation was compared using graph pad software. P>0.05 was considered statistically significant.