NOVEL STRATEGIES TO IMPROVE RECOMBINANT MEMBRANE PROTEIN PRODUCTION IN YEAST

Marvin Valentine Dilworth

Doctor of Philosophy

ASTON UNIVERSITY

May, 2014

ASTON UNIVERSITY

NOVEL STRATEGIES TO IMPROVE RECOMBINANT MEMBRANE PROTEIN PRODUCTION IN YEAST

Marvin Valentine Dilworth

Ph.D

2014

Thesis summary

Approximately 60% of pharmaceuticals target membrane proteins; 30% of the human genome codes for membrane proteins yet they represent less than 1% of known unique crystal structures deposited in the Protein Data Bank (PDB), with 50% of structures derived from recombinant membrane proteins having been synthesized in yeasts. G protein-coupled receptors (GPCRs) are an important class of membrane proteins that are not naturally abundant in their native membranes. Unfortunately their recombinant synthesis often suffers from low yields; moreover, function may be lost during extraction and purification from cell membranes, impeding research aimed at structural and functional determination. We therefore devised two novel strategies to improve functional yields of recombinant membrane proteins in the yeast *Saccharomyces cerevisiae*. We used human adenosine A_{2A} receptor ($hA_{2A}R$) as a model GPCR since it is functionally and structurally well characterised.

In the first strategy, we investigated whether it is possible to provide yeast cells with a selective advantage (SA) in producing the fusion protein $hA_{2A}R$-Ura3p when grown in medium lacking uracil; Ura3p is a decarboxylase that catalyzes the sixth enzymatic step in the *de novo* biosynthesis of pyrimidines, generating uridine monophosphate. The first transformant (H1) selected using the SA strategy gave high total yields of $hA_{2A}R$-Ura3p, but low functional yields as determined by radio-ligand binding, leading to the discovery that the majority of the $hA_{2A}R$-Ura3p had been internalized to the vacuole. The yeast deletion strain *spt3Δ* is thought to have slower translation rates and improved folding capabilities compared to wild-type cells and was therefore utilised for the SA strategy to generate a second transformant, SU1, which gave higher functional yields than H1. Subsequently $hA_{2A}R$-Ura3p from H1 was solubilised with n-dodecyl-β-D-maltoside and cholesteryl hemisuccinate, which yielded functional $hA_{2A}R$-Ura3p at the highest yield of all approaches used.

The second strategy involved using knowledge of translational processes to improve recombinant protein synthesis to increase functional yield. Modification of existing expression vectors with an internal ribosome entry site (IRES) inserted into the 5´ untranslated region (UTR) of the gene encoding $hA_{2A}R$ was employed to circumvent regulatory controls on recombinant synthesis in the yeast host cell. The mechanisms involved were investigated through the use of yeast deletion strains and drugs that cause translation inhibition, which is known to improve protein folding and yield. The data highlight the potential to use deletion strains to increase IRES-mediated expression of recombinant $hA_{2A}R$.

Overall, the data presented in this thesis provide mechanistic insights into two novel strategies that can increase functional membrane protein yields in the eukaryotic microbe, *S. cerevisiae*.

Key words: Yeast; *S. cerevisiae*; human adenosine A_{2A} receptor; selective advantage; IRES; Ura3p

I would like to dedicate this thesis to my amazing parents Cheryl and Valentine; you will never know how much I appreciate your support throughout my education, and for believing in me and encouraging me to push my limits. Also to my sister Bianca and nephew Marcellus for their encouragement love and support, and finally to my wonderful fiancée Dee, thanks for putting up with me for these last 4 years.

Acknowledgements

Firstly, I would like to give special thanks to my supervisor Professor Roslyn Bill for allowing me the opportunity to pursue a Ph.D and for all her time, guidance and support throughout the course of it, and the writing up of this thesis. My thanks also go to my associate supervisor Dr. Stephane Gross for his co-supervision, and his stalwart ability to raise questions and new ideas. Thanks also go to Dr. Sarah Routledge, Dr. Zharain Bawa, Dr. Stephanie Cartwright, Dr. Matthew Conner, Dr. James Barwell and Dr. Debasmita Sarkar for helping and teaching me many laboratory techniques and for many fruitful discussions and advice and also Charlie Bland for her help with confocal microscopy. My thanks also go to my lab colleagues, Michelle Clare, Anjana Patel, Lina Mikaliunaite, Jenn Lines, Sam Hebaishi, Andrew Poole, Bita Tajarodkhah and Gabriel Kuteyi for making this Ph.D an enjoyable experience. Finally I also would like to thank the BBSRC and Glycoform Ltd for funding this project.

Contents

Abbreviations

3´UTR	3´ Untranslated region
5´UTR	5´ Untranslated region
$A_{2A}R$	Adenosine A_{2A} receptor gene
ADP	Adenosine diphosphate
APS	Ammoniun persulphate
ATP	Adenosine triphosphate
AUG	Translational start codon
BCA	Bicinchoninic acid
B_{max}	Maximum binding capacity
CHS	Cholesteryl hemi-succinate
CSM	Complete synthetic medium
BSA	Bovine serum albumin
DDM	n-dodecyl-β-d-maltopyranoside
DNA	Deoxyribonucleic acid
DO	Drop out solution
E. coli	*Escherichia coli*
EC_{50}	Half the effective concentration
ECL	Extracellular loop
eEF	Eukaryotic elongation factor
eIF	Eukaryotic initiation factor
ER	Endoplasmic reticulum
GDP	Guanosine diphosphate
GEF	Guanine nucleotide exchange factor
GFP	Green fluorescent protein
GPCR	Guanine nucleotide-binding protein coupled receptor
GTP	Guanosine triphosphate
^3H	Tritium
$hA_{2A}R$	Human adenosine A_{2A} receptor protein
HIS3	Imidazoleglycerol- phosphate dehydrogenase gene
ICL	Intracellular loop
IRES	Internal ribosome entry sites
ITAFs	IRES trans-acting factors

K_d	Concentration of ligand required to bind half of the receptors
K_i	Concentration of competing ligand required to bind half of the receptors
LB	Luria-Bertani
min	minute
mRNA	Messenger RNA
OD	Optical density
ODCase	Orotidine-5´-phosphate decarboxylase
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline Tween 20
PDB	Protein Data Bank
pH	Negative logarithm of the hydrogen ion concentration
PIC	43S pre-initiation complex
pK_d	- log10 of K_d
pK_i	- log10 of K_i
P. pastoris	*Pichia pastoris*
s	second
S. cerevisiae	*Saccharomyces cerevisiae*
SA	Selective advantage
SAGA	Spt-Ada-Gcn5- acetyltransferase complex
SDS	Sodium dodecyl sulphate
SEM	Standard error of the mean
T	Time
TAE	Tris acetate EDTA
TEMED	N,N,N',N'-tetramethyl- ethane-1,2-diamine
TEV	Tobacco etch virus
TM	Transmembrane domain
TOR	Target of rapamycin
Tris	Tris(hydroxymethyl)aminoethane
TSD	Translational slowdown
Tween 20	Polyoxyethylene sorbitan monolaurate
uORF	Upstream ORF
UPR	Unfolded protein response

URA3	Orotidine-5´-phosphate decarboxylase (ODCase) gene
Ura3p	ODCase protein
v/v	Volume/volume
w/v	Weight/volume
YPD	Yeast peptone dextrose
YNB	Yeast nitrogen base
ZM241385	4-(2-(7-amino-2-(furan-2-yl)-[1,2,4]triazolo[1,5-a][1,3,5]triazin-5-ylamino)ethyl)phenol

Units

°C	Degree Celsius
g	Gram
g	Gravitational force
h	Hour
kDa	Kilo Dalton
kg	Kilogram
L	Litre
mg	Milligram
min	Minute
mL	Millilitre
mmol	Millimole
mol	Mole
nM	Nanomolar
nmol	Nanomole
pmol	Picomole
psi	Pounds per square inch
RCF	Reactive centrifugal force
rpm	Revolutions per minute
s	Second
U	Units
µg	Microgram
µM	Micromolar
µL	Microlitre

List of Figures

Chapter 1

Chapter 3

Chapter 4

List of Tables

Chapter 1

Chapter 2

Chapter 3

Chapter 1: Introduction

To structurally or functionally characterise a protein, the isolation of milligrammes to grammes of stable, active sample are required. For some of proteins this process is trivial; for membrane proteins, the majority are not naturally abundant in their native membranes (Bill et al., 2011). Consequently recombinant protein synthesis is required to generate appropriate samples for further study. In this thesis two novel strategies are described to increase the functional yield of a recombinant membrane protein in *S. cerevisiae*; human adenosine A_{2A} receptor ($hA_{2A}R$) was used as a model GPCR since it is functionally and structurally well characterised (Jaakola et al., 2008, Xu et al., 2011). The first strategy provides the host system with a selective advantage in expressing a recombinant protein in its cell membranes, the second explores the possibility of manipulating translational processes through the use of internal ribosome entry sequences (IRESes) and mutant strains of *S. cerevisiae* to increase and improve functional yields of membrane proteins.

1.1. Proteins

Proteins are the engines of normal cellular function. They are enzymes, which are responsible for catalysing reactions, antibodies, which bind antigens and stimulate the immune system, transport proteins, involved in transport of essential molecules in and out of cells, and regulatory proteins, such as cell receptors that allow the cell to communicate with its environment and neighbouring cells (Peeters et al., 2011). Overall, proteins are involved in practically every process that is carried out within an organism.

As proteins are involved in all processes that take place within the body, understanding their three-dimensional structures and functions is central to understanding the working of the cell in health and disease. For proteins associated with medical disorders, this knowledge should allow the design of drugs to be approached rationally. Established methods of drug discovery use high-throughput screening of thousands of compounds, and/or animal screening to select for inhibitory agents (Entzeroth, 2003, Koppitz & Eis, 2006, Rogawski, 2006). The common downside to these methods is that the mechanism of action is not known. This can often result in unwanted side-effects; for example, the anti-epileptic drug Levetiracetam can cause hostility, anxiety, hallucination or diarrhoea (Lyseng-Williamson, 2011). In contrast, rational drug design would result in compounds that act in a more specific manner, reducing side effects and increasing efficiency. For

example research involving the HIV virus discovered it uses the cell receptor protein, CCR5, to enter T-cell lymphocytes in order to propagate, resulting in destruction of the infected cell and release of more virus particles. As a result, a multitude of drugs have been designed to interfere with HIV-CCR5 interaction, usually by binding to CCR5 to prevent HIV doing so (Garcia-Perez et al., 2011).

1.1.1. Membrane proteins

Membrane proteins are proteins that span the cell membrane, are partially embedded within it or are associated with its surface (Fig 1.1) and have been highlighted as potential drug targets as it is known that they regulate essential cellular processes including cell signalling and transport (McCudden et al., 2004, Böhme & Beck-Sickinger, 2009, Moraes et al., 2014). This is evident by the fact that a wide range of diseases arise through malfunction of a membrane protein (Wu, 2010); if its function is known, the mechanism through which a disorder manifests can be discovered (Bartfai et al., 2004).

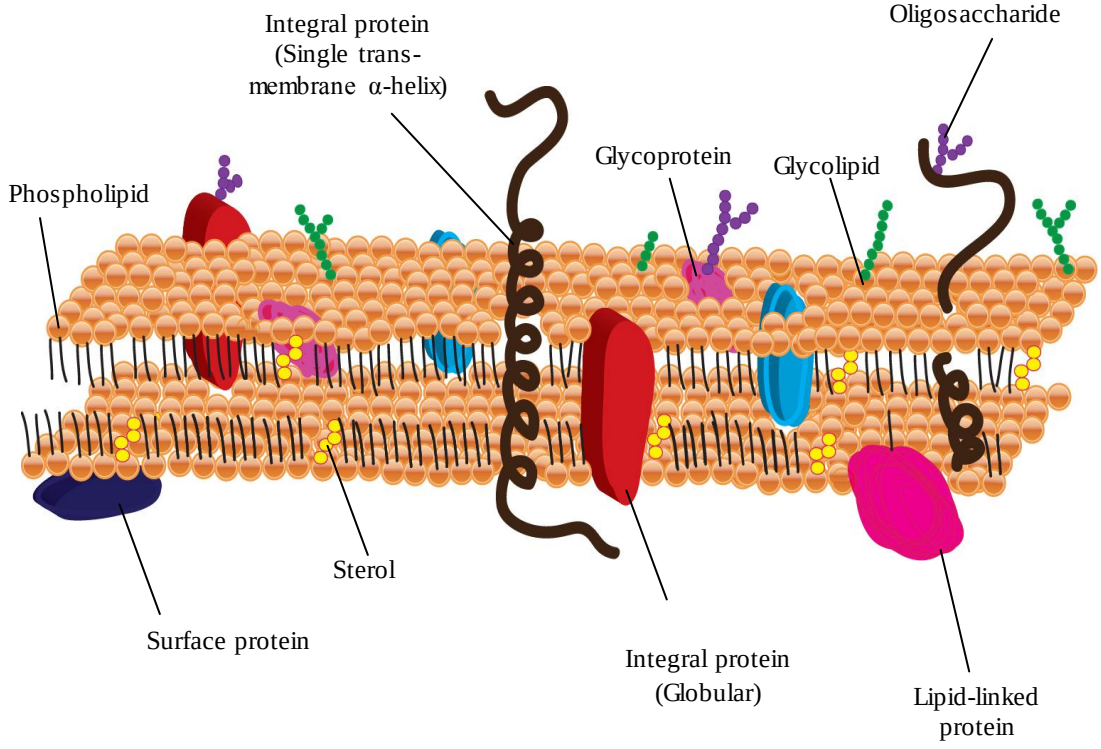


Fig 1.1 Illustration of membrane proteins in the cell membrane. *Proteins which reside and are present in a variety of different conformations throughout the lipid bilayer of the cell membrane are referred to as membrane proteins. The lipid bilayer is shown in orange, sterols in yellow, different types of membrane proteins in red, blue, pink, cyan and fuchsia, oligosaccharides in purple and glycolipids in green.*

An important group of membrane proteins are the integral membrane proteins, which receptors and transporters belong to, while other groups include membrane anchors, enzymes and structural proteins (McCudden et al., 2004, Böhme & Beck-Sickinger, 2009). The receptor and transporter groups are considered the most important by the pharmaceutical industry as the former is involved in cell signalling which regulates cellular pathways/processes, and the latter is responsible for transport of essential molecules in and toxins out (McCudden et al., 2004, Böhme & Beck-Sickinger, 2009, Bawa et al., 2011). It is therefore no surprise that membrane protein biology is an important field for study and an essential pre-requisite for drug design, reinforced by the fact that over 60% of drugs on the market target this essential class of proteins (Moraes et al., 2014).

Approximately 60% of pharmaceuticals target membrane proteins. However, while approximately 30% of the human genome codes for membrane proteins (Almén et al., 2009), only 1% of the total cellular protein population is membrane bound (Bernaudat et al., 2011). There are a few exceptions to this trend of low abundance, which include aquaporins, bovine and bacterial rhodopsins and light harvesting proteins (Bill et al., 2011). Yet these are an exception to the rule, and instead the vast majority of membrane proteins are not abundant in the cell membrane and are unstable when removed from it (Grisshammer, 2009). Notably, knowledge concerning how membrane proteins synthesis is regulated more generally has been lacking (Bill et al., 2011).

It is not surprising that naturally-abundant membrane proteins were amongst the first structures to have their structures solved via x-ray crystallography (Deisenhofer et al., 1985, Sugimoto et al., 1985). These exceptions aside, most membrane proteins must be synthesised recombinantly in bacteria, yeast, insect and mammalian host systems (Nettleship et al., 2010, Bawa et al., 2011), although generally for eukaryotic membrane proteins, bacterial cell lines are not preferred due to the lack of post translational modification attributed to eukaryotes, and the inherent problems that most eukaryotic membrane proteins produced in bacteria are non-functional (Newstead et al., 2007). Over the past quarter of a century there has been an exponential increase in the number of unique solved membrane protein structures deposited into the PDB, although the rate is less than that originally predicted in 2005 (Fig 1.2). This increasing trend is due to the

emergence of recombinant technology as the preferred tool for membrane protein production (Fig 1.3).

Fig 1.2 Graph illustrating the exponential trend of deposited solved unique membrane protein structures to the PDB from 1985-2013. *The bars in the graph are representative of the cumulative unique membrane protein structures (n), over time since the first membrane protein structure was solved in 1985. The red dotted lined showed the expected growth in terms of new structures, which was estimated in 2005. The actual growth as shown by the solid red line (best fit), indicates that membrane protein structures are not being solved as fast as expected.*

Since 2002 there has been a shift in the origin of membrane protein structures solved from natural sources to recombinant sources (Fig 1.3). The first structure solved in 1985 was bacterial rhodopsin (Deisenhofer et al., 1985) for which the researchers, Johann Deisenhofer, Robert Huber and Hartmut Michel, were awarded a Nobel Prize in Chemistry in 1988. For the next 12 years (1985-1997) only natural sources were used to obtain protein for the solving of structures; in 1998 the first structure from recombinant sources was achieved. Structural characterisation via crystallisation typically requires

milligrammes of purified, functionally active protein (Sarramegna, et al., 2003). By 2010, yeast was responsible for over 50% of eukaryotic membrane protein structures solved from eukaryotic recombinant sources (Fig 1.3).

Fig 1.3 Current progress in solving prokaryotic and eukaryotic membrane protein structures. *The graph shows a comparison of unique solved structures of membrane proteins derived from natural (black) and recombinant sources (orange) that have been deposited in the Protein Data Bank since the first deposit in 1985. The pie chart illustrates the distribution of recombinant host sources used to generate the recombinant membrane protein source material (reproduced from Bill et al., 2011).*

1.1.2. G protein-coupled receptors

Receptors are integral membrane proteins that regulate cellular process via cell signalling, the largest and most important family in mammalian genomes being the G protein-coupled

receptors (GPCRs)(Zhang et al., 2006), which make up 3% of the human genome (Gilchrist & Mazzoni, 2010) or 950 GPCRs (Takeda et al., 2002).

GPCRs in particular are known to regulate a diverse and complex array of essential physiological functions via cell signalling (Gilchrist & Mazzoni, 2010), mediated by several stimuli such as peptides, hormones, neurotransmitters, ions and even photons (Fredriksson et al., 2003). This is supported by research suggesting that abnormal or non-functioning GPCR pathways play a role in a wide array of diseases such as cardiovascular defects, blindness, allergies, depression, diabetes and some forms of cancer (Wu, 2010). This makes them excellent therapeutic targets, as knowledge of a protein's native structure can help us to understand its function, opening the avenue for new drug development, and increasing medical knowledge. Moreover, approximately 200 of the top selling pharmaceuticals target GPCRs (Table 1.1).

Table 1.1 Drugs that act by targeting GPCRs. *The table illustrates the breadth and scope of diseases that are treated by using GPCRs as drug targets, ranging from aliments of the respiratory, cardiovascular and neurological systems to cancer and HIV (Wu, 2010).*

GPCRs have the ability to be bound by ligands/agonists on the cell surface causing a signalling cascade within the cell. They are typically composed of an extracellular N-terminus, seven transmembrane domains, and an intracellular C-terminus that interacts with guanidine nucleotide binding proteins (G proteins) as shown on next page (Fig 1.4).

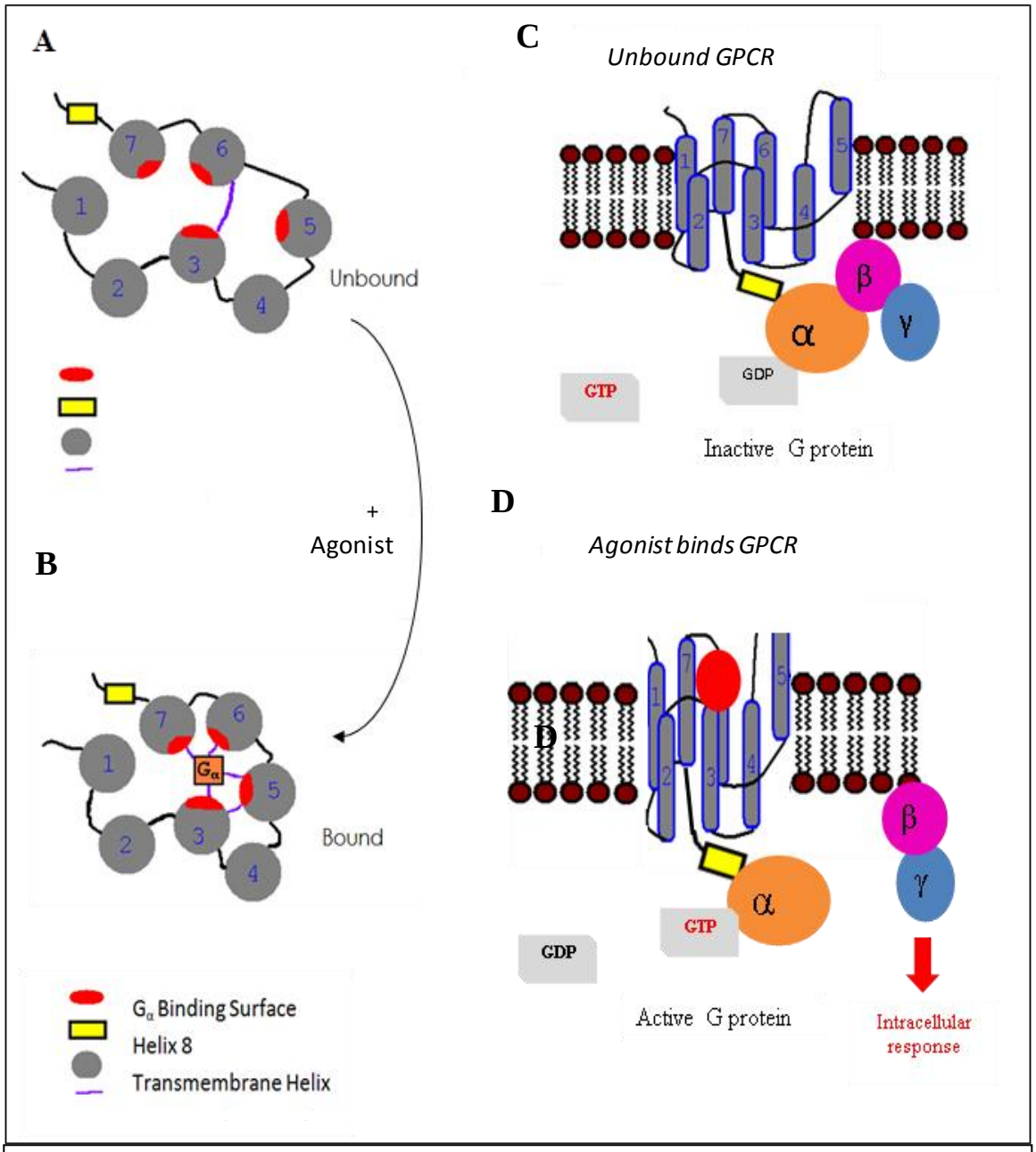


Fig 1.4 Schematic of a GPCR and the mechanism of G protein (G_a) binding. *Panels A and B illustrate the mechanism through which the seven transmembrane domains of a GPCR are bound at their respective binding surfaces, and the subsequent conformational change the receptor undergoes (refer to the inset box for a description of the coloured symbols). Panels C and D represent a side perspective depicting how the GPCR is integrated into the membrane showing the mechanism of G protein activation and intracellular response caused via agonist binding.*

The traditionally used classification for GPCRs divides them into 6 groups: Class A (rhodopsin-like); Class B (secretin-like receptor); Class C (metabotropic glutamate); Class D (Fungal mating pheromone receptors); Class E (Cyclic AMP receptors); and Class F (Frizzled/Taste2) (Attwood & Findlay, 1994, Kolakowski, 1994). Membership of a group is determined by >20% amino acid homology within the transmembrane domains (Attwood & Findlay, 1994, Kolakowski, 1994). This system was originally intended to cover vertebrates and invertebrates, and does not therefore include categorisation for mammalian GPCRs. Consequently, some classes do not exist in humans, which is true of class D and E (Fredriksson & Schiöth, 2005). Therefore another classification system, GRAFS, was developed (Table 1.2) which allows for an overall map of mammalian genomes (Fredriksson et al., 2003). The five main families in GRAFS are Glutamate, Rhodopsin, Adhesion, Frizzled and Secretin, with evidence suggesting they all share a common ancestor (Fredriksson et al., 2003).

Table 1.2 GRAFS classification of GPCRs. *The table details the Glutamate, Rhodopsin-like, Adhesion, Frizzled/Taste2 and Secretin-like members of the GRAFS family classification system of GPCRs* (Fredriksson & Schiöth, 2005).

The production of pharmaceutically-important integral membrane proteins is essential for further pharmacological research. For many years the only GPCR to have its crystal structure solved was bovine rhodopsin (Palczewski et al., 2000), due to it being readily available from bovine retina and being more thermostable than its counterparts. Consequently this was the only template for drug design and GPCR studies. Therefore many drugs were formulated during this era with limited knowledge concerning the intricacy of GPCR biology (Eisenstein, 2009). Fortunately recent breakthroughs in crystallography and expression strategies including protein engineering have allowed for the stabilisation and subsequent experimental determination of solved structures for the β_2 adrenergic receptor (Rasmussen et al., 2007), β_1 adrenergic receptor (Warne et al., 2008) and the adenosine A_{2A} receptor (Jaakola et al., 2008), which started a trend in the discovery of new unique membrane protein structures, the most recent being listed in Table 1.3.

Year	**GPCR structure**	Ref
2011	human histamine H1 receptor	Shimamura et al., 2011
2012	human M2 muscarinic acetylcholine receptor	Haga et al., 2012
2013	human smoothened receptor	Wang, Wu, et al., 2013
2013	human glucagon class B receptor	Siu et al., 2013
2013	human serotonin receptor	Wang, Jiang, et al., 2013
2013	human CCR5 chemokine receptor	Tan et al., 2013
2014	human $P2Y_{12}$ receptor	Zhang et al., 2014)
2014	human metabotropic glutamate receptor 1	Wu et al., 2014

Table 1.3 Most recently solved GPCR structures. *The table details the most recent successes for unique GPCR structures from 2011-2014.*

Despite these tremendous breakthroughs to date still less than 1% of GPCR structures have been determined (Sabbadin et al., 2014), and the ability to consistently and predictably produce milligrammes of stable functional GPCRs and membrane proteins in general remains a substantial challenge when compared to other categories of proteins that are of interest for study (Moraes et al., 2014).

1.1.3. Radio-ligand binding assays

Radio-ligand binding assays involve measuring the specific binding of a receptor to a known radio-labelled (labelled with a radioactive isotope) agonist or antagonist (McKinney & Raddatz, 2006, Hulme & Trevethick, 2010). Agonists are natural or synthetic compounds that bind and activate a receptor to cause a response, whereas antagonists bind

$$\text{Receptor} + \text{Ligand} \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \text{Receptor} \bullet \text{Ligand}$$

a receptor but do not cause a response; they can also prevent other agonists binding (Leach et al., 2010). The law of mass action provides the simplest explanation of receptor-ligand binding (shown below), and is the basis for radio-ligand binding assay calculations (Leach et al., 2010). In this equation, the ligand can be an agonist or an antagonist.

The ligand binds to the receptor forming a receptor – ligand complex; the rate at which this occurs is defined by the number of binding events per unit of time referred to as the association rate constant k_{on}. Since the process is reversible, the rate can also be defined by a dissociation rate constant k_{off} (dependent upon ligand-receptor affinity). Equilibrium between association and dissociation is reached when the rate of ligand-receptor complex formation equals the rate of ligand-receptor complex dissociation (Leach et al., 2010). During equilibrium, the ratio of the rate constants k_{on} and k_{off} can provide information regarding the potency of ligand-receptor interaction in the form of the equilibrium dissociation constant $K_{d;}$ is also the concentration of ligand that binds 50% of the receptors present. At equilibrium the ligand-receptor complex concentration is governed by the total receptor density [Receptor$_T$], the ligand concentration [Ligand] and the equilibrium dissociation constant of the ligand (Leach et al., 2010), which is referred to as the Hill-Langmuir binding isotherm equation:

Where [Receptor$_T$] = [Receptor] + [Receptor ● Ligand] and $K_a = k_{on} / k_{off}$.

$$[Receptor \bullet Ligand] = \frac{[\text{Receptor}_T] \times [\text{Ligand}]}{[\text{Ligand}] + K_a}$$

Several different types of radio-ligand binding experiments are possible. For saturation binding, the binding of increasing concentrations of radio-ligand [Ligand] is measured at equilibrium to determine the binding constant (dissociation constant K_d), additionally the concentration of specific binding sites for the radio-ligand [Receptor$_T$] is usually termed B_{max}. For competition binding experiments, one or more fixed radio-ligand concentrations is measured at equilibrium in the presence of increasing concentrations of unlabelled ligand. The data can allow the determination of the K_d for a compound for the un-liganded receptor and the co-operativity between the compound and the radio-ligand for the binding to the receptor. From these experiments the inhibitory constant K_i can be determined using the Cheng-Prustoff equation (Leach et al., 2010) (shown below).

Where K_i = dissociation constant; EC_{50} = half the effective concentration of the unlabelled agonist or antagonist to the receptor, K_d is the K_d of the radioligand and [Radioligand] = concentration of the radioligand.

$$K_i = \frac{EC_{50}}{1 + [\text{Radioligand}] \ / \ K_d}$$

1.1.4. Human adenonise-$_{2A}$ receptor ($hA_{2A}R$)

The adenosine $_{2A}$ receptor ($hA_{2A}R$) receptor is a GPCR belonging to the Adenosine Receptor Family, including A_1, A_{2A}, A_{2B}, and A_3, which are important targets for pharmaceuticals, being responsible for cell signalling in the most important organs; lungs, heart and brain (Jacobson, et al., 2000). $hA_{2A}R$ plays a part in the alleviation of cardiovascular and central nervous system disorders (Ongini, et al., 1996). It is antagonised by caffeine and theophylline (Jin & Fredholm, 1996). $hA_{2A}R$ is a well-studied GPCR, with a variety of ligands designed to bind it. Recombinant production of $hA_{2A}R$ has previously been achieved using yeast, specifically *S. cerevisiae* (Niebauer et al., 2004, Niebauer & Robinson, 2006, O'Malley et al., 2007) and *P. pastoris* (Fraser, 2006, Singh et al., 2008), due to the difficulties in using *E. coli* (Weiss & Grisshammer, 2002). In these studies the aim was to isolate and purify the protein for crystallisation to study its structure, multiple conformations and mechanisms depending on the ligand bound (O'Malley et al., 2007). However, its crystal structure was ultimately solved using recombinant protein produced in insect cells in complex with the high-affinity antagonist ZM241385 at a resolution of 2.6Å (Jaakola et al., 2008) (Fig 1.5).

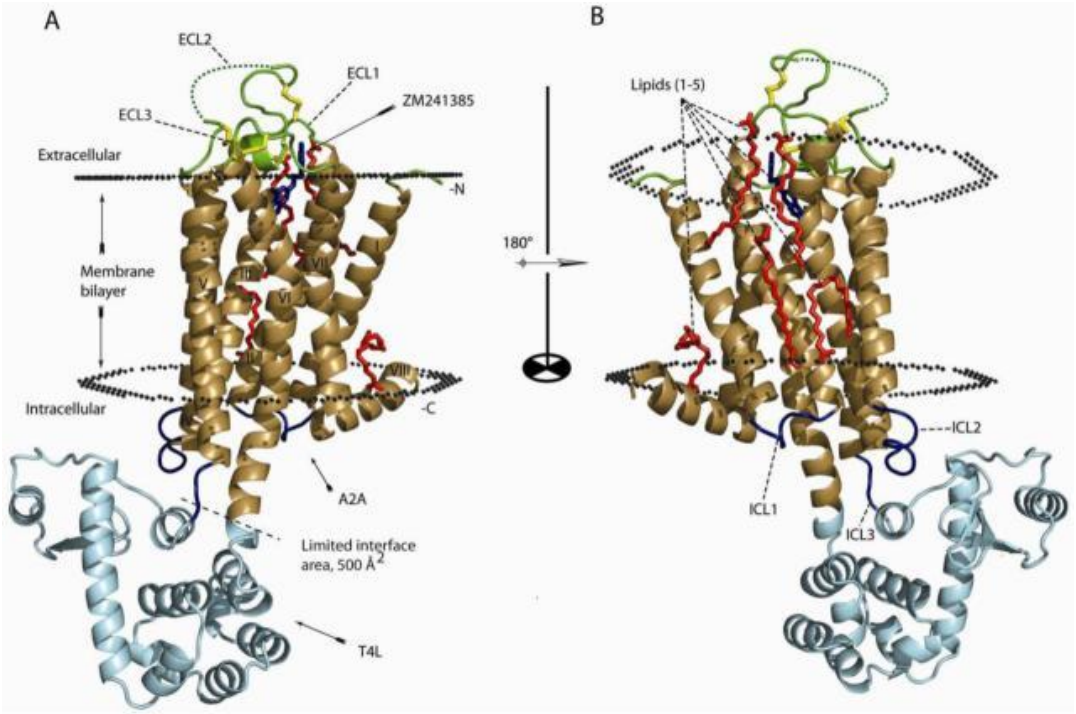


Fig 1.5 Diagram of $hA_{2A}R$-T4L crystal structure with antagonist ZM241385 bound. (A) *The structure is viewed perpendicular to the plasma membrane; with the transmembrane domain in brown, T4 lyzozyme (T4L) in cyan, ZM241385 in light blue, four lipid molecules bound to the receptor in red, the four disulphide bonds in yellow, the extracellular loops (ECL 1-3) in green, and the intracellular loops in blue.* ***(B)*** *The structure rotated by 180°around the x-axis* (Jaakola et al., 2008).

The structure shown in Fig. 1.5 was thermostabilised by removing the C-terminal tail and replacing it with T4 lysozyme (T4L) increasing the rigidity by replacing the intracellular loop 3(ICL3) (Jaakola 2008), since it had previously been reported that the C-terminal tail degraded when recombinantly expressed in *E. coli* (Weiss and Grisshammer, 2002). A subsequent crystal structure was obtained using agonist UK-432097 which when bound caused conformational changes leading to a more stable activated state at a resolution of 2.7Å (Xu et al., 2011). Additional efforts have been made to further thermostabilise $hA_{2A}R$ by using modified truncated variants and binding different ligands to induce different conformational states (Jaakola et al., 2008, Singh et al., 2008, Xu et al., 2011). Mutagenesis has also been employed by performing point mutagenesis to change amino acid residues in a systematic manner; consequently, thermostable mutants have been created which increase the chances of generating suitable crystals for characterisation (Lebon et al., 2011, Lee et al., 2014). However, a complete dynamic understanding of the

mechanism of $hA_{2A}R$ activation is still lacking, which requires wild-type protein in as native an environment as possible for further study. The yeasts *P. pastoris* and *S. cerevisiae* have been the most successful hosts for expression of $hA_{2A}R$ to date, producing the highest yields reported (*P. pastoris*; 2 mg/L (Singh et al., 2008) and *S. cerevisiae*; up to 6 mg/L (O'Malley et al., 2007) with near identical pharmacological properties as $hA_{2A}R$ from native membranes (Fraser, 2006, Niebauer & Robinson, 2006, O'Malley et al., 2007, Singh et al., 2010). Since yeasts are amenable to molecular manipulation, their use provides new opportunities to generate high yields of recombinant $hA_{2A}R$, and other GPCRs, by devising new strategies based on recent advances in our understanding of recombinant protein synthesis and the mechanisms of translation in eukaryotes (Bill, 2014).

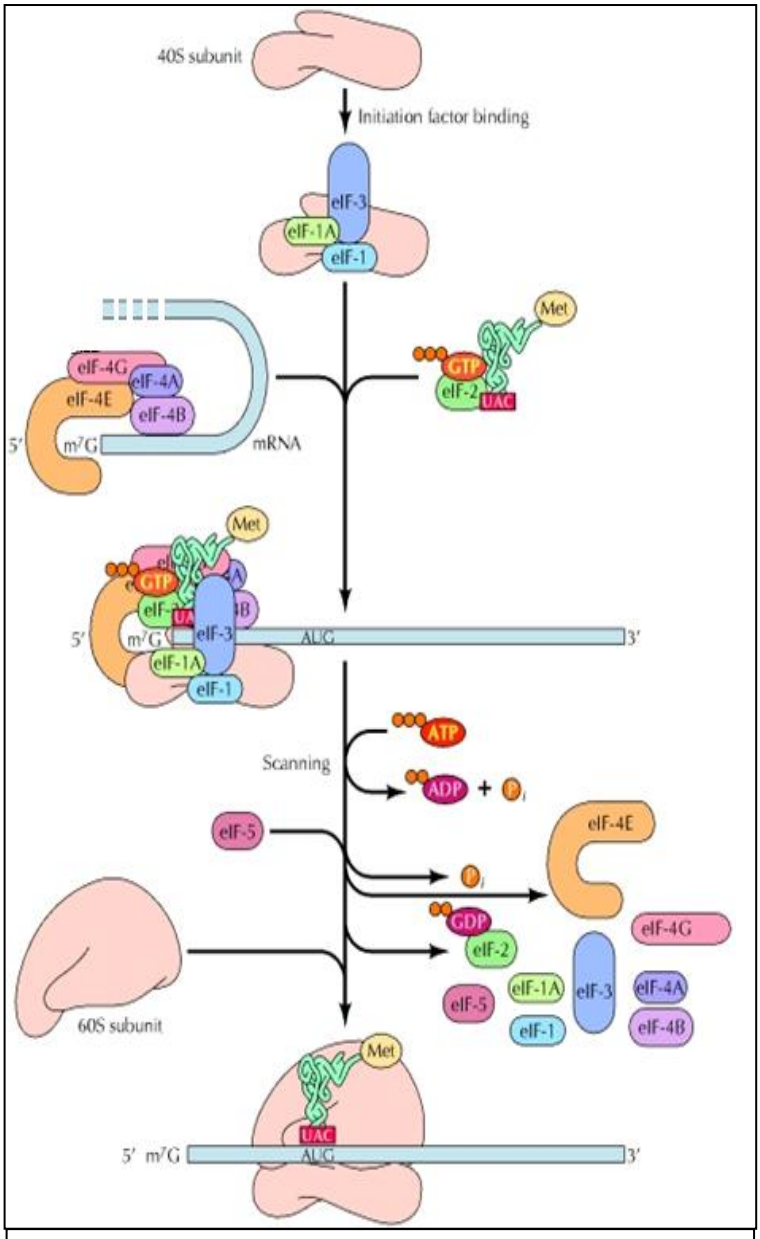


Fig 1.6 Translation initiation in eukaryotes. *The 40S subunit binds eIF1, eIF1A, eIF3 and eIF2-GTP-Met-tRNA, the resultant complex binds the eIF4 group at the 5′ (m7G) cap forming the 43S pre-initiation complex, which scans the mRNA until an initiation codon is found; eIF5 binds and causes the release of all eIFs, 60S then binds 40S to form the elongation-capable 80S (adapted from Cooper M, 2000).*

1.2. Mechanisms of translation in eukaryotes

Translation, the biosynthesis of proteins, is performed by ribosomes which are essentially large protein-ribosomal RNA multi-complexes. Ribosomes use messenger RNA (mRNA) as a template to synthesise polypeptide chains. The ribosomal subunits, 40S and 60S, associate to become the

functional 80S ribosome, which performs this process (Fig 1.6) in three distinct steps, referred to as initiation, elongation and termination. The initiation of translation (Fig 1.6) requires initiation factors called eukaryotic initiation factors (eIF). The factors, eIF1, eIF1A and eIF3 bind the 40S subunit, which in turn allows the association of the eIF2-GTP and Met-tRNA$_i$. Simultaneously eIF-4E recognises the 5´ 7-methylguanosine (m^7G) cap of the mRNA (Fig 1.6), and binds the other eIF4 factors. (Cooper M, 2000)

They then bring the mRNA to the ribosome where eIF4G binds to eIF3 (of the 40S, eIF1, eIF1A, eIF3, eIF2-GTP, Met-tRNA$_i$ complex), forming the 43S pre-initiation complex. This complex then scans the mRNA from 5´ to 3´, until the Met-charged-tRNA initiator recognises an initiation codon (AUG), which then recruits eIF5, which triggers the hydrolysis of the eIF2-bound GTP. This releases all the initiation factors and allows the 60S ribosome to bind the 40S subunit, generating the elongation-capable 80S ribosome. The 80S has three tRNA binding pockets; the peptidyl (P), aminoacyl (A) and exit (E). The A site is where new aminoacyl-tRNAs bind and are joined to previously-bound aminoacyl-tRNAs located in the P site where the growing peptide chain is located, and the E site is where the tRNA is released from its amino acid. At the start of the

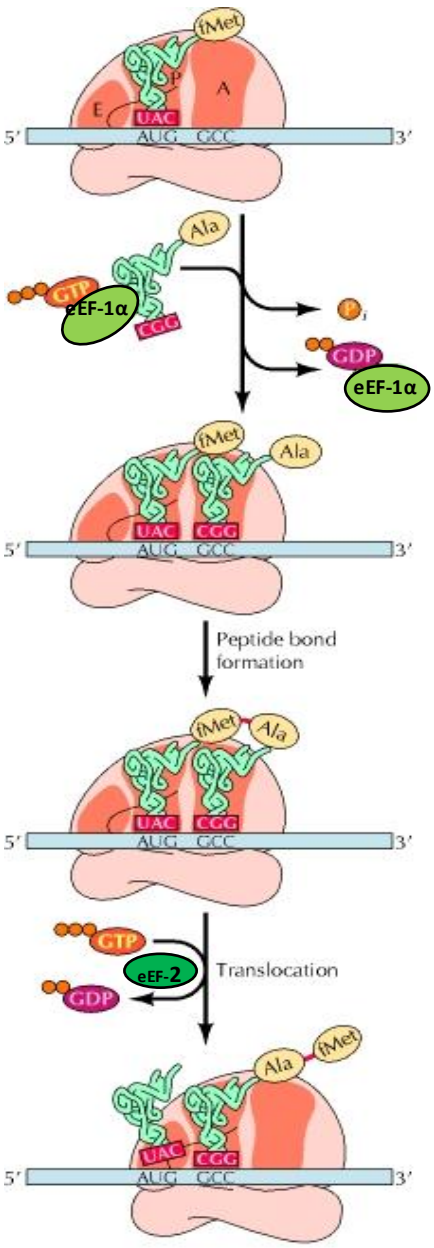


Fig 1.7 Translation elongation in eukaryotes. *The Met-tRNA$_i$ located in the peptidyl (P) site pairs with the initiation codon (AUG), while the eukaryotic elongation factor (eEF)1α recruits the next aminoacyl-tRNA to the amino-acyl (A) site which pairs to the next codon. A peptide bond is formed between the Met-tRNA$_i$ and the new aminoacyl-tRNA. The ribosome translocates along the mRNA to the next codon with the help of eEF2, the A site becomes vacant due to translocation, the last aminoacyl is now in the P site and the Met-tRNA$_i$ in the exit (E) site. With the A site now vacant another complementary aminoacyl-tRNA can be recruited and a peptide bond formed to add to the growing polypeptide; this process repeats until a stop codon is reached. (Adapted from Cooper M, 2000).*

elongation step (Fig 1.7) the Met-tRNA$_i$ is located in the P site and paired to the AUG, the next aminoacyl-tRNA is recruited by the eukaryotic elongation factor 1 alpha (eEF1α) GTP complex to the A site and pairs according to the next codon. During this process the GTP is hydrolysed, and a GDP-bound eEF1α is released and a peptide bond formed between the initiator methionyl tRNA residing in the P site and the second (newly arrived) aminoacyl at the A site, catalysed by the ribosome. The ribosome moves three nucleotides down the mRNA to the next codon, termed translocation, and requires eEF2 coupled with hydrolysis of GTP. This process translocates the A site over the new codon (the A site is now vacant), the peptidyl tRNA (Met-Ala-tRNA) from the A site into the P site, and the uncharged tRNA at the P site to site E which will be released upon recruitment of a new amino-acyl tRNA to the A site. This process is repeated forming a polypeptide until an A site translocates onto a termination codon, where the termination step takes place. The termination step (Fig 1.8) begins when a ribosomal A site moves to a termination codon having no complementary anticodon tRNA (UAA, UAG, UGA); in its place is a eukaryotic release factor (eRF-1.) This factor binds the A site and induces hydrolysis of the bond linking the tRNA with the polypeptide chain in the P site, resulting in completed-polypeptide release and mRNA-ribosome dissociation, terminating translation (Cooper M, 2000). Translation initiation may be the rate-limiting step in

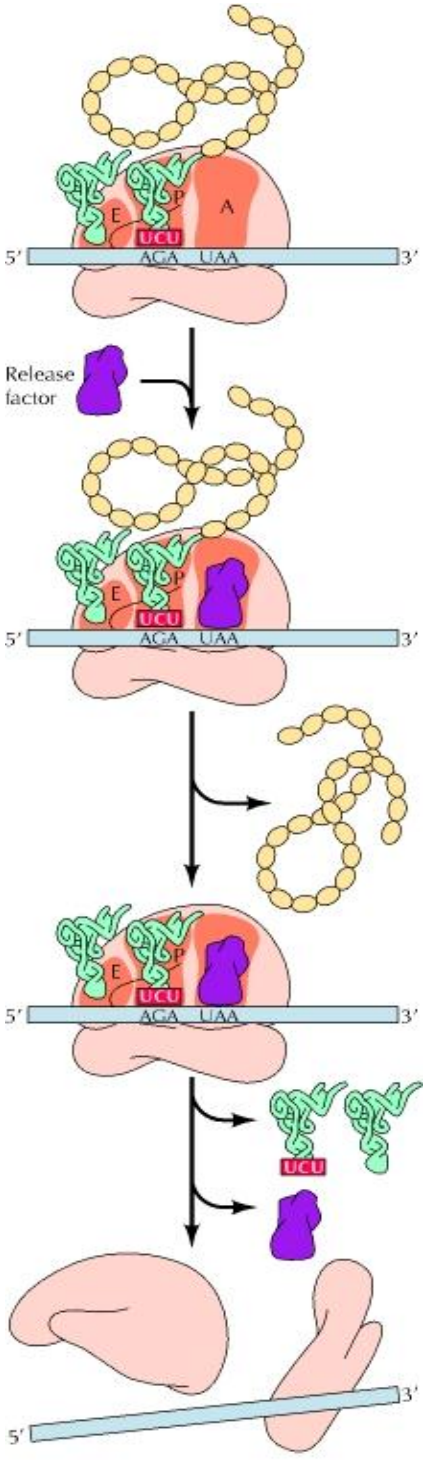


Fig 1.8 Translation termination in eukaryotes. *The ribosomal A site translocates to a stop codon, which has no complementary aminoacyl-tRNA; instead a release factor binds the codon. This causes the hydrolysis of the tRNA-polypeptide chain bond in the P site, release of the polypeptide and disassociation of the 40S and 60S subunits. (Adapted from Cooper M, 2000).*

recombinant protein production (Hershey & Cold Spring Harbor Laboratory., 1996), requiring the 5′ cap dependent mechanism of translation initiation. If this mechanism can be circumvented then the rate limiting step can be overcome and recombinant yield increased.

1.2.1. Internal ribosome entry sequences

The concept of an internal ribosome entry sequence (IRES) first came to light when encephalomyocarditis virus (EMCV) and poliovirus were observed to have the ability to maintain viral protein production even when 5′ cap-dependent ribosomal scanning was inhibited in infected cells as a stress response caused by the viral infection. This revealed the possibility of cap-independent initiation of translation, later attributed to the presence of specific secondary structures of mRNA now referred to as an IRES (Jang et al., 1988, Pelletier & Sonenberg, 1988, Jang et al., 1989).

Fig 1.9 Cap-dependent & Cap-independent models for translation initiation (reproduced from Kieft et al., 2010).

An IRES is a *cis*-acting nucleotide sequence that allows for the initiation of translation downstream of the 5′cap of an mRNA transcript independent of translation initiation factors (Jang et al., 1988, Pelletier & Sonenberg, 1988, Jang et al., 1989). It allows direct

recruitment of the 40S ribosome to the initiation codon independent of 5´ cap binding (Komar & Hatzoglou, 2011) by acting as a 'landing pad' for ribosomes (Pelletier & Sonenberg, 1988) (Fig 1.9). This is unique as standard translation is a complex process that requires recognition of the 7mG cap at the 5´ of mRNAs for initiation complex association, and subsequent 43S ribosomal subunit scanning until it 'stumbles upon' a start codon such as AUG. This triggers the assembly the 80S ribosome-initiation complex whereby translation begins. An IRES achieves this by being able to recruit ribosomes within the 5´ untranslated region (UTR) close to or at the start codon without the need for 5´cap binding and in some cases no initiation factors (Wilson et al., 2000).

Figure 1.10 Differing categories of IRES mechanisms compared to standard cap-dependent initiation of translation. *The diagram allows the comparison of viral IRES mechanisms according to the need for initiation factors (IFs), ranging from a reliance on all IFs except 4E exhibited by the poliovirus, to the cricket paralysis virus (CrPV) which requires none (reproduced from Kieft et al., 2009).*

There are various degrees of IRES activity and their reliance on initiation factors (Fig 1.10), as shown by the well-studied viral mechanisms above, ranging from the polio virus which needs nearly all eIFs, to the cricket paralysis virus (CrPV) and Plautia stali intestine

virus (PSIV) which need none (Wilson et al., 2000, Filbin & Kieft, 2009). It is believed that for most IRESes this is possible due to structural similarities between the IRES and the pre-initiation complex (Fig 1.11); the IRES essentially acts as an 'pre-initiation complex' mimic (providing similar conditions needed to attract ribosomes) (Stoneley & Willis, 2004). This is under debate, as native strong IRESes in yeast and the fruit fly have been shown to have a weak secondary structure, implying a common cap-independent mechanism that utilises unstructured RNA segments (Xia & Holcik, 2009). Additionally the recent crystal structures of the CrPV IRES bound to the ribosome of the yeast *Kluyveromyces lactis* revealed that it instead mimics a pre-translocation state (Fernández et al., 2014).

Cellular eukaryotic mRNA has also been found to possess IRES sequences; it is postulated these evolved to allow cap-independent translation of essential genes during mitosis or stress conditions, as translation is reduced or stopped at the initiation step (Sonenberg, 1994a, Han et al., 2001, Morris, 1995, Pain, 1996, Sonenberg, 1994b, Clemens & Bommer, 1999, Willis, 1999). Therefore cell survival requires this initiation block to be circumvented to allow essential genes to continue to be translated; IRESes have been the suggested mechanism to facilitate this. It has been shown in eukaryotes that under standard growth conditions, cap-dependent and cap-independent translation occur in unison, but

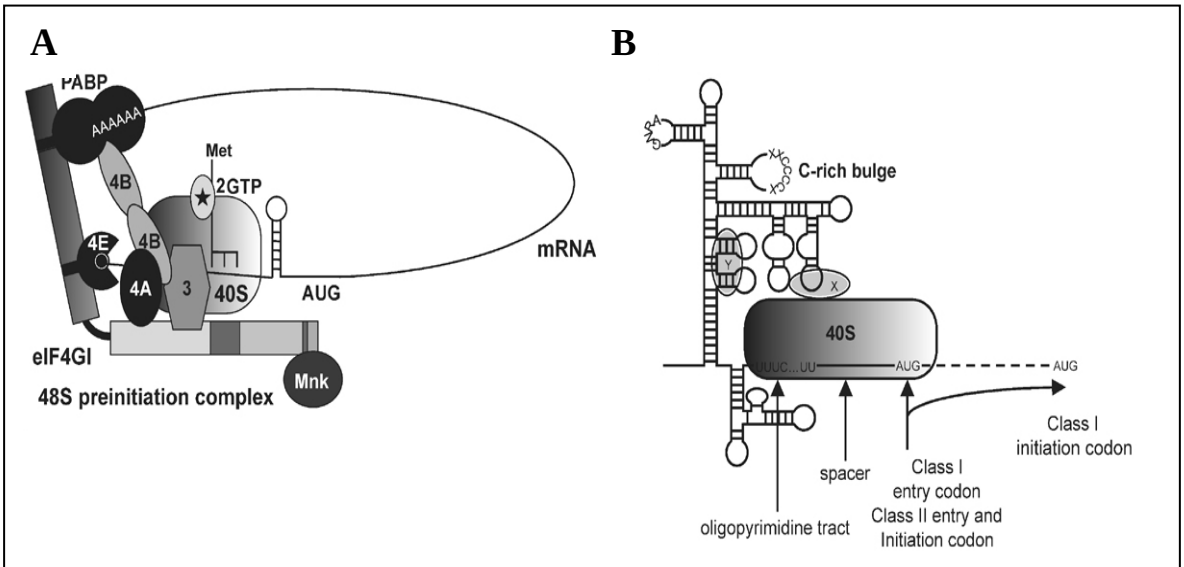


Fig 1.11 Picornaviral IRES structural mimicry of the 43S pre-initiation complex. (A) *The assembled 43S pre-initiation complex bound to mRNA, interacts with the ribosome and poly A-binding protein to allow circularisation of the mRNA.* ***(B)*** *Structural schematic depicting picornaviral IRES recruitment of the 40S ribosome directly to start codon (AUG), aiding by a polypyrimidine tract (adapted from Stoneley & Willis 2004 and Belsham & Jackson 2000).*

when stressed through low nutrient conditions, cap-dependent initiation is prevented while IRES-containing mRNA transcripts are still translated (Holcik & Sonenberg, 2005).

Approximately 10% of eukaryotic cellular mRNAs are thought to possess the ability to initiate translation via IRESes (Stoneley & Willis, 2004, Komar & Hatzoglou, 2011). As our knowledge of IRESes has improved, the potential to discover treatments for viral infections via inhibition of viral IRESes has become a reality with recent developments leading to the discovery of benzimidazole compounds that bind the HCV IRES and prevent the virus from replicating (Dibrov et al., 2012). Furthermore, a variety of oncogenes, growth factors and proteins involved in programmed cell death possess IRESes in their 5´ UTRs, because internal initiation allow genes to escape many regulatory mechanisms for cap-dependent translation. This could facilitate the survival of cancer cells under stress conditions (such as those caused by a lack of nutrients, hypoxia or therapy-induced DNA damage) and aid the progression of the cancer, thus targeting of IRESes might therefore result in effective future cancer treatments (Holcík, 2004).

1.2.1.1. Internal ribosome entry sequences in yeast

IRES activity in yeast, which is a lower eukaryote, has been specifically observed in *S. cerevisiae* mRNA for yeast adapter protein 1 (*YAP1*) and p150 (also called TIF4631), the yeast homologue of the cap-binding protein eIF4G (Zhou et al., 2001). Using luciferase expressing reporting vectors which allow only cap-independent translation by obstructing ribosomal scanning with mRNA hairpins, Zhou and colleagues were able to prove IRES activity in the 5´ UTRs of the mRNAs of both genes (Zhou et al., 2001). This was also confirmed using the *Renilla* luciferase reporter, with p150 showing a 10-fold and *YAP1* a 5-fold increase translational activity (Zhou et al., 2001) with similar findings being reported using fluorescent reporter genes (Edwards & Wandless, 2010). As of yet no native *P. pastoris* IRES have been confirmed due to the lack of UTR sequence information (Liang, et al., 2012a), although they have been theorised to be present in the 5´UTRS of the *KOG1* and *KOG2* mRNAs (Liang, et al., 2012b). Recently, a putative *S. cerevisiae* IRES from the 5´UTR of *GPR1* mRNA has been shown to initiate translation of a reporter gene in *P. pastoris* (Liang, et al., 2012a) .

The insect IRES of the CrPV was shown *in vitro* to bind the 40S ribosomal subunit, and upon the addition of the 60S subunit (with no other eIFs or GTP) the 80S ribosome was

formed (Hinnebusch, 2001). This demonstrates the ability of an IRES to recruit ribosomes without factors. Additionally *in vivo* studies using the CrPV IRES proved it functions in *S. cerevisiae*; uracil selection was used in conjunction with a *URA3* expressing vector under the control of the CrPV IRES and a tightly-regulated promoter. The yeast were able to grow sufficiently on uracil deficient plates, while cells expressing non-functional IRES mutant controls did not (Deniz et al., 2009). IRES efficiency was greatly increased when eIF2B was mutated to reduce its ability to bind 40S and thus increased the amount of free 40S (Deniz et al., 2009). In yeast the CrPV IRES does not mimic the pre-initiation complex with the help of other factors as the picornaviral IRESes do (Stoneley & Willis, 2004), but in fact mimics the Met-tRNA$_i^{Met}$ to directly recruit the 40S, which stimulates the binding of the 60S subunit, forming an elongation-capable 80S ribosome. (Deniz et al., 2009). Despite this, several studies have observed that cap-independent initiation in *S. cerevisiae* is unable to recruit sufficient ribosomes unless cap-dependent initiation is blocked or reduced (due to stress, starvation, viral infection) as there is competition with the cap-dependent mechanism (in particular for ternary complexes) (Deniz et al., 2009). It also has been speculated that IRESes might be employed to increase protein expression in eukaryotes (Chappell et al., 2000).

The literature discussed above provides a solid foundation for using IRESes to avoid rate limiting cellular control mechanisms in yeast, as this is regulated through the cap-dependent mechanism which IRESes do not. Recombinant synthesis has in some cases been shown to cause a translational block due to cellular stress. This is thought to be regulated by eIF-2 kinase, which is released to shut down protein synthesis/translation (Harding et al., 2000, Novoa et al., 2001) when the cell senses high amounts of denatured protein (a common occurrence during heterologous expression in recombinant hosts) (Mattanovich et al., 2004, Gasser et al., 2008). Modifying existing expression vectors with IRES sequences therefore has the potential to be used to circumvent any rate limiting events imposed by the cell in response to recombinant protein production. Secondly, the vector could allow potentially exclusive expression of a recombinant protein by artificially inducing stress upon host cells. IRES-augmented mRNA transcripts should be processed independently of most, if not all, translation initiation factors depending on the IRES element chosen, therefore exploiting a mechanism first highlighted by viruses (Jang et al., 1988, Pelletier & Sonenberg, 1988, Jang et al., 1989).

1.3. Recombinant protein production

Typically proteins of interest must be produced in the large quantities required for further study in a suitable host organism, as a higher yield can be acquired than from natural sources. This is particularly true for membrane proteins (Bawa et al., 2011). For example, human insulin for treating diabetes was originally extracted from pancreas glands of swine and cattle, with 8,000 pounds of gland needed to produce 1 pound of insulin (Noyd et al., 2013). Then in 1978 the protein hormone was produced recombinantly using *E. coli* and later with *S. cerevisiae* (Thim et al., 1986), improving yield dramatically while reducing time and costs. Recombinant production is usually performed through the introduction of plasmid DNA which encodes the protein of interest and a selection marker to allow the selection of cells that express the protein of interest. The plasmid vector has the ability to replicate and perform protein synthesis using the host cell machinery. The host cells therefore act as a recombinant protein factory which will be cultured at an exponential rate. Below is a table detailing properties of the major host systems (Table 1.4).

Properties	Bacteria	Yeast	Insect	Mammalian
Complexity of Growth Medium	Minimum	Minimum	Complex	Complex
Medium Cost	Low	Low	High	High
Expression Level	High	Low - High	Low - High	Low - Moderate
Yield (mg/L)	50-500	10-200	10-200	0.1-100
Protein Folding	Refolding Usually Required	Refolding May Be Required	Proper Folding	Proper Folding
Success Rate (%)	40-60	50-70	50-70	80-95
N-linked Glycosylation	None	High Mannose	Simple	Complex
O-linked Glycosylation	No	Yes	Yes	Yes
Phosphorylation	No	Yes	Yes	Yes
Project Cost	Low	Low	Medium	High
Advantage	Low cost, fast and easy to grow	Low cost, faster than higher eukaryotes, possess post translational modification (PTM)	Product closer to native state than microbes can manage, better PTM	Product is correctly folded via natural configuration, best PTM, more useful for study
Disadvantage	No PTM resulting in non-functional products	Suffers hyper-glycosolation, requiring the cleavage at a later stage	Requires specialised media and strict culture conditions, longer time, expensive.	Requires specialised media and strict culture conditions, longer time, highest cost

Table 1.4 Properties of each major host system with respect to recombinant protein production. *Adapted from http://www.genwaybio.com/technologies/protein-expression). Post translational modification (PTM).*

The majority of recombinant protein production is usually carried out using the four main host systems detailed in Table 1.4; bacteria (*E. coli*), yeast (*P. pastoris, S. cerevisiae*), insect cells (baculovirus/insect larvae) and mammalian cells (CHO, HEK). To date recombinant protein production is divided among these hosts as follows: *E. coli* 73%; *P. pastoris* 11%; Mammalian cell-lines 4%; Insect cells 4% and *S. cerevisiae* 2% (the remainder being accounted for by cell-free systems and other microbes). Yet of the 150 recombinant protein that are approved as drugs by the US Food and Drug Administration, 29% are from *E. coli*, 18% from *S. cerevisiae*, 12% from hybridoma cells, 40% from mammalian cells, 0.5% from insect and 0.5% from transgenic animals (Bill, 2014).

Each host system has a variety of unique accessory factors needed for the synthesis of membrane proteins of which not much is known (Bill et al., 2011). The significance of this is that although a membrane protein can be produced in a chosen host, there are subtle differences in factors such as chaperones, single peptides and translocon components that may result in low yields due to inefficient synthesis (Bill et al., 2011). Notably, it has been shown that higher yielding conditions do not always correlate with higher functional protein yield (André et al., 2006), and contrastingly production at a slower rate due to 'translational initiation blocks', allows proteins to fold properly improving functional yields (Bonander et al., 2005).This situation has been termed 'the bottleneck in membrane protein production', since acquisition of the membrane protein of interest is an important rate limiting step in research in this field (Bonander, et al., 2009).

The success of *E. coli* is due to the low cost and time involved in expressing soluble proteins such as insulin, human growth hormone and the insulin growth factor 1 (Ferrer-Miralles et al., 2009). Yet it does have disadvantages as a recombinant host (detailed in Table 1.4) such as a tendency to form inclusion bodies that need refolding at a later stage (Ferrer-Miralles et al., 2009). Furthermore, prokaryotic translation elongation occurs 4-10 times faster than eukaryotic translation elongation and the codon usage is different which can have unforeseen effect on the folding of eukaryotic proteins (Ferrer-Miralles et al., 2009). Additionally as shown in Table 1.4, *E. coli* does not perform adequate post-translation modification (PTM) such as glycosylation, phosphorylation, acetylation and disulphide bond formation, which can have adverse effects on eukaryotic proteins. In

contrast, insect and mammalian cells produce more authentic products when expressing proteins of human origin, and are used in the biopharmaceutical industry to produce erythropoietin, granulocyte colony stimulating factor and monoclonal antibodies (Demain & Vaishnav, 2009). The yeast species, *S. cerevisiae*, is used extensively for the production of therapeutics, possessing the properties of a prokaryotes (fast and easy culture, low cost) with the appropriate PTMs allowing correct eukaryotic expression and folding for human proteins (Nielsen, 2013).

Whilst products from yeast are not as authentic as those from mammalian or insect cells, the trade-off for high-density low cost cultures with adequate PTM make it a good mid-ground host (Demain & Vaishnav, 2009). *S. cerevisiae* performs high mannose-type N-linked glycosylation of recombinant proteins, where a chain of over 100 mannose residues is added during glycosylation, which is not typically found in mammals. This can adversely affect the function of recombinant human proteins, confer a short half-life *in vivo* and often requires modification of the protein remove glycosylation sites (Wildt & Gerngross, 2005, Nielsen, 2013). Consequently, much work has been performed to engineer *S. cerevisiae* (and *P. pastoris*) strains that that can carry out human N-linked glycosylation; this could see yeast host use become even wider (Wildt & Gerngross, 2005), especially since *P. pastoris* has recently been approved for use as a biopharmaceutical host (Berlec & Strukelj, 2013). *P. pastoris* is used in conjunction with the inducible alcohol oxidase 1 (*AOX1*) promoter, which is induced using methanol to turn on recombinant expression. Therefore the difficulties with high density culture do not apply to *P. pastoris*, and it can also express recombinant proteins without the hyperglycosylation typical of *S. cerevisiae* (Romanos et al., 1992). *P. pastoris* has been used extensively for the production of difficult targets, such as membrane proteins, as it is amenable to high-density cultures. However unlike *S. cerevisiae, P. pastoris* is not as well studied as a model organism and therefore is a less suitable candidate for genetic manipulation. In contrast to *P. pastoris*, *S. cerevisiae* cannot be grown to high cell yields unless under a complex feeding regime resulting in a production and subsequent build-up of ethanol (Verduyn et al., 1984). Interestingly the TM6* strain possesses mutated hexose transporters (Hx1p and Hx7p) responsible for glucose uptake and is an exclusively respiratory yeast, which does not undergo fermentation and can therefore be grown to higher cell densities than wild-type strains (Otterstedt et al., 2004).

While *E. coli* is responsible for the vast majority of recombinant protein production, when it comes to challenging targets, the yeasts *P. pastoris* and *S. cerevisiae* have substantial advantages, with over 50% of all the eukaryotic membrane protein structures deposited in the PDB being produced in yeast (Fig 1.3) (Bill et al., 2011). This is not surprising as a study using 103 GPCRs determined that only 50% could be expressed in *E. coli* when compared to 94% for yeast and 95% for mammalian cells (Lundstrom et al., 2006). Even so, membrane protein production still suffers from low functional yield (Bonander, et al., 2005) and cannot be produced predictably in the high-yields required (Bill et al., 2011), a problem that is further compounded by the fact that there is no universal host for recombinant membrane protein production (Bill, 2001, Sørensen, 2010).

1.4. *Saccharomyces cerevisiae*

The yeast species *S. cerevisiae* belongs to the Fungi kingdom and has historically been associated with bread and beer making. *S. cerevisiae* cells are around 2-10μM in size and are referred to as budding yeast, as the 'mother' cell divides to create a smaller 'daughter' cell or 'bud' (Fig 1.12). The typical doubling time of *S. cerevisiae* when grown in a complex medium containing its preferred carbon source, glucose, is approximately 1.6h (Werner-Washburne et al., 1993). As it is a eukaryote many cellular mechanisms are conserved between the yeast and humans, making it an indispensable tool for biological studies. It was the first eukaryote to have its genome sequenced revealing 6,200 genes on 16 chromosomes (Bassett et al., 1996). Additionally the *Saccharomyces* Genome Database (http://www.yeastgenome.org) is an open community source sharing molecular and microbial resources. The ease with which experiments can be performed using yeast has allowed a wide range of biological questions to be answered on translation, transcription and metabolism. For example, the geneticist

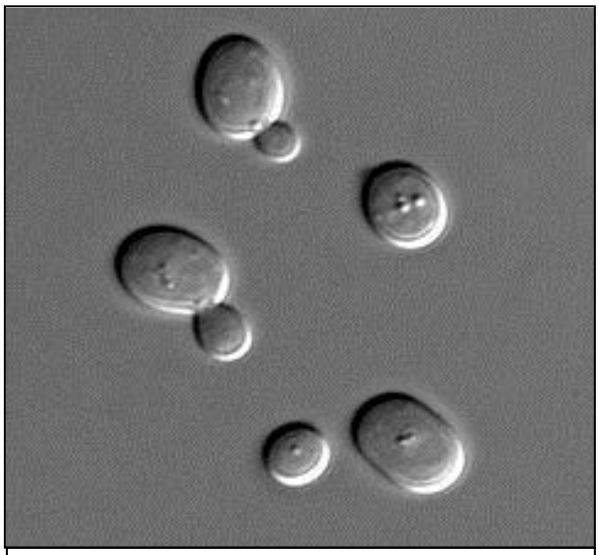


Figure 1.12 Light microscopy image of wild-type* S. cerevisiae *with budding daughter cells.

and cell biologist Sir Paul Nurse was awarded a Nobel Prize in 2001 for his discovery, using yeast to identifying the specific proteins that control cell division.

S. cerevisiae growth involves three phases (in a closed system under controlled conditions); lag, log, and stationary (Fig.1.13). Lag phase is when the yeast cells adjust their metabolism to utilise a new carbon source, such as glucose. Log phase is when growth is exponential and glucose is converted into ethanol and CO_2 via fermentation and some respiration (Verduyn et al., 1984). When the (fermentable carbon source) glucose is exhausted, a diauxic shift takes place (Fig.1.13), and the yeast metabolises the ethanol, cells grow at a reduced rate and increase their carbohydrate stores before entering stationary phase (Werner-Washburne et al., 1993).

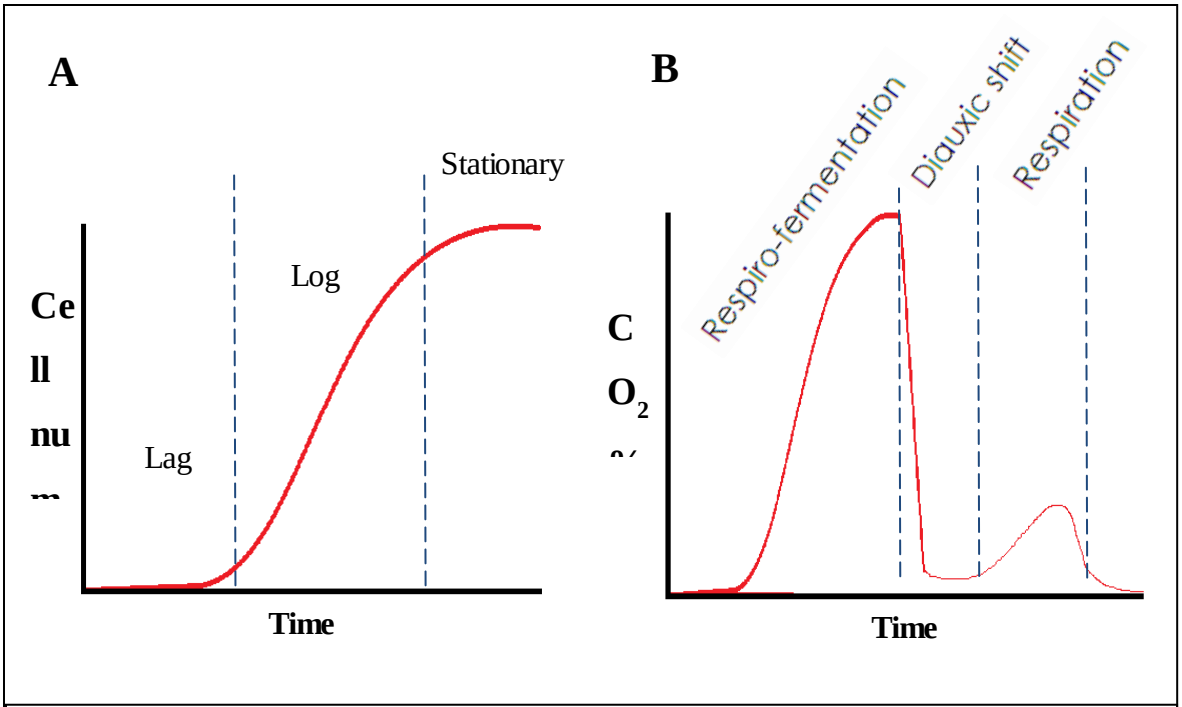


Figure 1.13 Growth and metabolism of S. cerevisiae. (A) *Growth curve highlighting the three distinct phases; lag, log, and stationary.* ***(B)*** *Metabolic curve illustrating the shift from respiro-fermentation to respiration indicated by the diauxic shift, which occurs when the preferred carbon source, glucose, is used up and ethanol that was produced during respiro-fermentation is consumed.*

When all the ethanol has been exhausted, the yeast enter stationary phase due to accumulation of toxic waste products and nutrient limitation, during which there is no increase in cell count and the cells differentiate to a stress resistant state where the cell wall thickens, cells adapts to be thermo-tolerant, and transcription is reduced. Cells in stationary phase have been shown to survive with practically 100% viability for at least 3 months (Werner-Washburne et al., 1993).

1.4.1 Recombinant membrane protein production in *S. cerevisiae*

A wide range of important yeast expression hosts are used in recombinant protein production including *S. cerevisiae*, *P. pastoris*, *Schizosaccharomyces pombe*, *Kluyveromyces lactis*, *Hansenula polymorpha*, *Yarrowia lipolytica* and *Arxula adeninivorans*, the most promising of which have proven to be *S. cerevisiae* and *P. pastoris* (Celik & Calık, 2012).Using *S. cerevisiae* for laboratory research involves genetically engineered strains such as BY4741, which is an auxotroph having non-functioning alleles for certain enzymes required for essential nutrient synthesis (Brachmann et al., 1998). This allows the selection of cells that harbour plasmids encoding a protein of interest. This is achieved by providing all the nutrients the organism requires in the growth medium excluding the nutrient that is encoded for by the plasmid selection marker gene, allowing the survival of plasmid-harbouring cells only. For example common selection markers are *URA3, HIS3, TRP1, LEU3 and MET15* which encode critical enzymes involved in the synthesis of uracil, histidine, tryptophan, leucine and methionine, respectively (Pronk, 2002).

S. cerevisiae is extremely well understood at a genetic level, possessing deletion libraries for 90% of it genes, and due to the high amount of conservation of expression and secretion pathways among lower and higher eukaryotes. Its higher eukaryote-like secretory pathways lead to correct processing of proteins (Mattanovich et al., 2012) and its machinery similar to mammalian cells enables post-translation modification such as disulphide bond formation and glycosylation (Böer et al., 2007). This allows the production of heterologous human proteins recombinantly in yeast, exploiting its microbial properties at the same time as its eukaryotic ones (Bawa et al., 2011). There is also huge potential for the modification of the complex systems that facilitate protein synthesis and

folding in yeast; this might lead to solutions for proteins that have proved difficult to express in their fully functioning form (Tate, 2001). Recent work on strain engineering to create improved yeast recombinant hosts (Bonander & Bill, 2012) has demonstrated that *S. cerevisiae* is particularly useful as a host organism for the production of difficult targets (Drew et al., 2008). The $P2Y_{12}$ receptor which is a GPCR and potential drug target for platelet aggregation, recently had its structure solved using protein synthesised using insect cell line Sf9 (Zhang et al., 2014).

1.5. Selective advantage

Practically every hostile environment is inhabited by unique organisms that have been selected for by pressure applied by their environment, giving them a selective advantage (SA) over invading, competing organisms. The bacterium *Shewanella oneidensis* has the ability to turn toxic metals into soluble, less unstable forms, and in doing so acquire the oxygen it needs for cellular processes. As this organism inhabits deep-sea and soil anaerobic environments, this ability confers significant advantage over other competing organisms (Swan et al 2009). The most extreme conditions on earth have selected for another of group of microbes known as Archaea. Their ability to adapt to chronic stress has given them a selective advantage over bacteria in these extreme environments (Swan et al 2009). They are organised into four main physiological groups; halophiles, thermophiles, alkaliphiles and acidophiles, which thrive in high salt, temperature, alkali and acid conditions, respectively. All the organisms mentioned have been selected for by their environment, therefore the same should apply to allow selection when the pressure applied is man-made/artificial. As mentioned in 1.4.1, strategies are employed routinely in microbiological research where an organism is transformed with a self-replicating double stranded DNA plasmid that codes for a protein of interest, allowing recombinant synthesis in the host. To guarantee that only organisms that possess this plasmid dominate a culture, a gene essential for survival of the host organism will be contained within the plasmid, allowing an organism to survive while their competitors perish (Fig 1.14).

Four previous attempts have been reported which utilised SA to increase recombinant protein yields of difficult targets. All have taken place using bacteria as the recombinant host on protein targets of prokaryotic or viral origin. This has been done through tagging

the protein of interest with another protein that will confer a SA on cells that synthesise it. In 1999, an attempt to obtain soluble HIV integrase was plagued by solubility issues when expressed in bacteria; an SA strategy was therefore designed by fusing HIV integrase with chloramphenicol acetyltransferase (CAT), an enzyme conferring antibiotic resistance, and expressing the fusion protein in *E. coli*. It was discovered that when cultured on plates that contained high levels of chloramphenicol, cells that had a higher resistance tended to express the more soluble form of the HIV-integrase (Maxwell et al., 1999) although the underlying mechanisms were not elaborated on.

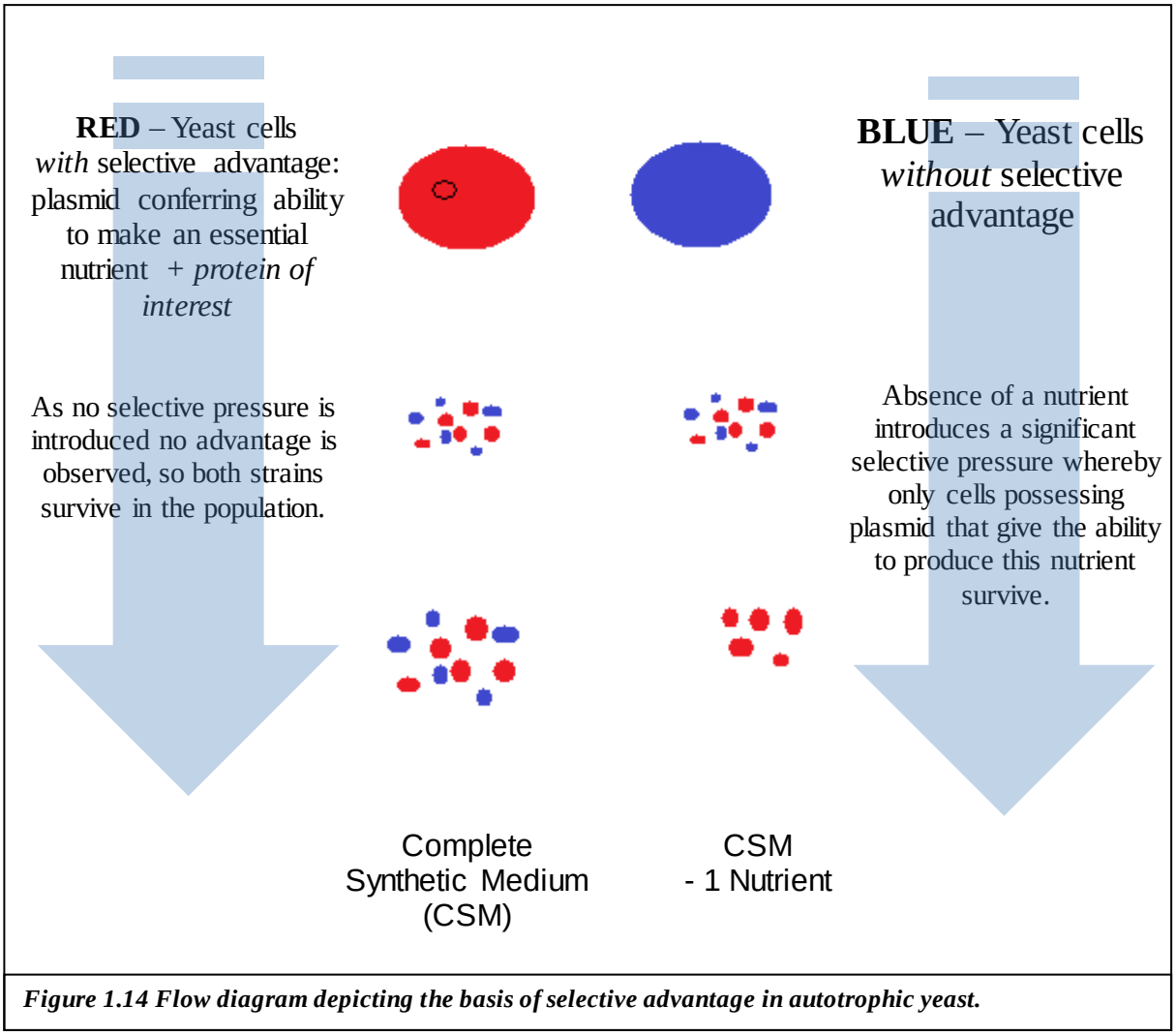


Figure 1.14 Flow diagram depicting the basis of selective advantage in autotrophic yeast.

In 2009, attempts were made to improve the yields of several membrane proteins from *Mycobacterium tuberculosis* (responsible for most cases of tuberculosis) including the membrane protein, rhomboid-Rv1337. A fusion between rhomboid-Rv1337and different antibiotic resistance enzymes resulted in the selection of strains that had up to a 75-fold

increase in yield (Massey-Gendel et al., 2009). Despite this success, the authors were only able increase yield of 10% of the membrane proteins tested.

In 2010 researchers attempted to increase expression of several membrane proteins in *Lactococcus lactis*, by fusing them to the erythromycin-resistance protein (ErmC) which conferred resistance to the antibiotic erythromycin and resulted in the selection of strains that had a 2-8 fold increase in yield for a number of the target proteins (Linares et al., 2010). In 2014, the same research group attempted a similar method in *E. coli*, in which they were able to select for strains that had an increase in membrane protein yield by up to 5-fold (Gul et al., 2014). In that study, GFP was used as a reporter of correct folding for the membrane proteins they were fused to it. While this is useful and routinely employed for high-throughput screening and optimisation of recombinant membrane protein expression (Newstead et al., 2007), no functional assays were performed on the recombinant proteins produced during the aforementioned strategies (Gul et al., 2014).

1.6. Strategies to increase recombinant protein yield

1.6.1. Employing Selective Advantage

In order to improve functional yields of recombinant $hA_{2A}R$ in yeast, we devised a strategy to provide yeast cells with an SA (Fig 1.15) in producing $hA_{2A}R$ in fusion with orotidine-5 phosphate decarboxylase (Ura3p). $hA_{2A}R$ was chosen since it is a well-characterised protein with a robust functional assay in the form of radio-ligand binding; this enabled the characterisation of total and functional recombinant protein. The fusion partner was Ura3p, which is essential in the catalytic pathways necessary for the production of uracil. Therefore cells that express the $hA_{2A}R$-Ura3p fusion have a selective advantage when grown in a medium lacking uracil. A detailed overview of the strategy is provided at the beginning of Chapter 3.

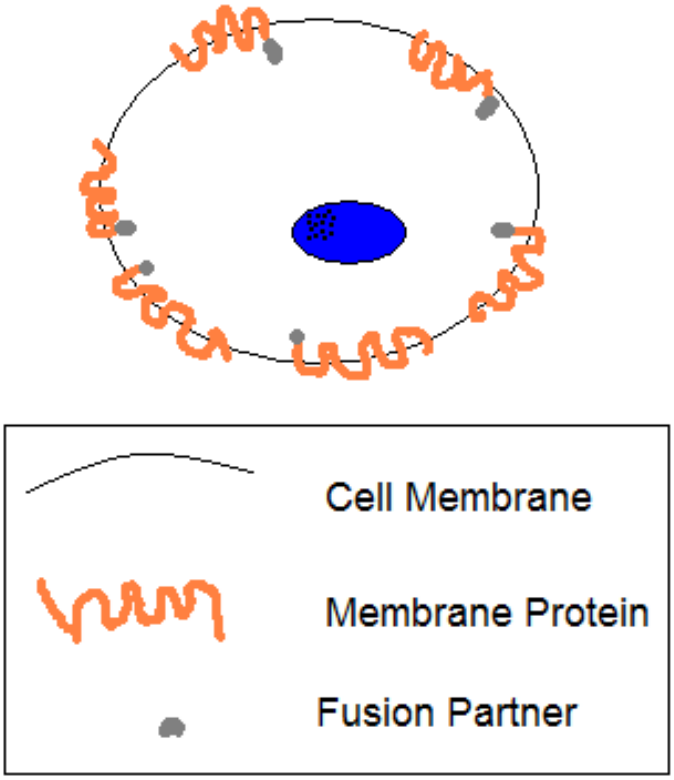


Figure 1.15 Synthesis of recombinant $hA_{2A}R$-Ura3p fusion protein. *With the membrane protein correctly localised in the plasma membrane, the Ura3p fusion partner is positioned at the C-terminus in the intracellular space anchored to the cell membrane, allowing the cell to benefit from the enzymatic activity of the fusion partner.*

1.6.2. Employing knowledge of translational processes

1.6.2.1. Translational slowdown

A reduction in the rate of translation has been shown to reduce amino acid incorporation errors and misfolding and thus lead to a higher functional yield, which is particularly important for membrane proteins; reductions in culture temperature have been employed to this effect for recombinant expression. It was decided to use deletion strains that had translational inhibitions, and also to administer drugs known to inhibit translation in yeast, in an attempt to reduce the rate of translational. Our model membrane protein was $hA_{2A}R$. A detailed overview of the strategy is provided at the beginning of Chapter 4.

1.6.2.2. IRES

Translation initiation independent of the canonical 5′ cap-binding mechanism enables genes downstream of an IRES to circumvent regulation. As previously described in 1.2.1.1, some yeast IRESes have also been shown to increase translational activity (Zhou et al., 2001, Edwards & Wandless, 2010), and they are thought to have increased activity when the host cell is in a stressed state as global translation initiation becomes compromised (Paz et al., 1999, Komar & Hatzoglou, 2005). Consequently, it was theoretically possible that modifying an existing expression vector by inserting an IRES into the 5′ UTR upstream of a low yielding target might result in increased yields, especially if IRES activity was increased by the stress of recombinant protein production or alternatively through expression in a deletion strain that exhibits a constitutively stressed state. It was decided to use $hA_{2A}R$ as the model membrane protein and the *YAP1*, p150 and CrPV IRESes (the most studied IRESes shown to have *in vivo* activity under physiological conditions in wild-type yeast cells), along with *spt3Δ*, which has been shown to exhibit a translation initiation block (Fig 4.6). A detailed overview of the strategy is provided at the beginning of Chapter 4.

1.7. Aims of project

I) To use the principle of SA in *S. cerevisiae* cells to increase the yield of recombinant $hA_{2A}R$ expressed in its membranes; specifically to increase the yield per cell of functional protein.

II) To use knowledge of translational processes that affect recombinant protein production to increase functional yield by:

a) the modification of existing expression vectors with an IRES inserted into the 5′ UTR upstream of the gene encoding $hA_{2A}R$, to circumvent limiting responses placed on recombinant translation by the host cell;

b) using *S. cerevisiae* deletion strains known to have translation initiation blocks, and drugs known to cause translational inhibition in an effort to slow down translation sufficiently to improve protein quality and therefore increase functional yield.

III) To gain insight into the underlying mechanisms that cause high functional recombinant membrane protein yield in *S. cerevisiae*.

Chapter 2: Methodology

2.1. Reagents

2.1.1. Culture reagents

2.1.1.1. MES (pH6)(1L)

This solution was prepared by adding 213.25g of 2-(N-Morpholino)ethanesulfonic acid (MES) monohydrate and made upto 1L with distilled water and adjusted to pH6.

2.1.1.2. Ampicillin

100mg/mL stocks were sterilised by syringe filter sterilisation into 1.5ml microcentrifuge tubes, and stored at -20°C until required. Stocks were diluted to a final concentration of 100µg/mL in the required media once cooled.

2.1.1. 3. Glucose (40%; 1L)

This solution was prepared by adding 400g of glucose by dissolving slowly in distilled water up to 1 L final volume, the solution was then filter sterilised and stored at room temperature.

2.1.1.4 10× Amino acid drop-out solution (DO; minus histidine)

This solution was prepared by adding 200 mg L-adenine hemi-sulphate salt, 200 mg L-arginine HCl, 300 mg L-isoleucine, 1000 mg L-leucine, 300 mg L-lysine HCl, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 200 mg L-tryptophan, 300 mg L-tyrosine, 200 mg L-uracil and 1500 mg L-valine and made up to 1 L with distilled water, and autoclaved then stored at 4°C.

2.1.1.5 10× DO (minus uracil)

This solution was prepared by adding 200 mg L-adenine hemi-sulphate salt, 200 mg L-arginine HCl, 200 mg L-histidine HCl monohydrate, 300 mg L-isoleucine, 1000 mg L-leucine, 300 mg L-lysine HCl, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 200 mg L-tryptophan, 300 mg L-tyrosine and 1500 mg L-valine and made up to 1 L with distilled water, and autoclaved then stored at 4°C.

2.1.1.6. 10× DO (minus uracil and histidine)

This solution was prepared by adding 200 mg L-adenine hemi-sulphate salt, 200 mg L-arginine HCl, 300 mg L-isoleucine, 1000 mg L-leucine, 300 mg L-lysine HCl, 200 mg L-methionine, 500 mg L-phenylalanine, 2000 mg L-threonine, 200 mg L-tryptophan, 300 mg L-tyrosine and 1500 mg L-valine and made up to 1 L with distilled water, and autoclaved then stored at 4°C.

2.1.2. Membrane preparation reagents

2.1.2.1. Breaking Buffer (pH7.4)

This solution was prepared by adding 50mM Na_2HPO_4, 50mM NaH_2PO_4, 2mM EDTA (pH7.4), 100mM NaCl and 5% Glycerol and made up to 1 L with distilled water and adjusted to pH7.4.

2.1.2.2. Buffer A (pH7)

This solution was prepared by adding 20mM HEPES, 50mM NaCl, 10% glycerol and made up to 1 L with distilled water and adjusted to pH7.

2.1.2.3. Bovine serum albumin (BSA) standard

BSA (Sigma) was diluted to a final amount in each well of 0-10 µg from a 1mg mL-1 stock for all protein determinations.

2.1.2.4. Lysis buffer

This solution was prepared by adding 11.3 mL 1M NaH2PO4, 38.7 mL 1M Na2HPO4, 1 mL 3 M NaCl and 10mL 1M imidazole (final concentration 50 mM NaH2PO4, 300 mM NaCl and 10 mM imidazole) which was made upto 1 L with distilled water adjusting to pH 8.0.

2.1.3. Immunoblot reagents

2.1.3.1. Western Tris buffer (1L)

This solution was prepared by adding 100mL 10×Tris buffer and 200mL methanol and made up to 1 L with distilled water.

2.1.3.2. Phosphate buffer saline (PBS; 1 L)

This solution was prepared by adding by 5 PBS tablets were dissolved in 1 L distilled water.

2.1.3.3. PBS-Tween 20 (PBST; 1 L)

This solution was prepared by adding 2mL Tween 20 (0.2%) and then made up to 1 L with PBS.

2.1.3.4 5×Laemmli sample buffer

This solution was prepared by adding 1.25mL 0.5M Tris-HCl (pH 6.8), 1mL 100% glycerol, 2mL 10% SDS, 0.5mL β-mercaptoethanol and 10µL bromophenol blue. Made upto 8mL with distilled.

2.1.3.5 PageRuler plus prestained protein ladder

A protein ladder (Thermo Scientific) used for SDS-PAGE, with a range of 10-250kDa.

2.1.4. Radio-ligand binding reagents

2.1.4.1. Binding buffer

This solution was prepared by adding 11.3 mL 1M NaH_2PO_4, 38.7 mL 1M Na_2HPO_4 and 1 mL 0.5 M EDTA, made up to 1 L with distilled water, and pH 7.4.

2.1.4.2. Tritiated ZM241385 ([^{3}H]ZM241385)

Tritiated ZM241385 was purchased from American Radio Chemicals (ARC Inc). Dilutions were made using binding buffer and stored at 4°C in a radiochemical laboratory.

2.1.4.3. Unlabelled ZM241385 (cold ZM241385)

Stock dilutions of cold ZM241385 (Tocris) were made with 100% DMSO to concentrations: 0.1mM, 10µM, 1µM, 0.1µM, 100nM, 10nM and 0.1nM. With the final concentration of cold ZM241385 being a 1:100 dilution of these stock solutions.

2.1.4.4. Soluene

Soluene®-350 (Perkin-Elmer) was used as a tissue solvent to solubilise total membrane pellets.

2.1.4.5 Scintillant

ScintiSafe (Fisher) scintillant was used in conjunction with the scintillation counter for the radio-ligand binding experiments.

2.1.5. Solubilising reagents

2.1.5.1. n-dodecyl-β-d-maltopyranoside (DDM)

DDM (Anatrace) was diluted to 5% (w/v) using distilled water to desired volume and stored at 4°C.

2.1.5.2. Cholesteryl hemi-succinate (CHS)

0.5% CHS (w/v) (Sigma) solution was made with 50 mM Tri-HCl pH 8.0 and sonicated for 10s repeated 3 times (using ice to keep the solution cool), and stored at 4°C.

2.1.6 Molecular biology reagents

2.1.6.1 Restriction enzymes

Several restriction enzymes were used for digestion of DNA and were acquired from New England BioLabs Inc. Enzyme details and reaction conditions were taken from the New England BioLabs Inc. website (http://www.neb.com/nebecomm/enzymefinder.asp).

2.1.6.2 *BamHI*

Source is an *E. coli* strain that carries the cloned *BamHI* gene from *Bacillus amyloliquefaciens* H Reaction temperature 37°C.

2.1.6.3. *NcoI*

Source is an *E. coli* strain that carries the cloned *NcoI* gene from *Nocardia corallina*. Reaction temperature 37°C.

2.1.6.4. *XmaI*

Source is an *E. coli* strain that carries the cloned *XmaI* gene from *Xanthomonas malvacearum*. Reaction temperature 37°C.

2.1.6.5. *NheI*

Source is an *E. coli* strain that carries the cloned *NheI* gene from *Neisseria mucosa heidelbergensis*. Reaction temperature 37°C.

2.1.6.6 T4 DNA ligase

T4 DNA ligate was used for all ligations (Promega).

2.1.6.7 *Pfu* DNA polymerase

DNA polymerase used for all PCR (Promega).

2.1.6.8 1kb plus DNA ladder

A DNA ladder (Thermo Scientific) used for DNA gels, with a range of 75bp to 20,000bp

2.1.6.9 MassRuler low range DNA ladder

A DNA ladder (Thermo Scientific) used for DNA gels, with a range of 80bp to 1,031bp

2.1.6.10 Primers

Primers were designed using GENtle software and ordered from Life Technologies.

2.2. Vectors

Control: pYX222-A_{2A}R (see Appendix 1.1)

Selective advantage: pYX222-A_{2A}R-URA3 (see Appendix 1.2).

Translation process: pYX222-YAP1(IRES)-$A_{2A}R$, pYX222-CrPV(IRES)-$A_{2A}R$ and pYX222-p150(IRES)-$A_{2A}R$ (see Appendix 1.3, 1.4 and 1.5).

2.3. Molecular biology

2.3.1. Miniprep (Fermentas GeneJET Plasmid Miniprep Kit)

A single colony of plasmid harbouring *E. coli* was added to a 15ml Falcon tube containing 5ml LB (2.4.3.5.4.) supplemented with 5µL 100mg/mL ampicillin (2.1.1.2.), and the sample was left to incubate at 37°C with shaking (220rpm) overnight. The next day the culture was centrifuged at 3,000rpm for 5min to pellet cells, the supernatant was discarded and tubes left to drain over paper towels. 250µl resuspension buffer was added to the pellet, and vortexed to resuspend the pellet. 250µl lysis solution was added to the tube, which was then inverted until the solution became clear and viscous. 350µl neutralisation solution was added and the mixture was mixed gently using a pipette, and pipetted into a labelled 1.5ml microcentrifuge tube. The sample was then centrifuged at 13,000rpm for 5min (all micro-centrifugation performed at 13,000rpm), a GeneJET minicolumn was placed into a collection tube. After centrifugation, the supernatant was then removed without disturbing the pelleted white precipitate, and it was pipetted into the minicolumn. The sample was then centrifuged for 1min and the flowthrough was discarded. 500µl wash buffer solution was added and the sample centrifuged for 1min, and flowthrough discarded. An empty column was centrifuged for 1min, the minicolumn was added to a labelled 1.5ml microcentrifuge tube. 50µl of ddH_2O was added directly to resin of the minicolumn, and incubated at 37°C for 4-6min, the spun at to elute the DNA which was stored at -20°C.

2.3.2. NanoDrop 1000

The NanoDrop 1000 spectrophotometer apparatus was used for DNA quantification after a miniprep or PCR, and prior to ligation, transformation or sequencing. The instrument was initialised with 1µl ddH_2O then blanked with 1µl of ddH_2O, then 1µl of DNA sample was added and the reading recorded in ng/µl, which was repeated 3 times to create a reliable average. As DNA absorbs at a wavelength of 260nm, the ratio of 260/280nm was noted to ensure the sample was in the >1.80 region to ensure the DNA is pure.

2.3.3. Polymerase Chain Reaction (PCR) (50µl)

PCR Mix

5µl 10X DNA polymerase buffer

2µl dNTPs (40mM)

15ng Template

1µl Forward primer (10pmol)

1µl Reverse primer (10pmol)

0.5 Herculase (or 1µl Pfu) DNA polymerase

Made upto 50µl with dH_2O

PCR Program

95°C	2min	
95°C	30s	
55°C	30s	10 cycles
72°C	60s/kb of target	
95°C	30s	
55°C	30s	
72°C	60s/kb of target plus 10s/cycle	20 cycles

2.3.4. DNA Restriction digest

Digest reactions were made up to a volume of 50µl and controls to 25µl, so all volumes added were halved for the controls with respect to the main digest. To a 1.5ml microcentrifuge tube, 33µl of water and 5µl of 10XBuffer suitable according to NEB were added. Then 10µl DNA and 1µl (1unit) of each restriction enzyme were added, and the reaction mixture was mixed gently with a pipette. The digest reactions were then left to incubate at 37°C for 2h. When the digest was complete the samples were analysed via agarose gel electrophoresis (1%).

2.3.5. DNA Ligation

Ligation reactions were made up to a volume of 25µl in a 1.5ml microcentrifuge tube, using the equation below.

$$\frac{\text{ng of vector x size of insert (kb)}}{\text{size of vector (kb)}} \text{ X molar ratio of insert/vector = ng of insert needed}$$

The volume of insert and vector DNA was added to dH_20 to a volume of 21.5µl. Then 2.5µl of 10× T4 DNA ligase buffer was added along with 1µl T4 DNA ligase, this reaction

mixture was mixed gently with a pipette and left to incubate at 22-25°C (room temperature) for 3 h. Once the ligation was complete the sample is heated in a heatblock at 65°C for 10min to inactivate the ligase, then 2µl of the reaction was used for subsequent transformations using *E.coli* competent strains DH5alpha, XL-2 Blue or XL-10 GOLD.

2.3.6. DNA Sequencing (Automated Fluorescence; University of Birmingham)

Sample preparation for sequencing; 200-500ng DNA, 3.2pmol primer then made up to 10µl with distilled water. The sample was loaded at the University of Birmingham into a 96-well plate, where the other relevant chemicals were added by staff. Results were obtained via the university's website the following day, and analysed using GENtle.

2.3.7. 1% Agarose gel

1g agarose was made up to 100ml using 1xTAE buffer, this was heated in a microwave until the agarose was completely dissolved. When the mixture was cool enough to touch 5µl ethidium bromide was added. This bound the migrating DNA allowing visualisation using UV. The mixture was poured into gel tray-cast containing combs which created sample loading wells. Once set the tray was placed into the gel tank, and covered in 1xTAE buffer. The combs were removed while submerged to prevent air bubbles. 5µl DNA was mixed with 1µl 6x loading buffer for each sample and loaded in subsequent wells, and 6µl of 1kb+ gene-ruler ladder was loaded. The gel was run at 90V for approximately 45min, after which the gel was visualised using a UV box-camera setup.

2.3.8. Gel Excision

Once the desired gel bands had been identified the gel was placed on a UV box, while taking due care to avoid direct UV exposure (wearing gloves, lab coat and perspex faceguard), and using a scalpel the bands were excised and put into a 1.5ml microcentrifuge tube and stored at -20°C.

2.3.9. Gel and PCR purification (Promega Wizard SV Gel and PCR Clean-up System)

In the case of gel purification, a 1.5ml microcentrifuge tube was weighed then the excised gel slice containing the band of interest was placed inside and weighed again to ascertain the weight of the gel slice. 10µl membrane binding buffer per 10mg of gel slice was added, vortexed and incubated at 50-65°C for 5-10 min or until the gel had dissolved. In the case of PCR, an equal volume of membrane binding buffer was added to the PCR product. A SV minicolumn was place into a collection tube, and the dissolved gel mixture or prepared PCR product was added to the column to incubate at room temperature (22-25°C) for 1 min. The column assembly was centrifuged at 13,000rpm for 1min and the flowthrough was discarded. 700µl membrane wash buffer was added, the sample centrifuged at 13,000rpm for 1 min and the flowthrough discarded. Then 500µl of membrane wash buffer was added, and the sample centrifuged at 13,000rpm for 5 min and flowthrough discarded. The sample was centrifuged again for 1min with no buffer added to allow for evaporation of residual ethanol. The minicolumn was removed from the collection tube used for the wash step, and placed in a new labelled 1.5ml microcentrifuge tube. 50µl nuclease-free water was added directly to the resin of the minicolumn, then left to incubate at room temperature for 1 min. The sample was then centrifuged for 1 min at 13,000rpm and the eluted purified DNA was stored at -20°C.

2.3.10. Genomic DNA extraction

50-100 µL packed volume of yeast cells was resuspend in 200 µL 1xTE and then 200 µL of glass beads was added. 400 µL of PCI (Phenol:Chloroform) and vortexed for 1min, and spin at 13,000rpm for 10 min. Take the aqueous layer, then add 1ml absolute ethanol and spin at 13,000rpm for 10min at 4ºC then recover the pellet. Resuspend the pellet in 200 µL TE, Add 500 µL absolute ethanol, and spin for 20min for 13,000rpm at 4ºC, and keep the pellet. Add 1 mL of 75% ethanol and invert 5-6 times, then spin at13,00rpm for 5min at RT (repeat the wash step).The pellet was left to dry then 100 µL ddH_2O added to resuspend pellet, the DNA was then stored at -20ºC or -80ºC.

2.4 Microbial strains, transformations and culturing conditions

2.4.1 Microbial strains

2.4.1.1 *E. coli*

DH5α was used for vector amplification and XL2-Blue for transformation of ligated products.

2.4.1.2 *S. cerevisiae*

BY4741 (*MATα, ura3Δ0, leu2Δ0, met15Δ0, his3Δ1*) haploid strain is the parental strain of the deletion mutants; *tor1Δ, gcn3Δ and spt3Δ* (from the yeast knockout selection: http://www.thermoscientificbio.com/non-mammalian-cdna-and-orf/yeast-knockout-collection).

2.4.2. Transformation

2.4.2.1 *E. coli*

1.5ml microcentrifuge tubes were pre-chilled on ice and the competent cells thawed on ice, which 50µl of competent cells was added to each chilled tube. Then 1.7µl β-mercaptoethanol was added to each tube, the content of the tube were swirled and left to incubate on ice for 10min, with swirling every 2min. After incubation 1µl plasmid DNA or 2µl ligation mixture (0.1-50ng) was added, tubes were swirled gently and incubated on ice for 30min. After incubation the tubes were heat shocked at 42°C for 45s in a water bath, then incubated on ice for 2min. Then add 0.9ml LB to each of the tubes and incubate at 37°C for 1hour with shaking. After incubation 100µl of the transformants was plated on an LB amp plate, the remaining sample to be stored at 4°C.

2.4.2.2. *S. cerevisiae* (LiOAC method)

5ml YPD was inoculated with one colony and grown till mid log phase, cells were pelleted at 5000rpm for 3min. Pellet was resuspended with 500µl sterile water, and pelleted again, then resuspended in 500µl 100mM lithium acetate (LiOAc) and transferred to an 1.5mL microcentrifuge tube. Cells pelleted at 13,000rpm for 15s, 100µl of 100mM LiOAc added and vortexed. 50µl of resuspension was taken and pelleted at 5000rpm for 2min. Then added in this order was; 240µl of 50% PEG, 36µl of 1M LiOAc, 25µl salmon testes DNA (boiled at 100°C then chilled prior), 50µl dH_2O and 3µl vector DNA. Incubated at 30°C for

30min and heat-shocked at 42°C for 20min, centrifuged at 6000rpm for 15s and supernatant removed. Pellet resuspended in 800µl dH_2O and 100µl of that used to plate on appropriate selection media plates, according to vector selection marker.

2.4.2.3. Glycerol stocks

50% glycerol was made by adding 50mL water to 50mL glycerol and the solution was autoclaved. 1mL overnight yeast culture was put in cryovial with 1mL 50% glycerol, mixed, and stored at -80°C.

2.4.3 Media

2.4.3.1. Complete Synthetic Medium (CSM; 1L)

This solution was prepared by adding 1.7g yeast nitrogen base (without amino acids), 5g $(NH)_2SO_4$, 20g agar (omit for liquid) was added and made up to 950ml with distilled H_2O (or 850ml and additionally 100ml 10xDO (2.1.1.4-2.1.1.6) for selective medium e.g. CSM -uracil, CSM -histidine-uracil and CSM –histidine -uracil), then autoclaved and 50ml of 40% glucose added.

2.4.3.2. YPD (1L)

This solution was prepared by adding 20g peptone, 10g yeast extract, 14g agar (omit for liquid) and made up to 950ml with distilled water, then autoclaved and 50ml of 40% glucose added.

2.4.3.3. L-Broth (LB; 1L)

This solution was prepared 5g NaCL, 5g yeast extract, 10g tryptone, 15g agar (omit for liquid and made up to 1 L with distilled water. For LB-Amp, add ampicillin to a final concentration of 100mg/L (to achieve 1000µL of 100mg/ml ampicillin was added).

2.4.4. Culturing conditions

2.4.4.1. Inoculation

A single colony of yeast was added to a 5ml universal tube containing 1ml YPD, grown overnight at 37°C with shaking at 220rpm.

2.4.4.2. Shake flask cultures (1:5 volume) (*S. cerevisiae)*

A single colony (as per 2.4.4.4) or inoculate was added to a 5ml universal tube containing 1ml YPD, then incubated at 30°C with shaking at 220rpm and grown until the required $0D_{600}$ was achieved.

2.4.4.3. Culturing (1:5 volume) (*E. coli*)

For a shake flask culture a single colony was added, then incubated at 37 °C with shaking at 220rpm overnight for approximately 16-19 h.

2.4.4.4 A1 and SA transformant culture procedure

For the A1 control; each experiment (immunoblot, ligand binding, confocal) was conducted at least 3 times, and for which a fresh transformation was performed and a colony randomly picked and the values from all the replicate were represented by the mean. For H1 and SU1 and U1, as they were generated through the SA strategy they were always obtained from glycerol stocks of the original selected colony that was stored at -80°C, experiments were also conducted at least 3 times and the values from all the replicates were represented by the mean.

2.4.4.5. Cell viability

The culture was diluted to OD_{600} and 2µL of culture was added to a microscope slide and mixed with 2µL trypan blue solution. Then 100 cells were counted at 100× magnification and the number of stained cells determined, each determination was done in triplicate.

2.4.4.6 Determination of uracil requirement

Dilutions of the standard 1,700µM concentration of uracil were made up (1,700µM (100%), 170µM (10%), 17µM (1%), 1.7µM(0.1%)), they were used to supplement 5ml CSM –uracil cultures. The cultures were inoculated at a starting O.D 0.01 and grown for 16h and the O.D recorded.

2.5. Protein expression and analysis

2.5.1. Membrane preparations

Membrane preparations, when 100ml flasks were grown, an Emulsiflex-C3 (C3) cell disrupter was used to homogenise the cells. Cells were resuspended in ice cold breaking buffer (2:1 buffer to cells). Protease inhibitor cocktail IV set was added to cells (1:2000 dilution), and the cells were passed through the C3 with a chilled heat exchanger for 20min at a homogenising pressure of 30000 psi. The cells were observed under a light microscope to assure the cells were homogenised, typically there was approximately 90% cellular disruption. The samples were at 10000 × g for 30 min to remove cellular debris and unbroken cells, the supernatant was transferred to ultra-centrifuge tubes and centrifuged at 100000 × g for 1 h. The pellet was then re-suspended in ice cold buffer A and stored at -80°C.

2.5.2. BCA assay (protein concentration determination; bicinchoninic acid assay)

The protein concentration from membrane preparation and cell lysates were determined using a BCA assay, using 1mg/ml bovine serum albumin (Sigma) at concentrations 0, 2, 4, 6, 8, 10µg as the linear standard. The protein quantification solution was made up using 1:50 4%(w/v) copper (II) sulphate solution (Sigma) added to BCA solution (Sigma) with 200µL of the solution added to each well in a clear plastic 96 well plate. Then 2µL of sample was added to the solution (repeated in triplicate to obtain the mean), and the plate was read at 570nm using a plate reader (if the reading was outside the range of the standard, the samples were diluted and the experiment repeated).

2.5.3. SDS-PAGE

Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate proteins from supernatant and membrane preparations sample according to their molecular weight. 10% SDS separating gels and 4% SDS stacking gels were used. TEMED (Tetramethylethylenediamine) and Ammonium persulphate (made fresh) added last. Isopropanol was added on top of the separating gel while it set to ensure the gel leveled out flat, and was washed away with distilled water. Samples were loaded using 5 x Laemmli buffer, and 25µg or 50µg of the protein sample (determined by BCA assay) and heated to 50°C for 5min on a heat-block, and then loaded into the gel with a prestained

protein ladder (Fermentas). A 1 x SDS running buffer was added and a current of 150V was applied for 1h.

2.5.4. Coomassie staining

The SDS-PAGE gel was submerged in 50ml Bio-Safe™ Coomassie Stain (Bio-Rad) and incubated while shaking for 1hr at room temperature (bands are visible after 20min). The stain was discarded and the gel was then rinsed in 200ml of distilled water for 30min. Images were then taken of the gel using a camera.

2.5.5. Immunoblot

Immunoblots (western blots) were then performed of the SDS-PAGE gels, transferring the proteins to nitrocellulose membranes. The nitrocellulose was blocked with PBS with 5% milk (Marvel milk powder), and incubated while shaking for 1hr at room temperature (RT). The nitrocellulose was then washed with PBST 3 times for 15min at RT, and then PBS with 5% milk was added along with primary rabbit anti-His6 monoclonal antibody (Serotec) to a dilution of 1:5000 and incubated for 1hr at RT. The nitrocellulose was then washed with PBST 3 times for 15min at RT, and then PBS with 5% milk was added along secondary mouse anti-rabbit HRP-conjugate antibody to a dilution of 1:5000 and incubated for 1hr at RT. The nitrocellulose was then washed with 4mL EZ-ECL Enhanced Chemiluminescence Detection Kit for HRP (Biological Industries) for 4min in a dark room. The nitrocellulose was then exposed for 15min, and viewed using the Chemidoc system (UVItech). The immunoblot images were analysed using ImageJ software.

2.5.6. Solubilisation of yeast membranes using DDM

Solubilisation with n-dodecyl-ß-D-maltoside (DDM) was performed with the following solubilisation buffer: 20 mM HEPES, pH 7.4, 50% glycerol, 250 mM NaCl, 1 µL protease inhibitors, 10% (w/v) DDM (2.1.5.1) and 2% (w/v) cholesteryl hemi-succinate (CHS)(2.1.5.2). The membrane fraction added to the solubilisation buffer for a 1:1 ratio where the starting concentration was ~ 1mg mL^{-1} and therefore the final concentration in the solubilisation was ~ 0.5mg mL^{-1}. After incubation with slow rotation at 4°C for 4 h, the sample was centrifuged at 100000 × g for 1 h. The supernatant contained DDM solubilised $hA_{2A}R$ and was stored at 4°C for further studies for a maximum of 3 days.

2.5.7. Gel filtration

Illustra G50 gel filtration columns (GE Healthcare) were used.

2.6. Radio-ligand binding assays

A radio-ligand binding involves labelling compounds with radioactive isotopes, the compound typically being a ligand that will bind the receptor of interest (Leach et al., 2010). Tritium [^{3}H] is the radiolabel used due to its having a long half-life of 12.3 years (Lucas & Unterweger, 2000). All experiments involving radio-ligand binding were carried out using tritiated ZM241385 ([3H]ZM241385) (ARC), a high-affinity $hA_{2A}R$ antagonist (Jaakola et al., 2008).

2.6.1. Single-point saturation

A single-point binding assay was used as initial experiment to determine if the $hA_{2A}R$ was folded correctly. A single, high concentration of 10nM [^{3}H]ZM241385 (therefore saturating the receptors with ligand) was used (Table 2.1), a concentration known to be at the top of a saturation curve for the $hA_{2A}R$. This allows calculation of the specific binding (total binding subtracted by the non-specific binding), but as a full curve has not been performed this is effectively a B_{max} estimate.

	Total Binding (T)			Non-specific binding (NS)			
Total membrane (µg)	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Binding buffer (µL)**	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Unlabelled ZM241385 (µM)**	**Binding buffer (µL)**
100	10	0.1	up to 500	10	0.1	1	up to 500

Table 2.1 Single-point saturation binding reaction preparation for membrane bound $hA_{2A}R$. The reaction uses 10nM [^{3}H]ZM241385, adenosine deaminase, unlabeled ZM241385 (for NS), and binding buffer.

2.6.1.1. Single-point binding for membrane bound $hA_{2A}R$

The binding reactions were prepared (as shown in Table 2.1) in 1.5mL microcentrifuge tubes, the reactions were mixed by inverting the tubes, then incubate for 1.5h at room temperature (RT) on the bench. Then centrifuged at 14000rpm for 5 min at 4°C (and supernantant removed), the pellets were washed carefully with tap water using pipette (with excess water removed carefully with tissue being careful not to disturb the pellet). Then 100µl soluene was added (in a fume hood) to dissolve the pellet, and incubated for 1.5h at RT on bench. Then transferred from microcentrifuge tubes to scintillation tubes, 4ml scintillant added and mixed well by inversion. The scintillation tubes were added to counting vials place in the scintillation counter (Packard 1600TR Liquid Scintillation Analyser) for counting.

2.6.1.2. Single-point binding for solubilised $hA_{2A}R$

For single-point binding analysis of solubilised $hA_{2A}R$ (2.5.6), 120µl (60µg) of the solubilised total membranes were used and the reaction prepared (Table 2.2) in 1.5mL microcentrifuge tubes. The reactions were mixed by inverting the tubes, then incubated for 1.5h at room temperature (RT) on the bench. The reaction was added to Illustra G50 gel filtration columns (GE Healthcare) (2.5.7) (which had been washed 3 times previously with 500µl binding buffer) and then centrifuged at 3000 rpm for 2 min and the eluate transferred to scintillation tubes. Then 4ml scintillant was added and mixed well by inversion, and the tubes were added to counting vials and placeed in the scintillation counter (Packard 1600TR Liquid Scintillation Analyser) for counting.

	Total Binding (T)			Non-specific binding (NS)			
Solubilsed membranes (µg)	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Binding buffer (µL)**	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Unlabelled ZM241385 (µM)**	**Binding buffer (µL)**
60	10	0.1	up to 200	10	0.1	1	up to 200

Table 2.2 Single-point saturation binding reaction preparation for solubilised $hA_{2A}R$ from total cell membranes. The reaction uses 10nM [^{3}H]ZM241385, Adenosine deaminase, unlabeled ZM241385 (for NS), and binding buffer.

2.6.2. Competition curve binding (homologous)

Competition binding assays allow the study of receptor interactions while a single constant radiolabelled ligand is in the presence of an increasing competition (unlabelled ligand) under equilibrium conditions (Leach et al., 2010). Homologous competition was used to calculate the specific binding (total binding subtracted by the non-specific binding), at various concentration of unlabelled ZM241385 while the radiolabelled [3H]ZM241385 stays at a low constant concentration (2nM). The specific binding measurements allow a homologous competition curve to be generated, to which the Cheng-Prusoff equation was applied to a one-site binding model. This allowed the calculation of the K_d and pK_a to determine $hA_{2A}R$ affinity (Leach et al., 2010).

$$K_d = K_i\, EC_{50} - [\text{Radio-ligand}]$$

With K_i = dissociation constant and is equivalent to K_d for homologous curves; EC50 = half the effective concentration of the unlabelled ZM241385 and [Radio-ligand] = concentration of the [3H]ZM241385.

The binding reactions were prepared as shown below in Table 2.3, using the method described in section 2.6.1.1.

Membrane (µg)	Final [^{3}H]ZM241385 concentration (nM)	Unlabelled ZM241385 (nM)	Adenosine deaminase (U)	Binding buffer (µL)
100	2	1000	0.1	up to 500
100	2	100	0.1	up to 500
100	2	10	0.1	up to 500
100	2	1	0.1	up to 500
100	2	0.1	0.1	up to 500
100	2	0.001	0.1	up to 500
100	2	0	0.1	up to 500

Fig 2.3 Homologous competition binding reaction preparation for solubilised $hA_{2A}R$ from total cell membranes. The reaction uses 10nM [^{3}H]ZM241385, Adenosine deaminase, unlabeled ZM241385 (for NS), and binding buffer.

2.6.3. Saturation binding curve

Saturation binding is used to discover by direct measurement the total amount of receptors present in the sample under investigation by determining the amount of occupied binding sites (B_{max}), and also to determine the affinity of said receptors known as the equilibrium constant (K_d) (Leach et al., 2010). This is achieved by measuring the specific binding (total binding subtracted by the non-specific binding) at various concentrations of radio-ligand [^{3}H]ZM241385. The binding reactions were prepared as shown in Table 2.4, using the method described in section 2.6.1.1. Non-linear regression was used to determine the B_{max} using GraphPad Prism® 4 software (2.8.2).

	Total Binding (T)			**Non-specific binding (NS)**			
Total membrane (µg)	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Binding buffer (µL)**	**Final [^{3}H]ZM241385 concentration (nM)**	**Adenosine deaminase (U)**	**Unlabelled ZM241385 (µM)**	**Binding buffer (µL)**
100	0.5	0.1	up to 500	0.5	0.1	1	up to 500
100	1	0.1	up to 500	1	0.1	1	up to 500
100	2.5	0.1	up to 500	2.5	0.1	1	up to 500
100	5	0.1	up to 500	5	0.1	1	up to 500
100	10	0.1	up to 500	10	0.1	1	up to 500
100	20	0.1	up to 500	20	0.1	1	up to 500
100	50	0.1	up to 500	50	0.1	1	up to 500

Table 2.4 Saturation curve binding reaction preparation for membrane bound $hA_{2A}R$. The reaction uses 10nM [^{3}H]ZM241385, Adenosine deaminase, unlabeled ZM241385 (for NS), and binding buffer.

2.7. Confocal Microscopy (Indirect Immunofluorescence Microscopy of Yeast Cells)

2.7.1. Cell Fixing and Permeabilisation

5 mL CSM/YPD was inoculated and grown for 19h . 500 µl of formaldehyde was added to the culture and left to shake for an additional 1.5 hours at 30°C. 1 OD600 (1ml of OD_{600}=1) was removed of cells and wash twice in 1 mL PBS/0.5% Tween-20 (PBST). Cells were resuspended in 0.5 mL 50 µg/mL zymolase diluted in PBST. (2 µl stock zymolase for every 0.5mL of PBST). Then incubate for 20 minutes at 37°C. Care was taken not to overincubate as proteolytic contaminants in the zymolase can ruin the sample. The washed three times in 1 mL PBST. From this point, cells to be spun no faster than 6,000 rpm to avoid breaking open the spheroplasts.

2.7.2. Cell Staining

The cells were resuspended in a dilution of primary antibody in 20 µl of PBS/4%BSA. (10 µg/mL his_6-anti-mouse [Clontech] (0.4 µl). Incubated for 1h at room temperature. Washed one time in 500 µl PBS/BSA then incubated for 15min in 500 µl PBS/BSA. Washed one more time in PBS/BSA then resuspended in secondary antibody diluted in 20 µl PBS/BSA. (Use 40 µg/mL of Alexa488-conjugated goat-anti-mouse antibody (0.4 µl). Then incubate 1h at room temperature in the dark. Then washed and incubated in PBS/BSA for 15min as described above. Then spun down, and resuspended in ~5 µl of PBS.

2.7.3. Slide Preparation

3µl of Fluoroshield™ mounting solution (Sigma) was added cover slip attached. Nail polish then applied to cement slip to slide.

2.8. Software packages

2.8.1. GENtle

GENtle was used for planning and design of molecular biology experiments.

2.8.2. Graphpad Prism

GraphPad Prism® 4 was used for radio-ligand binding analysis, statistical analysis and tabulation of results from yield analysis.

2.8.3. ImageJ

ImageJ software was used for quantification of immunoblots using densitometry.

2.9. Equipment

All equipment used was standard laboratory equipment, and is referred to in the methodology. The centrifuges are listed below.

- Beckman Coulter OptimaTM L-80XP Ultracentrifuge (Maximum revolutions per minute (rpm): 80,000)
- Beckman Coulter OptimaTM TLX Ultracentrifuge (Maximum rpm: 120,000)
- Beckman Coulter Allegra 25R Centrifuge (Maximum rpm: 15,000)
- Fisher Scientific AccuspinTM MicroR, benchtop (Maximum rpm: 13,000)

Chapter 3: Employing Selective Advantage to improve $hA_{2A}R$ Yield

3.1 Summary of experimental objectives

3.1.1 Rationale behind the selective advantage strategy

In order to give yeast cells a selective advantage (SA) in expressing $hA_{2A}R$ in its membranes, we fused it to a fusion partner that would confer the SA. The fusion partner chosen was the enzyme orotidine 5´phosphate decarboxylase (ODCase, which will be referred to as Ura3p) specifically the form from the yeast *S. cerevisiae*, encoded for by the *URA3* gene. This particular decarboxylase catalyses the reaction whereby orotidine 5-monophosphate (OMP) is converted to uridine 5-monophosphate (UMP)(Fig.3.1).

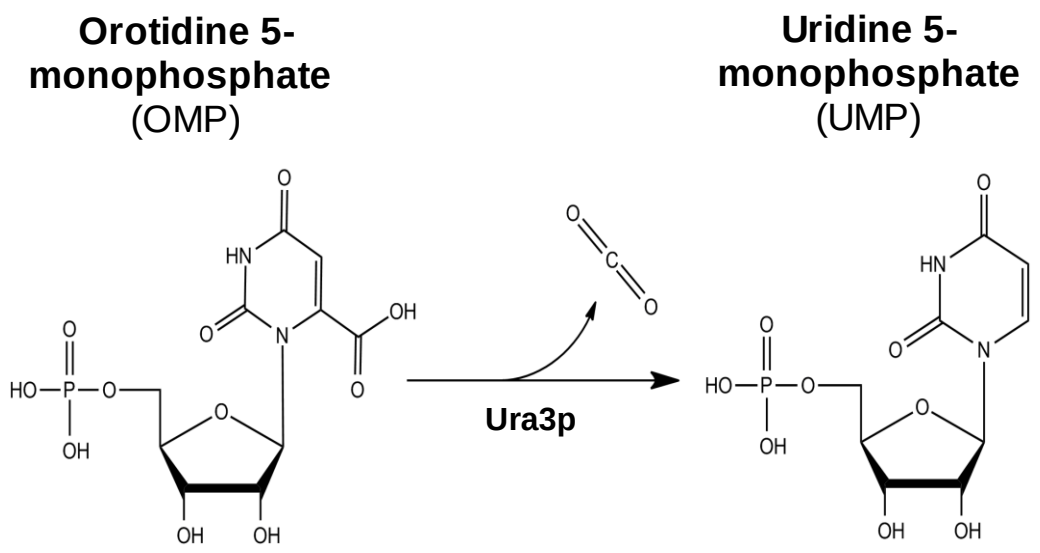


Figure 3.1 Mechanism of Ura3p. *Scheme depicting the decarboxylation of OMP to UMP, catalysed by the enzyme ODCase encoded for by* URA3*. This is the last step of* de novo *biosynthesis of the essential major uracil precursor UMP.*

The gene product of the *URA3* gene is a commonly used selection marker in yeast, essential for the production of uridine, a nucleoside of uracil (Houk et al., 2001, Raugei et al., 2004, Hu et al., 2008) which in turn is essential for RNA synthesis and thus survival and growth (Brachmann et al., 1998). In our SA strategy yeast cells lacking this essential enzyme must produce the fusion protein for survival when grown on nutrient media lacking uracil as is the case for the *S. cerevisiae* strain BY4741 which is auxotrophic for

histidine, leucine, methionine and uracil (Brachmann et al., 1998). Subsequently if all those nutrients are provided except uracil, a selection pressure is created which should drive the efforts of the cells to produce the fusion and thus facilitate the production of the needed uracil.

Ura3p is known to be able to enhance the rate of the reaction shown in Fig 3.1 by a factor of 10^{17} (Radzicka & Wolfenden, 1995). It also requires no co-factors or metals ions which is a common pre-requisite for decarboxylating enzymes, but relies entirely upon non-covalent bonding (Radzicka & Wolfenden, 1995, Miller & Wolfenden, 2002). The crystal structure revealed that Ura3p in its active form is a dimer, which is composed of two α/β-barrels with 2 active sites which are shared (Harris et al., 2000). It is believed that Ura3p possesses substantial flexibility in terms of its conformation provided by its several loops, allowing vital binding interactions (Miller & Wolfenden, 2002, Wu et al., 2000). These findings suggested that fusing Ura3p to $hA_{2A}R$ would not affect its function and ability to confer SA. Furthermore, when Ura3p was expressed from a *HIS4C-URA3* fusion in *S. cerevisiae*, the enzyme was shown to function correctly allowing growth (Alani & Kleckner, 1987). The aim of that study was to ascertain which mutations decreased or increased Ura3p function, whereas the experiments carried out throughout this thesis use Ura3p to drive protein production yields. Nonetheless this previous result sets a precedent for the use of a Ura3p fusion to select for specific phenotypes.

The fusion was expected to be expressed in the manner illustrated in Fig 3.2, with Ura3p being located on the intracellular C-terminus of $hA_{2A}R$. This is an essential characteristic as Ura3p is a cytosolic enzyme, and can only catalyse the reaction shown in Fig 3.1 when in contact with the intracellular space (Metzger et al., 2008). This configuration leaves the Ura3p C-terminus free of any steric hindrance, as fusion at the C-terminus of Ura3p has been reported to increase instability and prevent *S. cerevisiae* cells from expressing the selection marker when grown in a medium lacking uracil (Gilon et al., 1998).

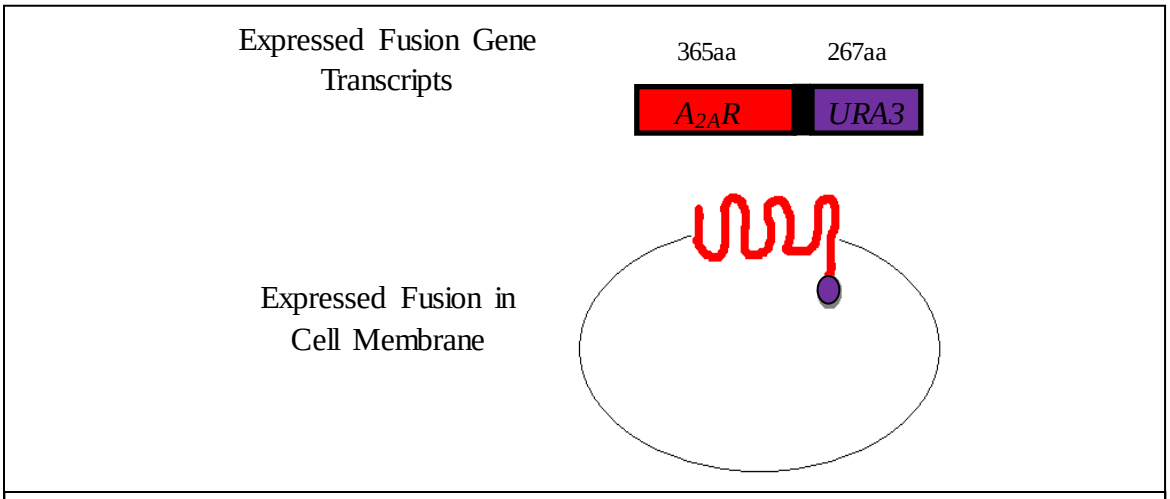


Figure 3.2 Expected expression trait selected for by SA. *The fusion protein was designed to be expressed from* A_{2A}R-URA3 *mRNA as a* $hA_{2A}R$*-Ura3p fusion. The fusion protein was expected to comprise the* $hA_{2A}R$ *partner (red) inserted into the yeast cell membrane and the Ura3p partner (purple) fused to its C-terminus and being presented intracellularly to confer the SA.*

3.1.2 $A_{2A}R$ construct

The $A_{2A}R$ gene construct used encoded a truncated glycosylation deficient form of $hA_{2A}R$ designed by Niall Fraser (Fig 3.3, red), which had the glycosylation site Asn154 mutated to Gln, which prevents hyper-glycosylation in yeast (Fraser, 2006). The $A_{2A}R$ construct was previously used by researchers to produce sufficient quantities of $hA_{2A}R$ to begin crystallisation studies (Fraser, 2006) so was a validated variant. The $A_{2A}R$ gene was preceded by a yeast consensus sequence (Fig 3.3, dark grey) thought to enhance translation initiation (Wang et al., 1998, Robbins-Pianka et al., 2010), and a precursor of the yeast mating pheromone α-factor secretion signal (Fig 3.3, light grey), which is routinely employed when expressing recombinant proteins in yeast (Brake et al., 1984). The α-factor signal allows the translocation of the fusion protein to the cell membrane, as it is involved in facilitating export from the site of synthesis to the site of release in the cell membrane (Bitter et al., 1984, Brake et al., 1984). For purification, a sequence encoding a His_{10} tag was inserted as shown (Fig 3.3, green).

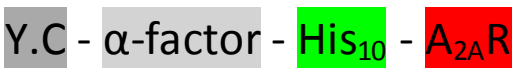


Fig 3.3 Schematic illustrating the $A_{2A}R$ ***construct cloned into pYX222 which was used for the SA strategy.*** *The* $A_{2A}R$ *variant was devised by Niall Fraser and is a truncated form that has had the glycosylation site removed (red). It is preceded by a yeast consensus sequence (Y.C) (dark grey) for enhanced translation initiation, α-factor secretion signal (light grey) for export of the recombinant protein to the cell membrane, and a sequence encoding a* His_{10} *tag (green) for purification.*

3.1.3 Objectives

The specific objectives of Chapter 3 were therefore:

1. To design and construct a SA vector;
2. To generate SA transformants;
3. To examine the $hA_{2A}R$ yield and characteristics from the SA transformants.

3.2 Vector construction

3.2.1 Cloning strategy: pYX222-hA_{2A}R-URA3

The cloning strategy is illustrated in Figure 3.4. The pYX222 backbone contains a *HIS3* selection marker that can be used to select for colonies maintaining the plasmid in histidine deficient medium. Introduction of the *URA3* sequence in fusion with the *$A_{2A}R$* coding sequence additionally allowed for application of SA on uracil deficient medium.

3.2.2 Construction of pYX222-A2AR-URA3

In order to insert the *URA3* gene downstream of the *$A_{2A}R$* coding region in plasmid pYX222-A_{2A}R (the kind gift of Dr Richard Darby) to create a fusion protein, it was necessary to disrupt the *$A_{2A}R$* stop codon (as illustrated in Fig 3.4). Extensive attempts were made to use conventional cloning approaches to remove the stop codon in pYX222-A_{2A}R. Unfortunately, errors in the vector map for pYX222-A_{2A}R resulted in three independent strategic approaches yielding incorrect inserts. It was therefore decided, due to the low cost of emerging gene synthesis technologies, to use gene synthesis to expedite the vector construction. The *$A_{2A}R$* sequence (3′ minus TGA)-linker-*URA3* insert (Fig 3.4) was therefore designed using GENtle software (www.gentle.magnusmanske.de), synthesised by GeneArt (Life Technologies) and was delivered pre-cloned into a stock vector designated pLAU. For the new insert's target site to replace the stop codon of the *$A_{2A}R$* coding region, *XmaI*, which was the most extreme unique restriction site 3′ of *$A_{2A}R$*, was chosen being located 241bp upstream of the stop codon. The next unique restriction site after the *$A_{2A}R$* stop codon was *NheI*, located in the backbone of the plasmid 352bp away. The pLAU plasmid, was transformed into XL2-Blue supercompetent *E. coli* to amplify the DNA and acquire a stock; analysis of pLAU was performed via gel electrophoresis which confirmed a product of approximately 3,000bp (Fig 3.5A).

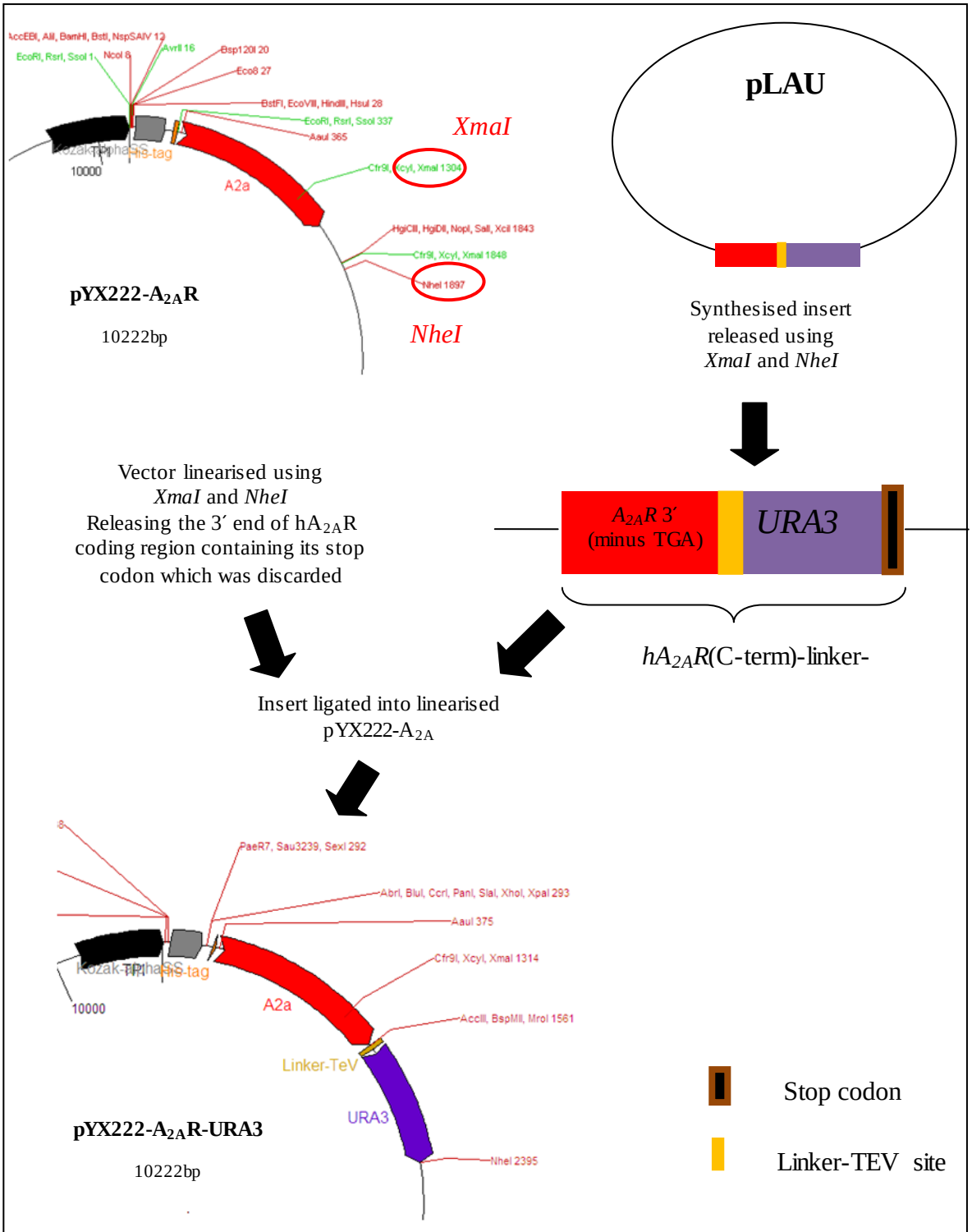


Fig 3.4 Schematic illustrating the cloning strategy devised to create the SA vector pYX222-A_{2A}R-URA3. *An insert was synthesised (GeneArt from Life Technologies) and inserted into a standard vectors (designated pLAU). The insert consisted of the 3´A_{2A}R with its stop codon removed, and* URA3. *The A_{2A}R (3´minus TGA)-linker-URA3 was released from pLAU using* XmaI *and* NheI. *The vector containing the Fraser variant pYX222-A_{2A}R was cut with* XmaI *and* NheI *to release the 3´end of the original A_{2A}R (containing its stop codon), the synthesised A_{2A}R (3´minus TGA)-linker-URA3 insert was then ligated into pYX222-A_{2A}R producing pYX222-A_{2A}R-URA3 (with the stop codon removed, the A_{2A}R coding region will be read-through until the stop codon present in the URA3, producing a hA_{2A}R-Ura3p fusion protein with a linker-TEV portion between the two proteins).*

The pLAU miniprep product was double digested using *XmaI* and *NheI* to release the *$A_{2A}R$* (3′ minus TGA)-linker-*URA3* insert at 1087bp (Fig 3.5B, lane 2), which was validated by comparison with an uncut control (Fig 3.5B lane 1). The released insert was excised and purified. The pYX222-A_{2A}R was digested with *XmaI* and *NheI* which released a 700bp (Fig 3.5C, lane 2) insert, creating a suitable accepting backbone for the *$A_{2A}R$* (3′ minus TGA)-linker-*URA3* insert (as per the cloning strategy shown in Fig 3.5), against an uncut control (Fig 3.5C, lane 1).Double digested pYX222-A_{2A}R was excised and purified ready for ligation with the purified *$A_{2A}R$* (3′ minus TGA)-linker-*URA3*.

The purified *$A_{2A}R$* (3′ minus TGA)-linker-*URA3* and pYX222-A_{2A}R(double digested) were ligated using a 3:1 vector to backbone ratio, and the subsequent ligation mixture used to transform XL-2 blue super-competent bacteria (as described in 2.4.2.1). Four colonies from the transformation were grown and minipreps performed to obtain the vector DNA. A restriction digest was then performed on the vector DNA using the restriction enzymes used to sub-clone the insert (*XmaI* and *NheI*) to confirm the insert's presence, correct size and ensure the ligation has been successful (Fig 3.5D). All colonies released an insert of 1,087bp which was the correct size for *$A_{2A}R$* (3′ minus TGA)-linker-*URA3* (Fig 3.5D, black arrow). In the case of colony 2 the backbone DNA retrieved was 2,500bp in size (Fig 3.5D, lane 2, red arrow) when it should have been approximately 10kbp and therefore this colony was discarded. The vector DNA from each of colonies 1, 3 and 4 was sequenced and found to be correct. The DNA from colony 1 was taken forward as the pYX222-A_{2A}R-URA3 selective advantage vector.

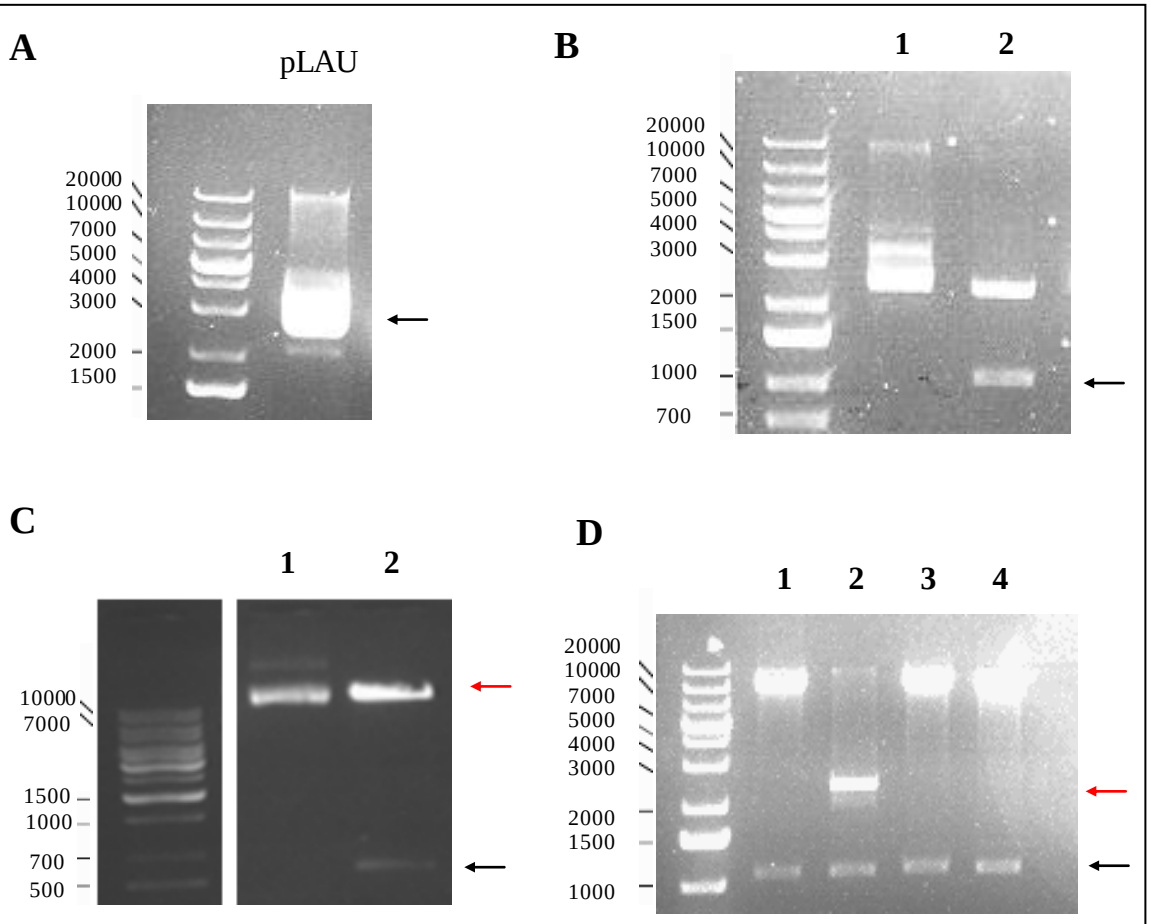


Fig 3.5 (A) Construction of pYX222-A_{2A}R-URA3. *A 1% agarose gel DNA electrophoresis gel was performed to validate the synthesised plasmid and to ensure correct retrieval of the* A_{2A}R *(3´minus TGA)-linker-*URA3 *insert.* ***(A)*** *The pLAU plasmid which contained the synthesised* A_{2A}R *(3´minus TGA)-linker-*URA3 *insert (GeneArt, Life Technologies) was transformed into XL2-Blue supercompetent* E. coli *and three separate minipreps performed. A band of approximately 3,000bp (arrow) representing the pLAU miniprep product was observed (confirmed using supplementary documentation supplied by Life Technologies).* ***(B)*** A_{2A}R *(3´minus TGA)-linker-*URA3 *insert was released from pLAU plasmid via double digestion using* XmaI *and* NheI*, confirmed as a product of 1,087bp. The insert was excised from the gel (arrow), the gel slices were pooled and purified ready for ligation into pYX222-A_{2A}R.* ***(C)*** *A 1% agarose DNA electrophoresis gel was performed to analyse whether the pYX222-A_{2A}R vector was correctly prepared for ligation with the* A_{2A}R *(3´minus TGA)-linker-*URA3 *insert. The vector was digested with* XmaI *and* NheI *and a correct insert release of 700bp confirmed correct digestion (black arrow); the backbone of the cut vector (red arrow) was excised and purified ready for ligation with the* A_{2A}R *(3´minus TGA)-linker-*URA3 *insert* ***(D)*** *Transformants from the newly created pYX222-A_{2A}R-URA3 were analysed via restriction digest for correct insert release of 1,087bp to confirm correct insertion resulting from the ligation. All transformant colonies produced the correct result except 2 (red) which had an erroneous starting plasmid size of 2,500bp. Samples 1, 3 and 4 were sequenced and found to be correct.*

3.3 Employing SA to generate transformants

3.3.1 Generating transformants by SA using pYX222-A_{2A}R-URA3

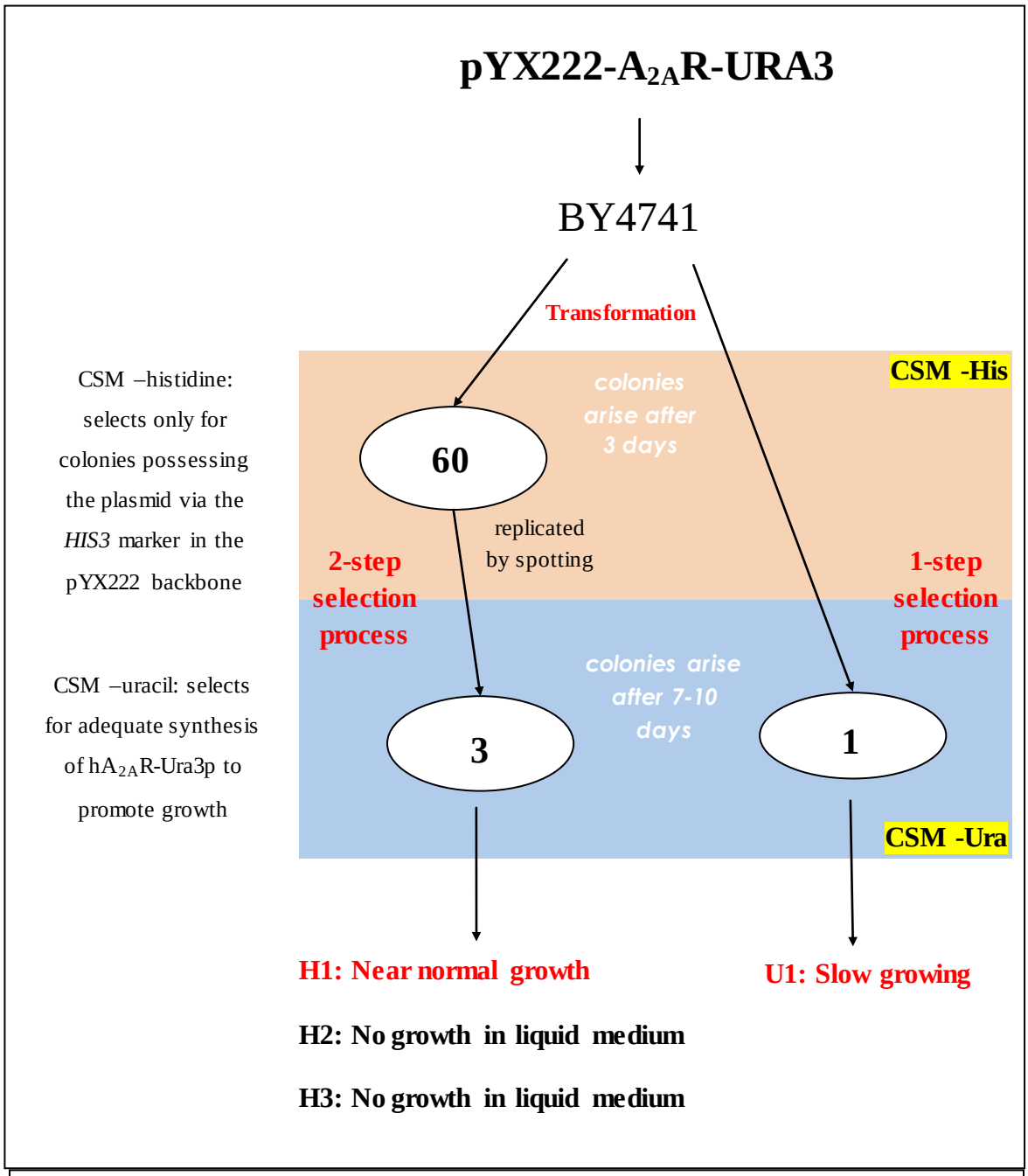


Fig 3.6 Scheme depicting the generation of SA transformants using pYX222-A_{2A}R-URA3. *The selection process through which transformants were generated was achieved using both a 1-step and a 2-step process. In the 1-step process, yeast were grown on uracil-deficient medium (CSM –uracil) immediately following the transformation process. In the 2-step process, yeast were grown on histidine-deficient medium (CSM–histidine) after transformation and then colonies were spotted from CSM –histidine to CSM –uracil. As the transformation process is stressful to cells, we expected the two-step to produce more viable transformants. The 1-step process generated 1 colony (U1) which was slow growing, while the two-step process generated 3 colonies (H1, H2 and H3); H2 and H3 were not viable in liquid medium while H1 had normal growth.*

The 1-step process generated 1 colony designated U1 (uracil 1); this transformant grew particularly slowly (Fig 3.6); two further biologically-independent attempts to generate colonies in this manner yielded no colonies. The two-step method generated 3 colonies yet only one of them (Fig 3.6), which was designated H1 (histidine 1), could be cultured (Fig 3.6), as such H2 and H3 were not used for further study. H1 and U1 were therefore taken forward for further studies to ascertain their yield characteristics together with the control, A1 (Fig 3.7).

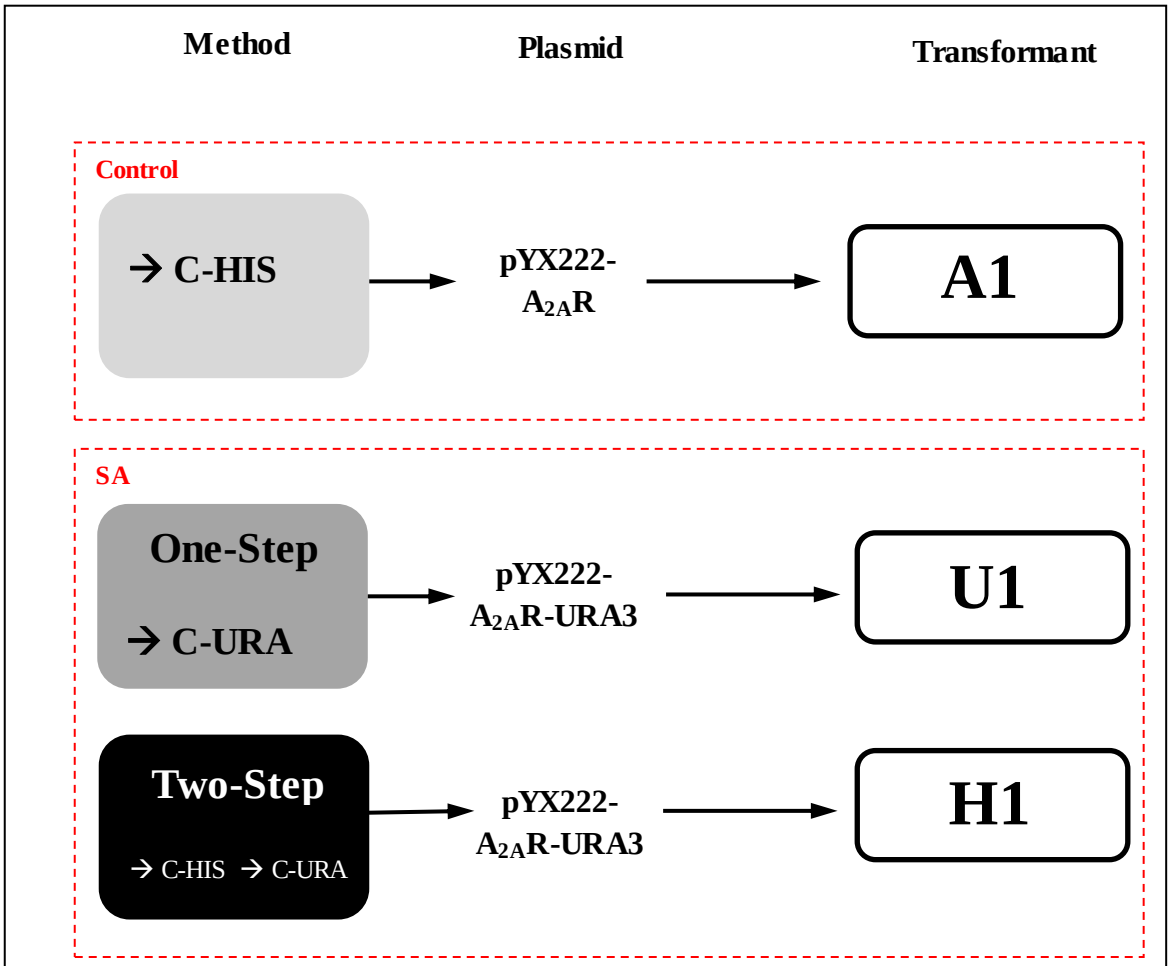


Fig 3.7 Scheme illustrating the generation of the 3 transformants A1, H1 and U1 that were used for the SA strategy study. *Plasmid pYX222-A_{2A}R was transformed into* S. cerevisiae *BY4741 WT to produce A1 which was the non-SA control. pYX222- A_{2A}R-URA3 was used to generate transformants U1 and H1 using one-step and two-step methods respectively, as shown in Fig. 3.6. For the A1 control fresh transformations were performed and a biologically independent colony picked randomly for each experiment. For H1 and U1, the glycerol stocks were prepared of these transformants and used in all subsequent experiments.*

3.3.2 Determination of the uracil requirement of H1

An experiment was devised to assay the uracil requirement of H1, with A1 as the control.

	Growth OD_{600} at 16h	
Uracil (µM)	**A1**	**H1**
1,700µM (100%)	3.0	3.1
170µM (10%)	2.7	2.8
17µM (1%)	1	2.9
1.7µM (0.1%)	0.3	2.7
0.0µM (0%)	0.0	2.8

Table 3.1 Analysis of A1 and H1 growth in varying concentrations of uracil. *The pYX222-$A_{2A}R$ transformant, A1, and the pYX222-$A_{2A}R$-URA3 transformant, H1, were cultured in the CSM –uracil drop out medium supplemented with uracil as shown; 1,700µM is the typical concentration of uracil added (100%) to complete CSM. 5ml cultures were started at an OD_{600} 0.01 and grown for 16h. H1 was able to sustain growth in all condition, most importantly in the absence of uracil (0.0%), whereas the control A1, which lacks the* URA3 *gene, cannot grow in the absence of uracil. Surprisingly the results suggest a huge excess of uracil is used in drop out medium because A1 had near normal growth at 170µM uracil (values are the mean of n=2).*

Table 3.1 suggests H1 produced adequate $hA_{2A}R$- Ura3p to sustain growth in medium lacking uracil, while A1 showed no growth under the same conditions (Table 3.1). Interestingly it appeared that the uracil typically supplemented in CSM (1,700 µM) is in large excess because A1 could grow in 17µM uracil (albeit to a lower biomass yield than in 1,700 µM uracil). This excess may be to prevent uracil becoming rate-limiting in high-density culture, especially in the case of recombinant protein production. The data in Table 3.1 demonstrated that H1 could be used for further study to determine its $hA_{2A}R$- Ura3p yield characteristics. U1 was not taken forward on account of its slow growth phenotype.

3.3.3 Growth characteristics of A1 and H1

It has been previously reported that to achieve maximal expression of membrane proteins from *S. cerevisiae* that cells should be harvested in the late exponential phase of growth, just prior to glucose exhaustion and the diauxic shift (Bonander et al., 2005). All experiments were therefore done on samples after 19h of growth at which time the cells were at an approximate OD_{600} 4-5. Fig 3.8 (left) shows typical growth curves for H1 and A1 and residual glucose concentration in the culture medium (Fig 3.8, right).

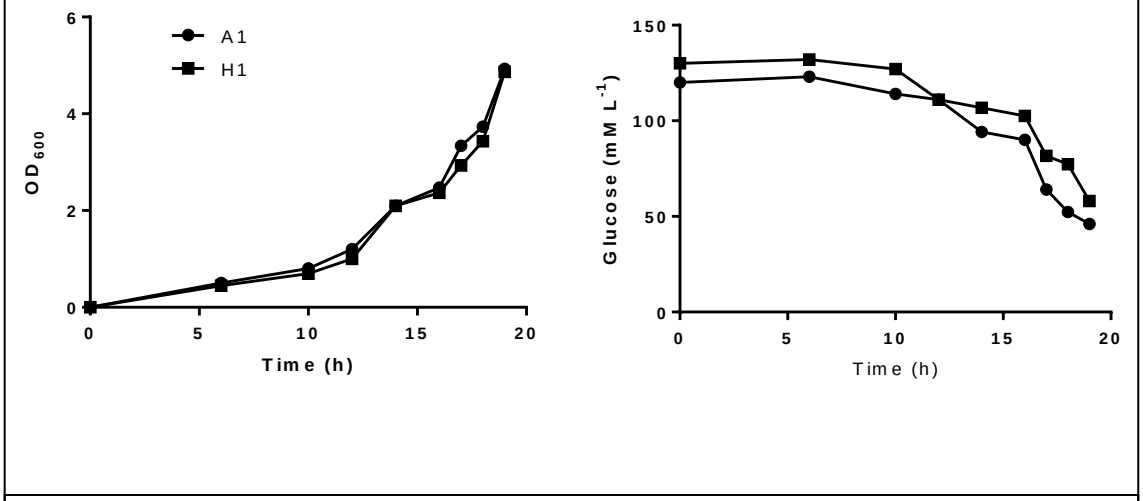


Fig 3.8 growth curves and glucose concentration for A1 and H1. *Measurements of the optical density (left) and residual glucose concentration (right) were taken periodically over 19h of growth for the SA transformants A1 and H1 (data representative of typical growth profiles).*

3.3.4 Viability of A1 and H1

The viability of A1 and H1 was assessed using trypan blue dye exclusion to determine whether the SA strategy and associated recombinant synthesis had impacted it. Table 3.2 shows essentially identical viability for A1 and H1 compared to the untransformed parental strain, BY4741. This suggested that the SA strategy and recombinant expression had no effect on viability.

Viability (%)			
Transformant	**YPD**	**CSM-HIS**	**CSM-URA**
BY4741	99.99	—	—
A1	—	99.90	—
H1	—	—	99.95

Table 3.2 The effect of SA on the viability of A1 and H1. *A viability assay was performed using trypan blue dye exclusion (as described in 2.4.3.4). The yeast transformants were grown for 19h at 30°C. Cell viability is reported as the % viability of 100 cells. The viability data suggest that the SA strategy has no observable effect on the viability of the cells when compared to the BY4741 untransformed control (values are the mean of n=3).*

3.3.5 Reproducibility of H1-like colony generation

Fig 3.6 shows that the 1-step process generated only 1 colony, designated U1; this transformant grew particularly slowly and two further biologically-independent attempts to generate additional colonies in the same manner yielded no colonies. Since the two-step selection process that had generated H1 had resulted in only 1 culturable colony, the two-step procedure was repeated to determine the reproducibility of H1-like colony generation and colony survival rates.

Comparison of reproducibility of the two-step selection					
Transformation attempt	**Selection (no. colonies)**		**Initial Survival rate (%)**	**Viable in liquid CSM -ura**	**Overall survival rate (%)**
	CSM -his 1st step	**CSM -ura 2nd step**			
1 (original)	60	3	5.00	Yes (1)	1.66
2	45	5	11.11	No	0.00

Table 3.3 Repeat of the two-step selection that generated H1. *The two-step selection method was performed again and the data compared with the previous attempt that generated H1.The first selection round gave 60 colonies on CSM –histidine, of which 3 survived on CSM –uracil; of the 3 only 1 could be cultured in liquid CSM –uracil medium (H1). The second attempt gave 45 colonies on CSM -histidine, of which 5 survived on CSM –uracil medium, but could not be cultured.*

Table 3.3 shows that during the original selection, 60 colonies formed after step 1, yet only 3 of them presumably synthesised adequate $hA_{2A}R$-Ura3p to support growth on CSM – uracil; a survival rate of 5%. Since only 1 out of the 3 was culturable in liquid CSM – uracil, the overall survival rate was 1.7%. A second, biologically-independent attempt to generate H1-like colonies yield only 45 colonies on CSM -histidine plates, yet 5 colonies survived on CSM- uracil; a survival rate of 11.1%. However, none of the colonies could be cultured in liquid CSM –uracil, giving an overall survival rate of 0%. A biologically-independent third attempt yielded no colonies. These data suggested that the generation of H1-like colonies is a rare event and so it was decided to focus on characterising the yield characteristics of H1.

3.4 Employing SA to increase recombinant $hA_{2A}R$ yield

3.4.1 Immunoblot analysis of expression profile using anti-His_6 specific antibody

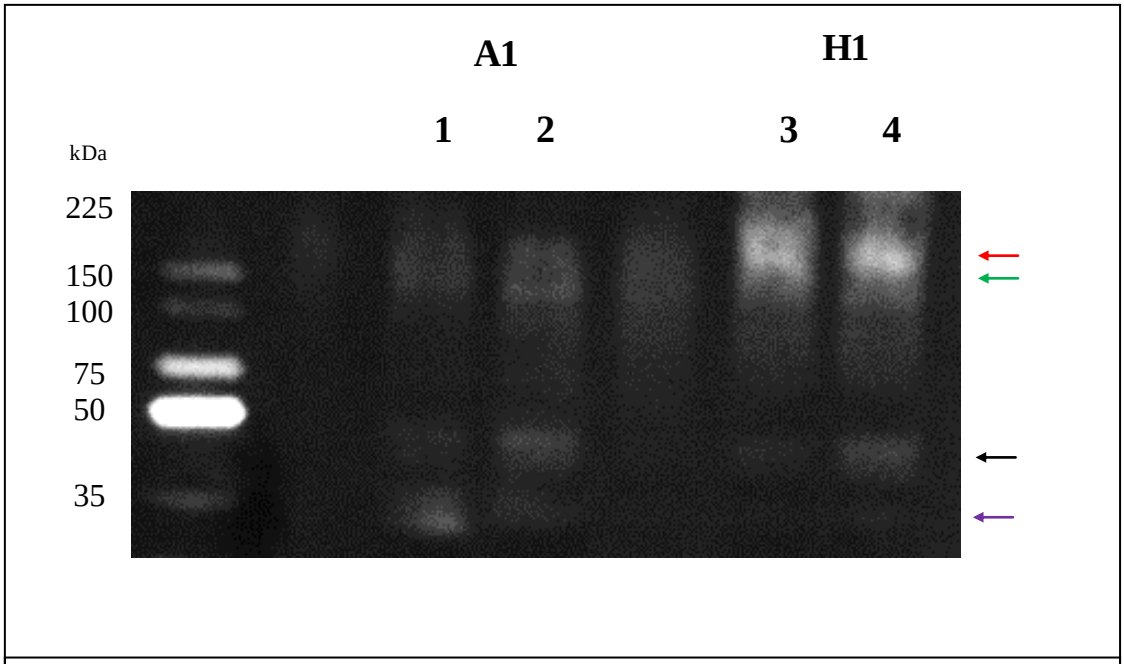


Fig 3.9 Immunoblot performed using anti-His_6 antibody [Clontech]. *Transformants A1 (grown in CSM-histidine) and H1 (grown ins CSM-uracil) were grown to an OD_{600} 4-5 in 100ml culture. Cells were harvested, a membrane preparation performed and the concentration of total membrane protein determined using a BCA assay. A1 $hA_{2A}R$ (1) 25ng, (2) 50ng. H1 $hA_{2A}R$-Ura3p (3) 25ng, (4) 50ng.*

The A1and H1 transformants were grown in 100ml CSM-histidine and CSM-uracil cultures respectively, until an OD_{600} 4-5, harvested and a membrane preparation was performed (described in 2.5.1) to isolate total cell membrane protein presumed to contain

$hA_{2A}R$ (from A1) or $hA_{2A}R$-Ura3p (from H1). An immunoblot was performed with 25µg or 50µg total membrane protein as determined by BCA assay (described in 2.5.2), using an anti-His_6 antibody (Clontech, 1:2000 dilution), as shown in Fig 3.9. An anti-$hA_{2A}R$ antibody (Santa Cruz Biotech) was evaluated, but gave inconsistent and unreliable results and therefore was not used further.

Lanes 1 and 2 show membranes from A1, with 25µg and 50µg of total membrane protein loaded, respectively. A band at approximately 45kDa (black arrow) was observed in both lanes corresponding to the mass of a monomer of $hA_{2A}R$, plus a higher band of around 150kDa (red arrow) that could be either an oligomeric or an aggregated form of $hA_{2A}R$. Lanes 3 and 4 were loaded with membranes from H1, with 25µg and 50µg of total membrane protein added, respectively. Both lanes had bands of around 45kDa (black arrow) representing a monomer of $hA_{2A}R$, and a higher band of 170kDa (green arrow) in lanes 3-4 which could represent aggregated $hA_{2A}R$-Ura3p and was brighter than the presumed oligomeric form of $hA_{2A}R$ from A1 cultures. No 75kDa $hA_{2A}R$-Ura3p monomer band was observed. As $hA_{2A}R$ is thought to homodimerise in the plasma membrane (Canals et al., 2004), and importantly Ura3p is only active as a dimer (Hu et al., 2008), this result was not unexpected.

In addition a band of 35kDa (purple arrow) was seen in all wells and was thought to correspond to $hA_{2A}R$ monomer that had suffered C-terminal degradation which has been previously documented in the literature (Weiss & Grisshammer, 2002, O'Malley et al., 2007, Singh et al., 2008).

3.4.2 Quantification via immunoblot of total $hA_{2A}R$-Ura3p expression yields from H1 grown in different culture media

To determine whether the increase in $hA_{2A}R$-Ura3p yield over the yield of $hA_{2A}R$ seen in Fig 3.9 was due to SA through the use of a selective medium, the yields expressed from H1 cultured in CSM-histidine, CSM -histidine -uracil and CSM –uracil were compared, along with that from U1 cultured in CSM-uracil. The data from triplicate immunoblots was analysed via ImageJ software to compare relative expression judged by band intensity of the whole lane. $hA_{2A}R$-Ura3p yields were calculated relative to the $hA_{2A}R$ control from transformant A1 as shown in Fig 3.10.

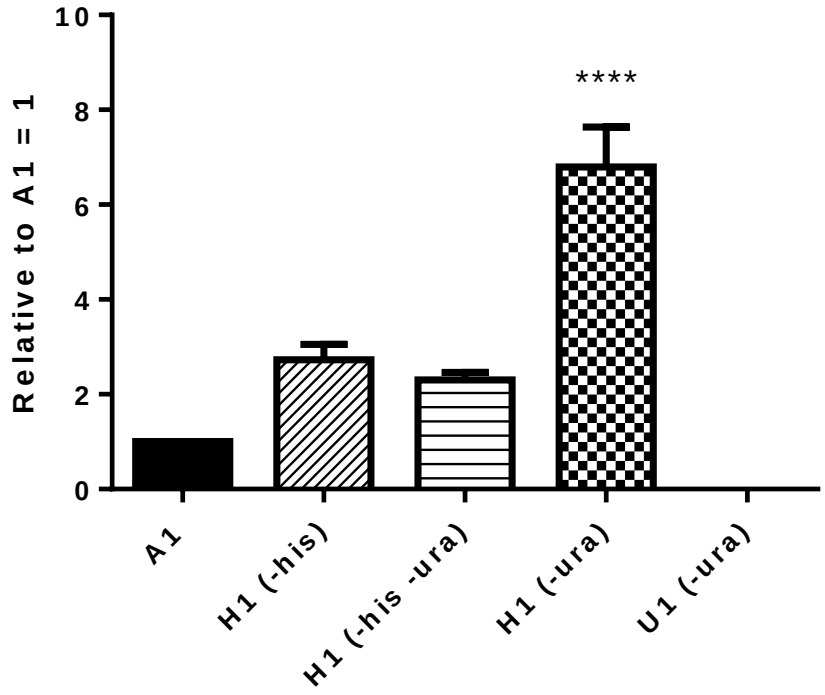


Fig 3.10 Quantification of $hA_{2A}R$/$hA_{2A}R$-Ura3p yields via ImageJ analysis of immunoblots. *Transformants generated through SA, H1 and U1, were cultured in uracil deficient selective medium to encourage high expression of $hA_{2A}R$-Ura3p. In addition H1 was cultured in medium deficient in histidine and medium doubly deficient in histidine and uracil. The control, A1, was cultured in medium deficient in histidine. The graph shows mean data for the combined data sets of immunoblots performed using Clontech anti-His_6 antibodies. All wells had 55µg total membrane proteins loaded as determined by BCA assay. The results are the mean of 3 independent experiments. A 1-way ANOVA with a Dunnett's multiple comparison test against the control group A1 indicated a significance with a p= 0.0001 (****) for H1 (CSM -uracil).*

H1 growth in CSM-uracil selected for cells that express adequate $hA_{2A}R$-Ura3p encoded by pYX222-$A_{2A}R$-URA3, while growth in CSM -histidine selected only for cells containing the plasmid (through its *HIS3* selection marker in the pYX222 backbone); this was identical to the A1 control growth conditions. Growth in CSM -histidine -uracil selected simultaneously for adequate expression of $hA_{2A}R$-Ura3p and for possession of the plasmid through the *HIS3* selection marker.

Fig 3.10 indicates that H1 cultured in CSM -uracil had the highest increase in yield compared to the A1 control showing approximately a 7-fold increase. When H1 was cultured in CSM-histidine and CSM -histidine – uracil, there was only a 2-fold yield increase over A1. The U1 transformant cultured in CSM -uracil had no observable recombinant expression which supports our hypothesis that a one-step selection on CSM -uracil was too stressful for the cells.

Interestingly H1 cultures grown on CSM -histidine were also higher yielding than A1, despite them both being cultured in the same conditions (i.e. no SA). However it has been documented that the addition of tags can increase yield, protein stability and function (Waugh, 2005), which could be true of Ura3p and would explain the unexpected increase over the A1 control. H1 cultured in CSM -histidine -uracil, had comparable yields to the A1 control most probably as a result of the metabolic stress attributed to the double auxotrophy which appeared to have a negative effect on recombinant yield. From this point it was obvious that H1 should be cultured exclusively in CSM –uracil in all subsequent experiments.

3.4.3 Confirmation of the identity of the recombinant proteins synthesised by the A1 and H1 transformants by mass spectrometry

To confirm the identity of the bands in Fig 3.9 as $hA_{2A}R$-Ura3p, 50µg of total membrane protein (determined by a BCA assay) from each of A1 and H1 was separated by SDS-PAGE and stained with Bio-safe Coomassie stain. The bands of interest at 150kDa (Fig 3.9, green arrow) and 170kDa (Fig 3.9, red arrow) were excised from the gel and sent to the University of Birmingham's mass spectrometry facility for analysis (Table 3.4). The 170kDa band from H1 membranes was shown to contain both $hA_{2A}R$ and Ura3p, consistent with a $hA_{2A}R$-Ura3p dimer of approximately 140kDa. The 150kDa band from A1 membranes yielded no data from the analysis, possibly due to an inadequate sample concentration. However, the band had been identified in Fig. 3.9 with an anti-His_6 antibody and an anti-$hA_{2A}R$ antibody (Santa Cruz Biotech) had also previously given one positive signal (data not shown on account of the inconsistency of this antibody). Since the H1 band had been identified as a $hA_{2A}R$-Ura3p dimer, A1 and H1 membranes were subject to radio-ligand binding assays to confirm the presence of a correctly-folded $hA_{2A}R$ moiety.

Sample	Accession	Description	Score	Coverage	# Protein	# Peptides	# AAs	MW [kDa]
A1 150kDa	-	-	-	-	-	-	-	-
H1 170kDa	**C9JQD8**	**ADORA2A ($hA_{2A}R$)**	**9.9**	**7.12**	**2**	**2**	**365**	**40.1**
		High	VLAAHGSDGEQVSLR	2	2	C9JQD8	0	0
		Low	QMESQPLPGER	1	2	C9JQD8	0	0.164
	207346070	**YEL021Wp (Ura3p)**	**12.87**	**25.84**	**4**	**7**	**267**	**29.2**
		High	TVDDVVSTGSDIIIVGR	2	4	207346070	0	0
		High	SDKDFVIGFIAQR	1	4	207346070	0	0
		High	LQYSAGVYR	1	4	207346070	0	0.001
		High	QTNLcASLDVR	1	4	207346070	0	0.001
		High	YNFLLFEDR	1	4	207346070	0	0.001
		Medium	YNFLLFEDRK	1	4	207346070	0	0.045
		Low	MsKATYKER	1	4	207346070	0.63	

Table 3.4 Mass spectrometry of A1 and H1 immunoblot bands. *A 10% SDS-PAGE gel was performed with 50µg total membrane protein loaded (determined by a BCA assay) for both A1 and H1, and bands of interest were excised and sent for mass spectrometry as the University of Birmingham. The results show hits for both $hA_{2A}R$ and Ura3p in the 170kDa band from H1 confirming the presence of the $hA_{2A}R$-Ura3p dimer. A1 returned no results from recombinant sources.*

3.4.4 Radio-ligand binding analysis of $hA_{2A}R$ and $hA_{2A}R$-Ura3p in A1, H1 and U1

To determine whether the $hA_{2A}R$-Ura3p we had produced was correctly folded and to estimate the amount of functional $hA_{2A}R$ moiety (complementing total $hA_{2A}R$ yield estimates by immunoblot), a radio-ligand binding assay was performed (Singh et al., 2012). A single-point saturation radio-ligand binding analysis was performed using the well know antagonist [^{3}H]ZM241385 (Jaakola et al., 2008) using 100µg of total membrane extract from A1, H1 and U1 (Fig 3.11). The binding data in Fig 3.11 indicate that H1 had only a minimal increase (1.6 ± 0.1 pmol mg^{-1}) in $hA_{2A}R$-Ura3p that is in a correctly-folded state and able to bind the antagonist compared to the A1 control (1.3 ± 0.1 pmol mg^{-1}). This is in contrast to the earlier quantification by immunoblot (Fig 3.10), which showed a 7-fold increase. While U1 had a negligible yield as expected (0.2 ± 0.1 pmol mg^{-1}).

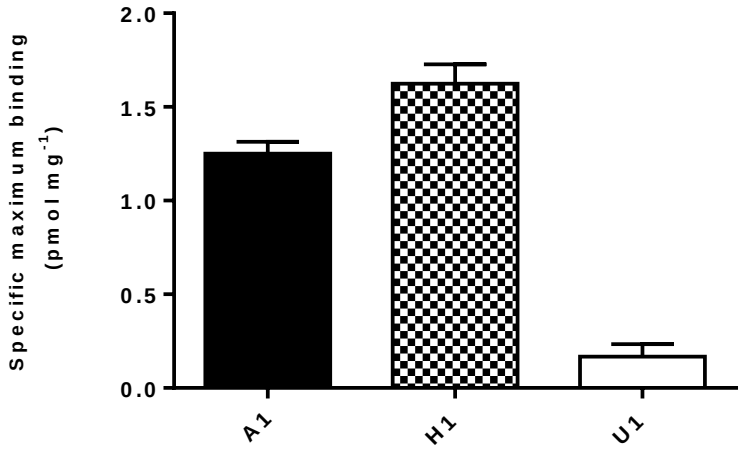


Fig 3.11 Single-point saturation binding analysis of transformants generated through SA. *Single-point binding using the antagonist [^{3}H]ZM241385 was performed on total membrane extracts containing 100µg total membrane protein harvested from A1, H1 and U1. The transformants were cultured in media according to their respective selection criteria; A1 was cultured in CSM –histidine thereby selecting for yeast using the selection marker* HIS3 *of the plasmid it harbours (pYX222-$A_{2A}R$). H1 and U1 were cultured using CSM –uracil which selects for expression of the fusion protein $hA_{2A}R$-Ura3p encoded in the pYX222-$A_{2A}R$-URA3. The data are the mean of at least 3 independent experiments and the errors represent SEM.*

This suggested that the majority of $hA_{2A}R$-Ura3p produced in H1 was not correctly folded. Confocal microscopy was therefore employed to establish $hA_{2A}R$/$hA_{2A}R$-Ura3p localisation within the cells and whether any differences could be seen between H1 and the A1 control.

3.4.5 Confocal microscopy of N-terminally His_{10}-tagged hA2AR/hA2AR-Ura3p in A1 and H1

To investigate the 7-fold increase in recombinant yield for H1 compared to A1 shown by immunoblot and the significantly smaller increase shown by radio-ligand binding, we decided to visualise the A1 and H1 cells by confocal microscopy. The cells were therefore grown to an OD_{600} of 4-5 in line with previous cultures. Confocal microscopy was performed using Clontech rabbit anti-His_6 primary antibodies in conjunction with Alexa-Fluor488 conjugated anti-rabbit secondary.

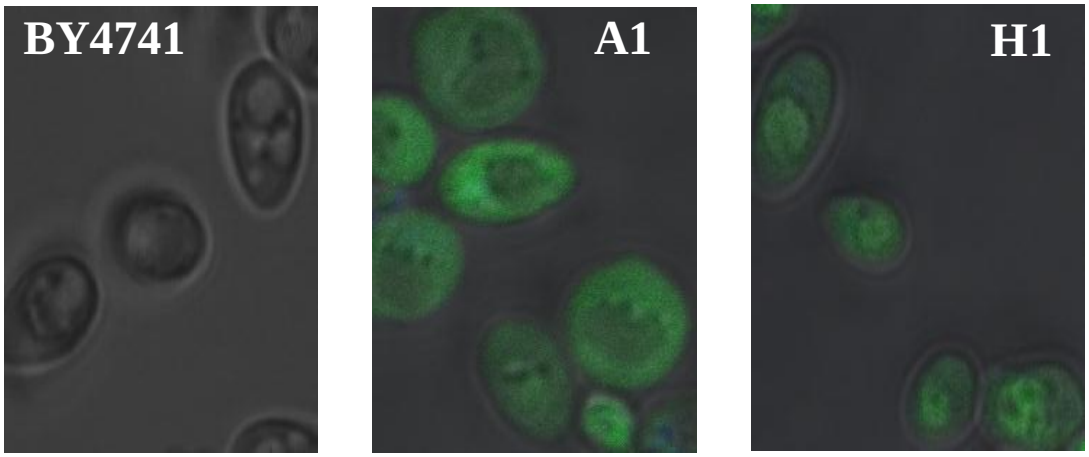


Fig 3.12 Confocal microscopy visualisation of recombinant $hA_{2A}R$/$hA_{2A}R$-Ura3p in transformed* S. cerevisiae *using AlexFluor488 antibodies. *The representative images depict the general localisation of recombinant $hA_{2A}R$/$hA_{2A}R$-Ura3p in green in BY4741, A1 and H1 grown to an OD_{600} of 4-5. Using Clontech rabbit anti-His_6 as the primary antibody, the Alexa-Fluor488 conjugated anti-rabbit secondary was used to allow visualisation under a confocal microscope. The BY4741 control displayed no non-specific binding; the green fluorescent signal for A1 and H1 was therefore due to His-tagged recombinant proteins. The A1 control displayed expression of the recombinant protein throughout the cell excluding the vacuole, while H1 displayed a high concentration of recombinant protein in its vacuoles.*

Fig 3.12 shows that the A1 transformants exhibit expression throughout the cell and that the vacuole was devoid of recombinant protein. In comparison, H1 appeared to have the majority, if not all, of the recombinant protein internalised in the vacuole. The untransformed BY4741 parental strain did not show any signal, so we were able to rule out non-specific binding. We concluded that the internalisation event in H1 cells may be responsible for the discrepancies between total recombinant $hA_{2A}R$-Ura3p yield (determined by immunoblot) and functional recombinant $hA_{2A}R$-Ura3p yield (determined by radio-ligand binding assay).

3.4.6 Strategies to avoid vacuolar internalisation of recombinant $hA_{2A}R$-Ura3p in H1

Localisation of $hA_{2A}R$-Ura3p in the vacuole due to an apparent internalisation in H1 cells (Fig. 3.12) appeared to have a negative effect on functional yield as determined by radio-ligand binding. We therefore decided to investigate 3 vacuolar mutants of BY4741, *apm3Δ*, *vps1Δ* and *pep3Δ*. The two main pathways for protein trafficking in *S. cerevisiae* are the alkaline phosphatase pathway (ALP) and the vacuolar hydrolase carboxypeptidase Y pathway (CPY) (Conibear & Stevens, 1998), which can both be disrupted through the deletion of *APM3* and *VPS1* (Fig.3.13). Since *PEP3* is responsible for vacuolar biogenesis (Srivastava et al., 2000) and its deletion prevents vacuole formation, this mutant was also investigated (Fig 3.13).

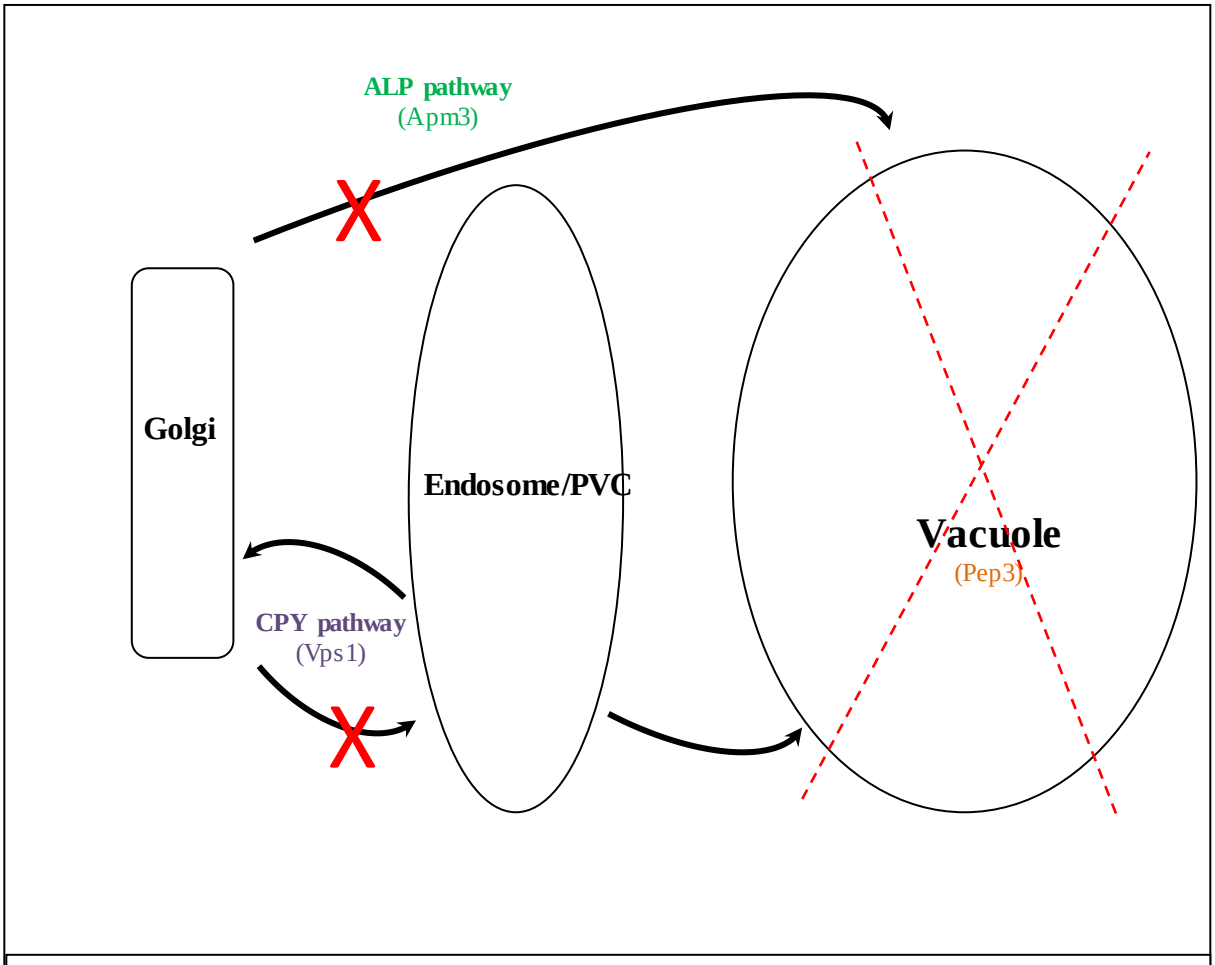


***Fig 3.13 Schematic illustration depicting the effect of deletions of the vacuolar genes,* APM3, VPS1 *and* PEP3, *on vacuolar trafficking pathways and vacuolar biogenesis in* S. cerevisiae.** *Newly synthesised proteins are trafficked to the vacuole via two pathways, the vacuolar hydrolase carboxypeptidase (CPY) pathway which involvestransit through the pre-vacuolar compartment, and the alkaline phosphatase (ALP) pathway. Deletion of the Vacuolar Protein Sorting-1* (VPS1) *gene or the clathrin Adapter Protein complex Medium chain (*APM3*) gene, cause a disruption to the CPY and ALP pathways, respectively. Additionally deletion of the carboxyPEPtidase Y-deficient protein (*PEP3*) gene prevents vacuolar biogenesis.*

The clathrin Adapter Protein complex Medium chain protein (Apm3p) is a subunit of clathrin associated protein complex (AP-3), responsible for trafficking to the vacuole *via* the ALP pathway (Nothwehr et al., 1995, Cowles et al., 1997). The Vacuolar Protein Sorting-1 protein (Vps1p) is a dynamin-like GTPase which is involved in vesicle budding from the Golgi, facilitating trafficking along the CPY pathway (Ekena et al., 1993). The carboxyPEPtidase Y-deficient protein (Pep3p) protein is a component of the CORVET tethering complex which is a peripheral membrane protein responsible for playing an important role in vacuolar biogenesis (Srivastava et al., 2000).

3.4.7 Cell morphology and localisation of $hA_{2A}R$/$hA_{2A}R$-Ura3p in vacuolar mutant *apm3Δ, pep3Δ and vps1Δ*

Confocal microscopy was performed to investigate whether vacuolar accumulation could be disrupted through the use of the vacuolar mutant strains, *apm3Δ, pep3Δ and vps1Δ*, as host cells for recombinant protein synthesis. The 3 mutant strains were therefore transformed with pYX222-A_{2A}R-URA3 (the SA plasmid) and pYX222-A_{2A}R (the control plasmid). The two-step method was used to generate vacuolar mutant transformants; 3-8 colonies were generated on CSM-uracil plates from 15-25 colonies on CSM-histidine plates and 1 culturable colony was picked at random for each strain. The vacuolar control transformants were generated in the same fashion as A1.

Figure 3.14A shows that the *apm3Δ* transformants displayed a similar localisation for both $hA_{2A}R$ and $hA_{2A}R$-Ura3p as the A1 control, with a relatively empty vacuole. In the case of the *vps1Δ* transformants, both *vps1Δ*: $hA_{2A}R$ and *vps1Δ*: $hA_{2A}R$-Ura3p appeared to have small vesicles containing recombinant protein together with a signal throughout the cell and no visible vacuole (Fig 3.14B). The cells also exhibited a non-typical spherical morphology. The *pep3Δ*: $hA_{2A}R$ transformant (Fig 3.14C), which has no vacuole, appeared to have small vesicles which contained recombinant $hA_{2A}R$-Ura3p. In contrast, *pep3Δ*: $hA_{2A}R$-Ura3p gave a relatively low signal throughout the cell and no visible concentration in vesicles (Fig 3.14C) indicating that expression was relatively low for this transformant. The *pep3Δ* cells also had the non-typical spherical morphology.

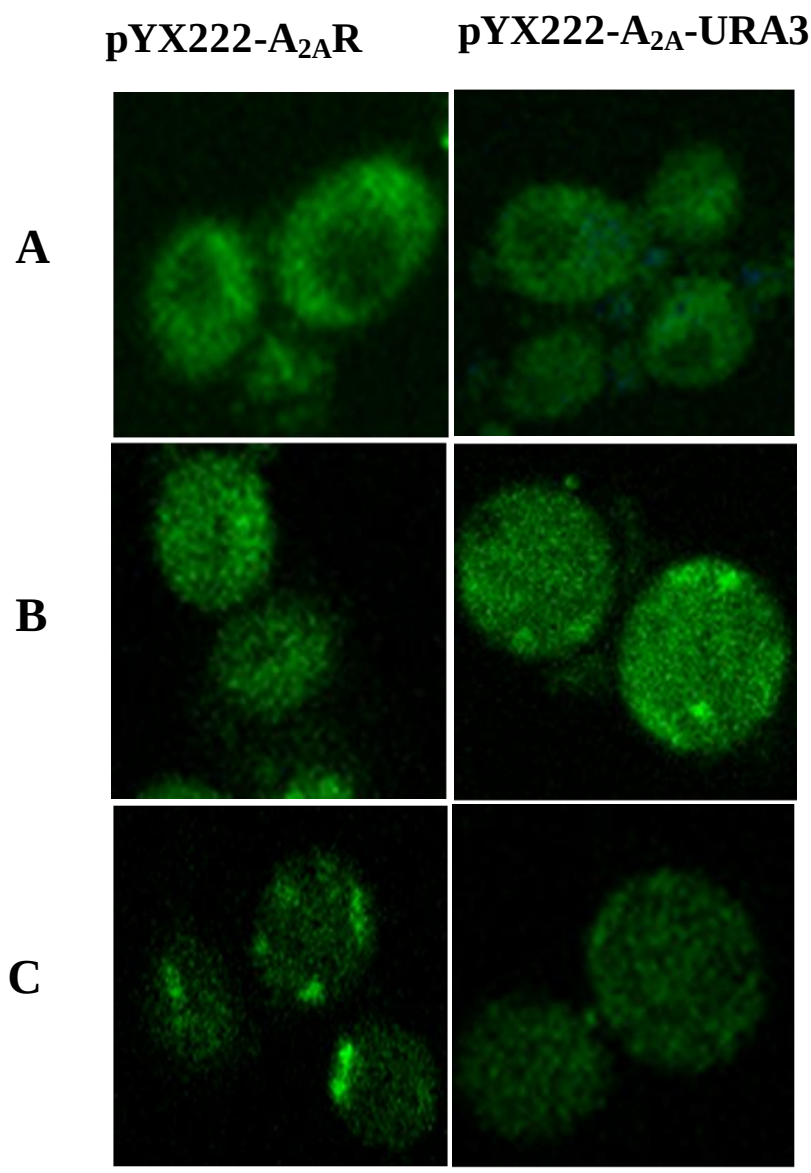


Fig 3.14 Confocal microscopy of* S. cerevisiae *BY4741 vacuolar mutants expressing $hA_{2A}R$ and $hA_{2A}R$-Ura3p. *The images above are representative images depicting the general localisation of recombinant $hA_{2A}R$ in green in yeast transformants that were grown to an OD_{600} of 4-5. Clontech rabbit anti-His_6 was used as the primary antibody, with Alexa-Fluor488 conjugated anti-rabbit as secondary which allowed visualisation under a confocal microscope. (A)* apm3Δ*: $hA_{2A}R$, (B)* apm3Δ*: $hA_{2A}R$-Ura3p, (C)* pep3Δ*: $hA_{2A}R$, (D)* pep3Δ*: $hA_{2A}R$-Ura3p, (E)* vps1Δ*: $hA_{2A}R$, (G)* vps1Δ*: $hA_{2A}R$-Ura3p.*

Radio-ligand binding assay was performed to determine whether the use of these mutant strains had improved the amount of functional recombinant protein.

3.4.8 Analysis of $hA_{2A}R/hA_{2A}R$-URA3 yield by radio-ligand binding in vacuolar mutant strains

Single-point radio-ligand binding analysis was performed using [^{3}H] ZM241385 on total membrane extracts containing 100µg total membrane protein. Vacuolar mutants, *apm3Δ, pep3Δ and vps1Δ* expressing $hA_{2A}R/hA_{2A}R$-Ura3p were analysed to quantify functional yield (Fig 3.15).

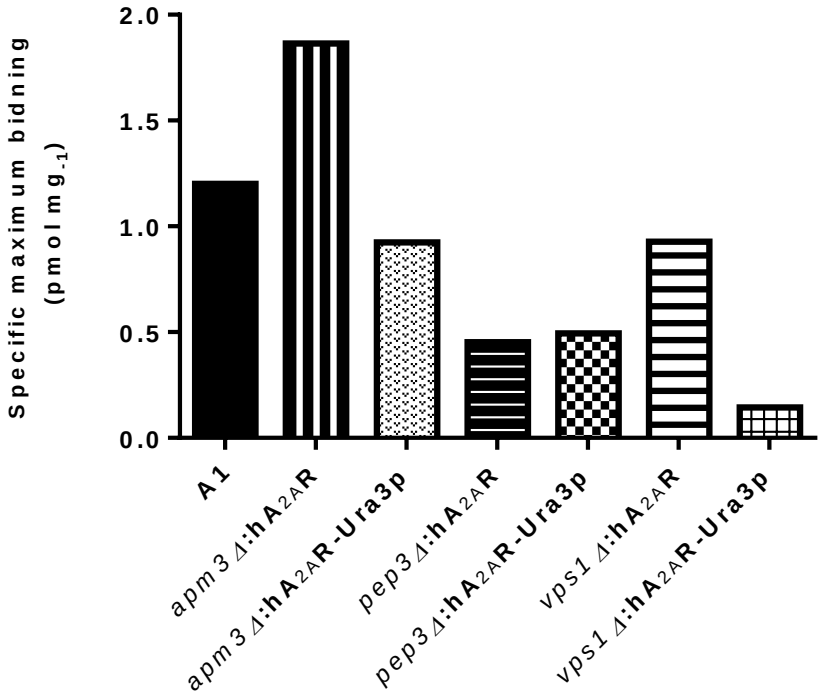


***Fig 3.15 Single-point binding analysis of vacuolar mutants* apm3Δ, pep3Δ *and* vps1Δ**. *Single-point radio-ligand binding analysis was done using the antagonist [^{3}H] ZM241385 on 100µg total membrane preparations harvested from vacuolar mutants which expressed $hA_{2A}R$ or $hA_{2A}R$-Ura3p; this experiment was performed once.*

The analysis showed that for *apm3Δ* the SA strategy (*apm3Δ*:$hA_{2A}R$-Ura3p) caused a reduction in yield when compared to its control (*apm3Δ*: $hA_{2A}R$). For *pep3Δ* there was no observable difference in yield. Notably, *vps1Δ*: $hA_{2A}R$-Ura3p had an extremely low yield compared to its control *vps1Δ*: $hA_{2A}R$ which had a yield comparable to A1. Interestingly

the $hA_{2A}R$ yield from *apm3Δ* (*apm3Δ*: $hA_{2A}R$) was approximately 1.9 pmol mg^{-1} which was higher than A1 control levels (1.3 pmol mg^{-1} from the BY4741 strain). Although there was no vacuolar internalisation in these 3 mutant strains, there was also no increased $hA_{2A}R$-Ura3p yield using the SA strategy. We therefore devised an alternate strategy to alleviate vacuolar accumulation by investigating the use of the *spt3Δ* strain which has been reported to have improved recombinant protein quality (Bonander et al., 2005), possibly due to the possession of a translation initiation block (unpublished data by Dr. Stephanie Cartwright, Aston University); Fig 4.6). Such translationally inhibited states have been suggested to be amenable to producing higher quality protein (Siller et al., 2010, Meriin et al., 2012, Sherman & Qian, 2013).

3.4.9 Use of the *spt3Δ* strain to improve functional yields with the SA strategy

The *SPT3* gene encodes a transcription factor that is a component of the SAGA complex, which is composed of 20 subunits and is involved in transcriptional activation of 10% of genes in yeast, many associated with stress (Jacobson & Pillus, 2009). Previous studies have shown that the *spt3Δ* strain can be used to improved recombinant protein quality and has shown increased yields of the yeast membrane protein Fps1, a glycerol facilitator (Bonander et al., 2009). It also has be shown to have a translational initiation block (unpublished data by Dr. Stephanie Cartwright, Aston University);Fig 4.6). Slowing the rate of translation through translational inhibition has been shown to decrease the proportion of misfolded recombinant proteins expressed in host systems by enhancing folding efficiency, possibly due to the increased availability of chaperones and a reduced load on the ER machinery (Siller et al., 2010, Meriin et al., 2012, Sherman & Qian, 2013). A one-step selection method was therefore used to generate *spt3Δ* SA transformants; only one colony formed from the process which was designated SU1 (*spt3Δ* uracil 1; Fig 3.16). Unlike U1 which also was generated through a one-step selection, SU1 did not acquire a severe growth defect as a consequence of being selected via the more stressful method, instead exhibiting the same growth characteristics as the parental strain *spt3Δ*. Consequently a two-step process was not used to generate further transformants.

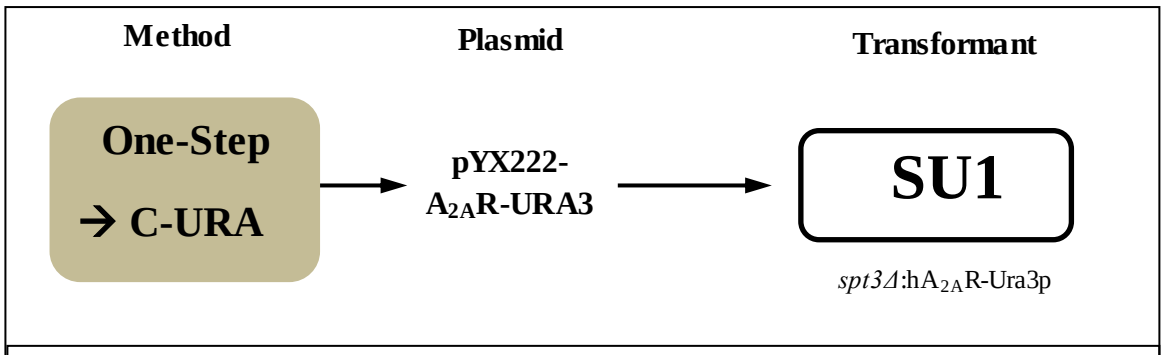


Fig 3.16 Schematic illustrating the generation of the SU1 (spt3Δ:hA$_{2A}$R-Ura3p). *The pYX222-A$_{2A}$R-URA3 SA vector was used to generate transformant SU1 using a one-step method.*

An immunoblot was performed on total cell membranes containing 50µg total membrane protein to determine the yield of recombinant hA$_{2A}$R-Ura3p from SU1, with A1 as the control. The immunoblot results were analysed using ImageJ to compare hA$_{2A}$R /hA$_{2A}$R-Ura3p expression quantitatively between the control, A1, and SU1, respectively. Fig 3.17 shows that SU1 has a reduction in yield scoring only 0.6 times as much as A1.

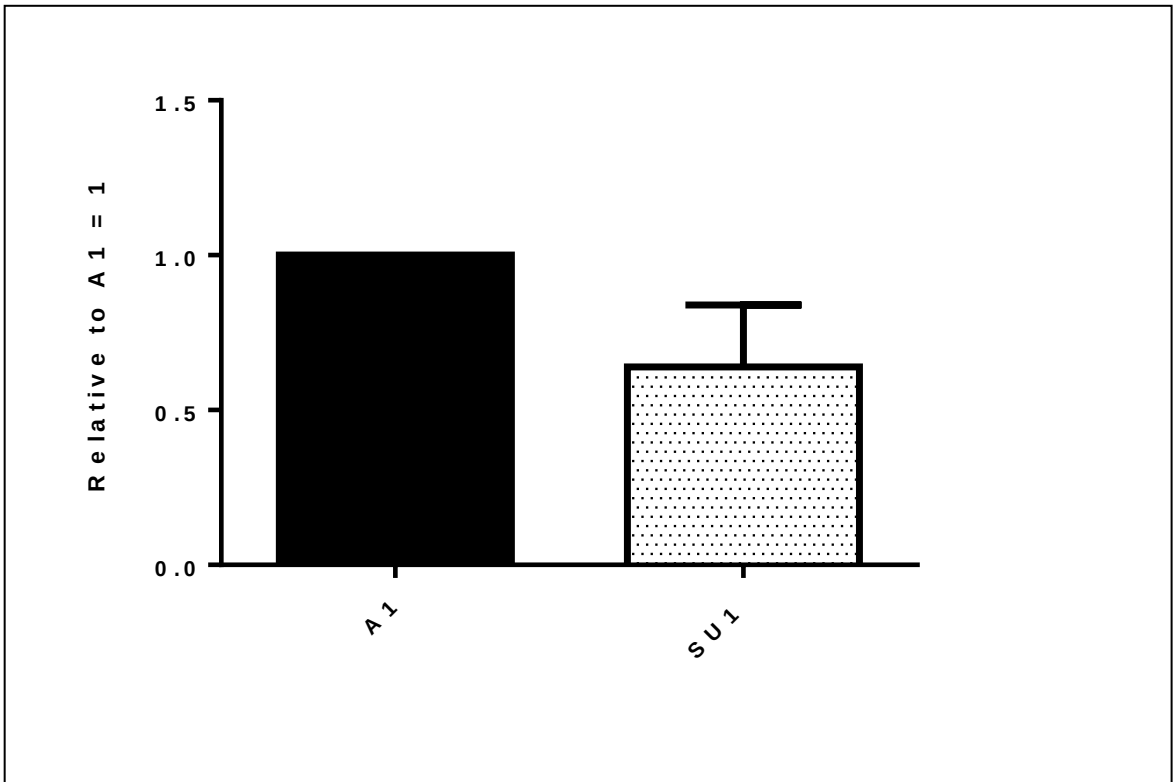


Fig 3.17 Immunoblot quantification of A1 and SU1 recombinant yields by ImageJ analysis. *Transformants were grown to an OD$_{600}$ of 4-5. Using rabbit anti-His$_6$ (Clontech) as the primary antibody, His-tagged hA$_{2A}$R and hA$_{2A}$R-Ura3p were detected in A1 and SU1 membranes and the bands quantified by ImageJ. Data are the mean of triplicate determinations; with error ± SEM.*

Single-point radio-ligand binding analysis was performed using [^{3}H] ZM241385 on total cell membrane extracts containing 100µg total membrane protein (determined by BCA assay), to determine the functional $hA_{2A}R$-Ura3p yield from SU1 (Fig 3.18). The data show that the SU1 transformant has a functional yield of 3.3 ± 0.2 pmol mg^{-1} $hA_{2A}R$-Ura3p, a 2.5-fold increase over the A1 control (1.3 ± 0.1 pmol mg^{-1}). Since *spt3Δ*:$hA_{2A}R$ had a lower yield (0.5 ± 0.1 pmol mg^{-1}) than the control A1 and SU1, this suggested that the increase in functional yield shown by SU1 over A1 is a result of the implementation of the SA strategy rather than being a result of the specific characteristics of the *spt3Δ* strain alone.

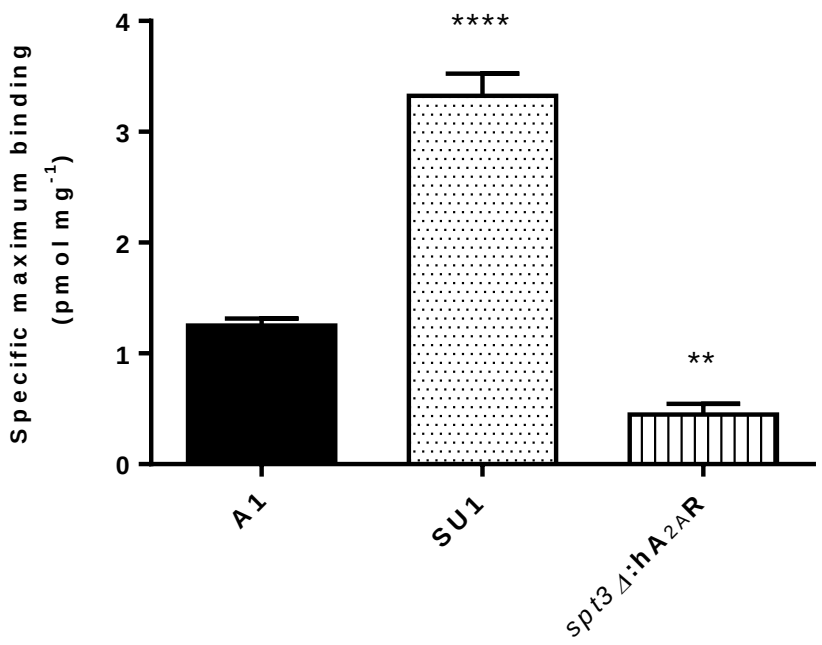


Fig 3.18 Single-point binding analysis of SU1 (spt3Δ:hA$_{2A}$R-Ura3p) and spt3Δ:hA$_{2A}$R transformants compared with the A1 control. *Single-point binding using the antagonist [^{3}H] ZM241385 was performed to quantify functional yield of $hA_{2A}R$/$hA_{2A}R$-Ura3p from total cell membranes containing 100µg total membrane protein harvested from SU1 and spt3Δ:$hA_{2A}R$ with A1 as the control. SU1 was shown to have a $hA_{2A}R$ yield of 3.3 pmol mg^{-1}, and spt3Δ:$hA_{2A}R$ only 0.5 pmol mg^{-1}. Data are the mean of triplicate determinations; error bars represent SEM. A 1-way ANOVA with a Dunnett's multiple comparison test with the control group A1 was performed; p = of 0.0001 (****) for SU1 and 0.01 (**) for spt3Δ:$hA_{2A}R$. Data are representative of at least 3 independent experiments with error ± SEM*

Immunoblot analysis suggested SU1 had a lower total yield of $hA_{2A}R$-Ura3p than the yield of $hA_{2A}R$ in the A1 control (Fig 3.17). In contrast, Fig 3.19 demonstrates higher specific

activity in SU1 than A1 when the membranes were assayed by radio-ligand binding analysis. Confocal microscopy was therefore used to investigate these seemingly contradictory results.

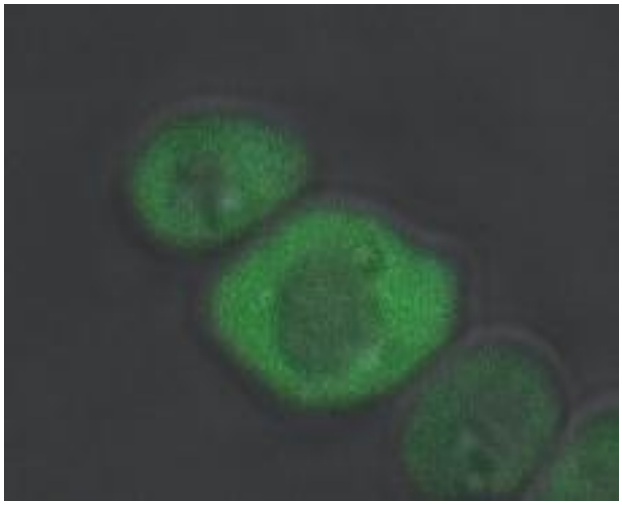


Fig 3.19 Confocal microscopy visualisation of recombinant $hA_{2A}R$-Ura3p expressed in SU1 using AlexFluor488 antibodies. *The images above are representative images and depict the general localisation of the recombinant $hA_{2A}R$ in green in the SU1 transformants. The cells were grown to an OD_{600} of 4-5 and visualised using rabbit anti-His_6 (Clontech) as the primary antibody and Alexa-Fluor488 conjugated anti-rabbit as secondary. The SU1 transformant had empty vacuoles and did not appear to contain internalised $hA_{2A}R$-Ura3p.*

The confocal images are reminiscent of the A1 control confocal images (Fig 3.12) where the recombinant protein is found to be located throughout the cell, with no vacuolar accumulation of recombinant protein occurring as it did in H1 (Fig 3.12); SU1 has empty dark vacuoles devoid of the green signal associated with recombinant protein (Fig 3.19). It was clear that the SU1 transformant did not suffer from vacuolar accumulation despite the SA pressure put upon the cells. This could be attributed to the *spt3Δ* strain's ability to fold proteins more slowly and efficiently due to its translational initiation block; it has been

documented in other such mutants (that suffer an inhibition of translation) that they have better protein folding capabilities (Bonander et al., 2005, Sherman & Qian, 2013).

A viability assay was performed as shown in Table 3.5, SU1 had identical viability to H1 and the controls (A1 and BY4741), suggesting that recombinant expression had no effect on viability.

Viability (%)			
Transformant	**YPD**	**CSM-HIS**	**CSM-URA**
BY4741 WT	99.99	—	—
A1	—	99.90	—
H1	—	—	99.95
SU1	—	—	99.88

Table 3.5 The effect of SA on the viability of SU1*. The viability data suggest that the SA strategy had no observable effect on the viability of SU1 cells when compared to the other SA transformant (H1) and the controls (A1 and BY4741); all have identical viability (values are the mean of n=3).*

Overall, it was unclear why the comparative immunoblot (Fig. 3.17) and radio-ligand binding data (Fig 3.18) were contradictory for SU1. Consequently the pharmacological profiles of $hA_{2A}R$-Ura3p produced by H1 and SU1 were compared with the profile of $hA_{2A}R$ produced by A1.

3.4.10 Pharmacological profile of $hA_{2A}R$-Ura3p produced by H1 and SU1 compared with $hA_{2A}R$ produced by A1

It had been established that H1 had a small increase and SU1 a substantial increase over the A1 control in terms of functional yield of $hA_{2A}R$-Ura3p. The question still remained whether or not the Ura3p fusion partner affected receptor affinity. Homologous competition curve assays were therefore performed using ZM241385 (as described in 2.6.2) for A1, H1 and, SU1 (Fig 3.20). The pK_d values observed for H1 and SU1 were 8.4 ± 0.2 and 8.3 ± 0.2 respectively which is in line with the values for the A1 control (8.6 ± 0.2). All values were within the nanomolar range expected for membrane-bound $hA_{2A}R$ expressed from various constructs in the literature (Singh et al., 2010).

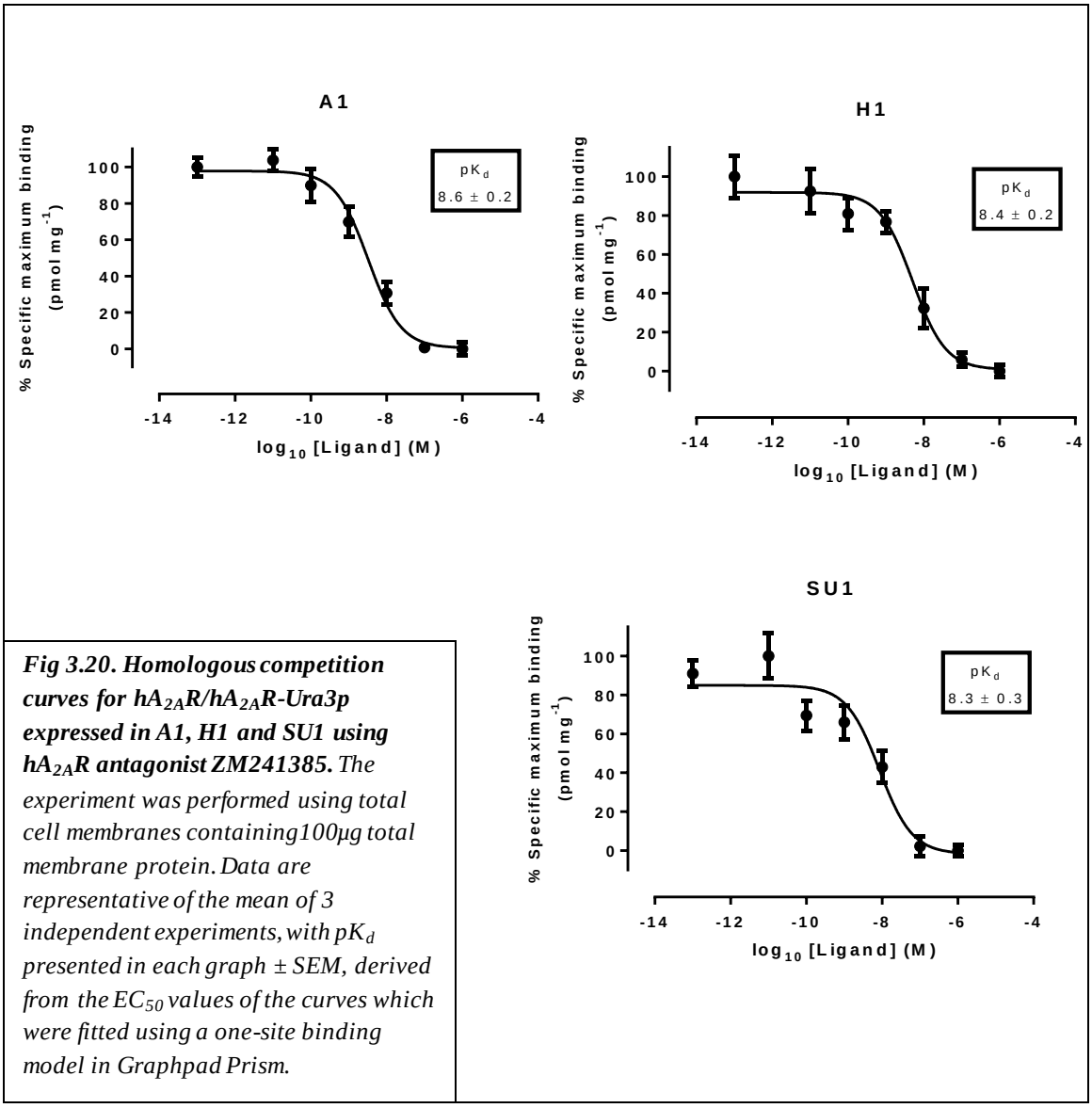
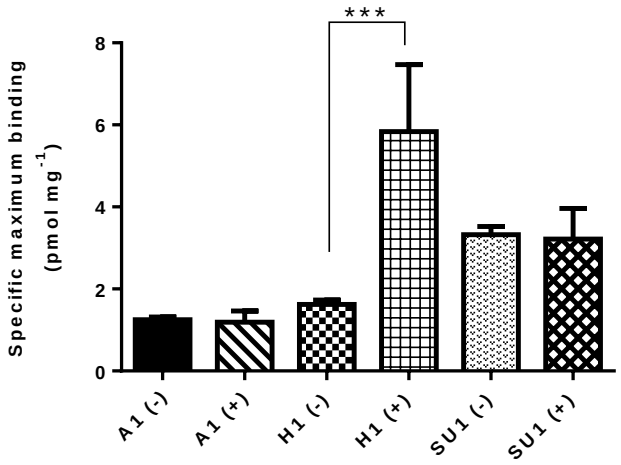


Fig 3.20. Homologous competition curves for $hA_{2A}R$/$hA_{2A}R$-Ura3p expressed in A1, H1 and SU1 using $hA_{2A}R$ antagonist ZM241385. *The experiment was performed using total cell membranes containing100µg total membrane protein. Data are representative of the mean of 3 independent experiments, with pK_d presented in each graph ± SEM, derived from the EC_{50} values of the curves which were fitted using a one-site binding model in Graphpad Prism.*

We were therefore confident that expression in an SA-driven manner did not affect the affinity of the $hA_{2A}R$ and neither did expression in *spt3Δ* membranes. The existence of a heterogeneous receptor population in H1 (Fig 3.12) also suggested that misfolded or mislocalised $hA_{2A}R$-Urap3 was not able to bind the antagonist and therefore did not affect the binding data. This did not appear to be the case for the immunoblot assays, because there would be no discrimination between receptors as long as the His_{10}-tag was presented.

3.4.11 Solubilisation of $hA_{2A}R$-Ura3p / $hA_{2A}R$ from A1, H1 and SU1 membranes

A solubilisation study was carried out to determine if it was possible to recover misfolded protein from H1(Fig 3.21), where a 7-fold increase in total $hA_{2A}R$-Ura3p yield compared to the A1 control from immunoblots (Fig 3.10) was matched with a 1.3-fold increase from functional data using radio-ligand binding (Fig 3.11). As confocal microscopy showed $hA_{2A}R$ in H1 appeared to be accumulating in the vacuole rather than throughout the cell and the cell membrane, as seen in the A1 control and SU1, this might have a negative effect on the structure or folding of the receptor. It was hypothesised that the function of the misfolded $hA_{2A}R$-Ura3p expressed in H1 could be restored by solubilisation in n-dodecyl β-D-maltopyranoside (DDM), a detergent known to retain GPCR function when supplemented with cholesteryl hemisuccinate (CHS) (Weiss & Reinhard Grisshammer, 2002, O'Malley et al., 2007, Singh et al., 2010).



Functional yield (pmol mg^{-1})		
	Solubilisation	
	–	+
A1	1.3 ± 0.1	1.2 ± 0.3
H1	1.6 ± 0.1	5.8 ± 1.6
SU1	3.3 ± 0.2	3.2 ± 0.7

Fig 3.21Effect of solubilisation on $hA_{2A}R$/$hA_{2A}R$-Ura3p harvested from A1, H1 and SU1. *A single-point saturation binding assay for $hA_{2A}R$/hA2AR-Ura3p using antagonist [^{3}H]ZM241385 and total cell membranes preparations harvested from A1, H1 and SU1 that had been solubilised with 10%DDM and 2%CHS that contained 60µg total membrane protein. Depicted above are the mean of at least 3 experiments for A1, H1 and SU1. The previous $hA_{2A}R$ single-point saturation values in total membranes (-) and solubilised $hA_{2A}R$ single-point saturation values (+) were compiled to analyse the effect of solubilisation with respect to the increase in functionality of the receptor. Only H1 was observed to have an increase in functionality after solubilisation. A 1-way ANOVA with a Holm-Sidak's multiple comparison test with samples grouped into three preselected groups A1, H1 and SU1 gave significance with p = of 0.001 (***) for H1(-) versus H1(+). A table containing the numerical data calculated for the solubilisation study is shown on the left (data are representation of 3independent experiments with error ± SEM).*

The total cell membranes from A1, H1 and SU1 were solubilised using 10%DDM supplemented with 2%CHS (as described in 2.7), and a single point radio-ligand binding assay was performed to assess whether or not function had been recovered for $hA_{2A}R$-Ura3p or $hA_{2A}R$ (for the A1 control).

Comparison of the binding data for solubilised (+) and un-solublised (-) $hA_{2A}R$-Ura3p (Fig 3.21), it is clear that $hA_{2A}R$-Ura3p activity could be recovered from the solubilisation of H1 membranes. An increase from 1.6 to 5.8 pmol mg^{-1} was observed, which represented a significant 5.2-fold relative increase in functional $hA_{2A}R$-Ura3p (also compared to the A1 controls) strongly indicating that the use of detergent appeared to have rescued receptor functionality thereby increasing functional yield. It is possible that the $hA_{2A}R$-Ura3p internalised in the vacuoles was present in vesicles but these were not sufficiently lysed during preparations of the H1 membrane samples that were used for binding assays (-). When the membrane preparations were solubilised using DDM (+), this might have resulted in permeabilisation of the vesicles, allowing access of the radioligand to these previously inaccessible receptors, thus increasing the binding.

For the A1 control and SU1 membranes, solubilisation appeared to have no effect. It was most likely that A1 and SU1 saw no increases in functional yield because they did not have any substantial misfolded $hA_{2A}R$/$hA_{2A}R$-Ura3p to be recovered. In contrast for H1, only 24% of total recombinant $hA_{2A}R$-Ura3p was functionally active as determined by radioligand binding assay (Fig 3.11), leaving a potential 76% for recovery; when this recovery was done with DDM this increased the total functional yield to 60% (an increase of 36%)(Fig 3.21).

3.4.12 Quantifying receptor yield of A1, H1 and SU1 from saturation binding curve data

Three saturation curves were performed on total cell membranes containing 100µg total membrane protein from A1, H1 and SU1 membranes (Fig 3.22A) using $hA_{2A}R$ antagonist [^{3}H]ZM241385. Single-point binding assays were performed previously to estimate the B_{max} since this required a relatively low amount of radio-ligand (Fig 3.11 and 3.18). A full saturation curve is required to accurately determine the B_{max} because during the single-point assays the receptors might not have been fully saturated.

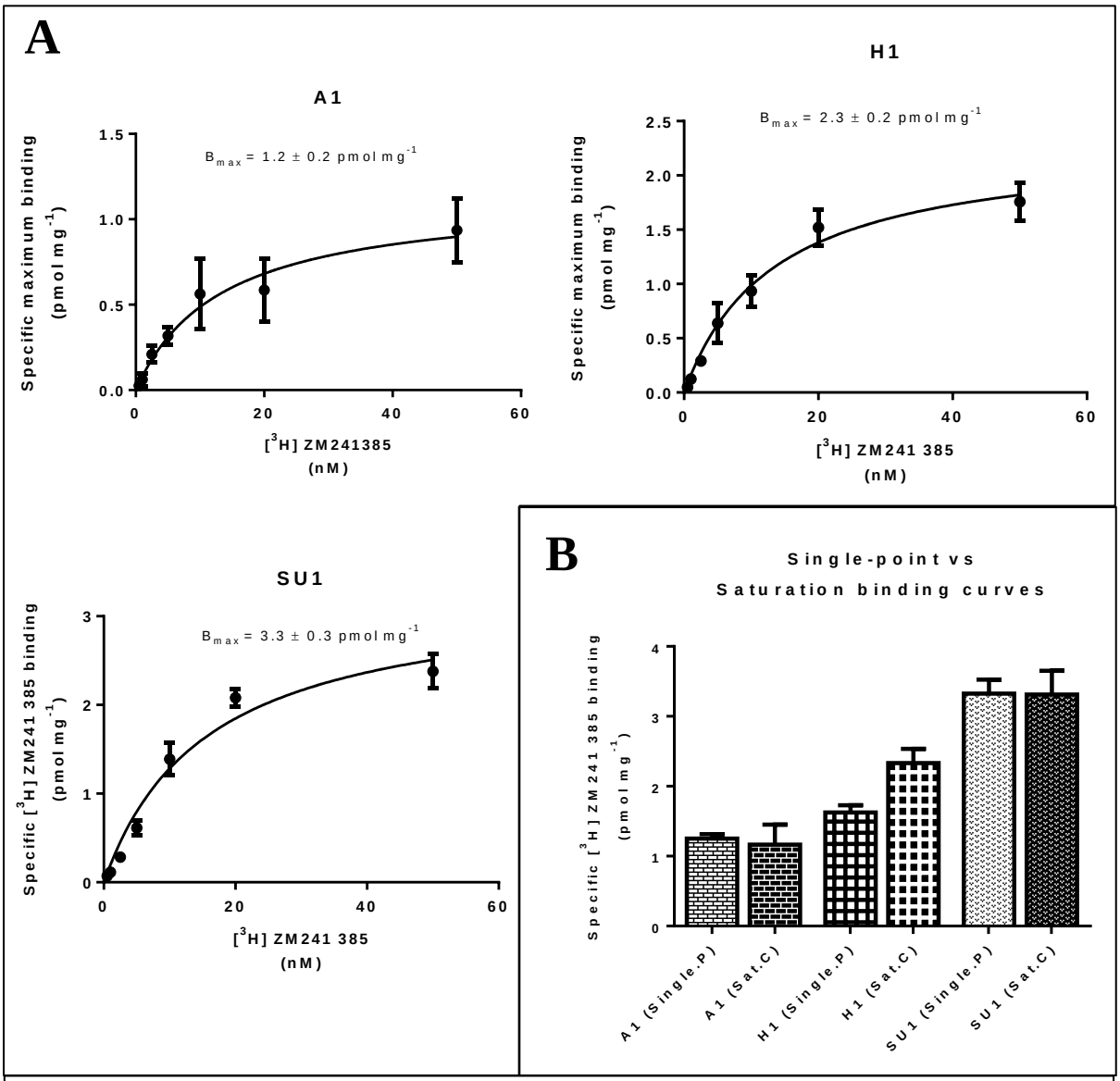


Fig 3.22 (A) Saturation binding curves for $hA_{2A}R$/$hA_{2A}R$-Ura3p expressed in A1, H1 and SU1 using antagonist [^{3}H]ZM241385. *Saturation binding curves were performed using the antagonist [^{3}H]ZM241385 to quantify functional yield of $hA_{2A}R$/$hA_{2A}R$-Ura3p in a more robust manner than the initial experiments (single-point). Assays used total cell membranes containing 100µg total membrane protein. Data are the mean of 3 independent experiments. The B_{max} values were derived from non-linear regression of the saturation curves using Graphpad Prism.* ***(B) Comparison between single-point (Single.P) and saturation binding curve (Sat.C) data.*** *To validate whether single-point assays are suitable for determining B_{max} estimates, the values from both assays were compiled in a single graph. All data are representative of at least three independent experiments with error ± SEM.*

A mean B_{max} of 1.2 pmol mg^{-1} was determined for A1 which was in line with single-point binding data (1.3 pmol mg^{-1}), and showed the single-point method to be accurate in obtaining the B_{max} in respect to A1 (Fig 3.22). This value was the control value for comparting increases found for the SA transformants, H1 and SU1. A mean B_{max} of 2.3 pmol mg^{-1} was determined for H1 (Fig 3.22), which did not correlate as well with the single-point binding results (1.6 pmol mg^{-1}). This might be accounted for by the observed the vacuolar internalisation/accumulation (Fig 3.12), suggesting that saturation curves are more reliable in obtaining the B_{max} values for to H1. The B_{max} values from the saturation curve suggest that there was a 1.8-fold increase in functional hA_{2A}R-Ura3p yield from H1 membranes over the A1 control, indicating that in the case of H1, the SA strategy has resulted in a transformant that produced nearly twice the amount of functional hA_{2A}R as the standard expression vector strategy. A mean B_{max} of 3.3 pmol mg^{-1} was determined for SU1 which was exactly the same value obtained via single-point binding (3.3 pmol mg^{-1}) (Fig 3.22B). This indicates that a single-point analysis was suitable for investigating the functional yield with respect to SU1. Using the saturation curve B_{max} values, SU1 was seen to have a 2.5-fold increase in functional hA_{2A}R-Ura3p yield over the A1 control, indicating that in the case of SU1, the SA strategy in conjunction with the *spt3Δ* strain resulted in a transformant that produced nearly 2.5-times the amount of functional hA_{2A}R as the standard expression vector strategy.

3.5 Summary

3.5.1 Generation of high-yielding transformants through SA

We established that it is possible to select for *S. cerevisiae* transformants that express sufficient Ura3p (via expression of a hA_{2A}R-Ura3p fusion) to support growth in CSM-uracil. Using a two-step method of selection (selecting first for the *HIS3* marker on CSM-histidine, and then for expression of *URA3* and synthesis of the recombinant fusion hA_{2A}R-Ura3p on CSM-uracil) one colony was generated; H1 (Fig 3.6). The one-step method also only generated one colony (U1) (Fig 3.6), which exhibited poor growth and negligible hA_{2A}R-Ura3p yield when analysed via immunoblot and radio-ligand binding (Fig 3.10 and 3.11). H1, specifically cultured in CSM –uracil was therefore used as the basis for further investigation.

3.5.2 Increasing recombinant $hA_{2A}R$ synthesis through SA

Immunoblot analysis revealed that H1 had a 7-fold increase in total yield of recombinant $hA_{2A}R$-Ura3p compared to the A1 control (Fig 3.10), but a single-point radio-ligand binding assay indicated only a 1.6-fold increase (Fig 3.11) suggesting the SA method might have negatively affected the function of $hA_{2A}R$ produced. Confocal microscopy was performed to assess any differences between the A1 and H1 transformants with respect to the localisation of the recombinant $hA_{2A}R$-Ura3p. The images revealed that H1 suffered vacuolar internalisation of $hA_{2A}R$-Ura3p, whereas the A1 control exhibited evenly-distributed expression throughout the cell with a vacuole devoid of $hA_{2A}R$ (Fig 3.12).

3.5.3 Preventing vacuolar internalisation of $hA_{2A}R$-Ura3p

Since we had observed vacuolar accumulation in H1 and the associated reduction in functional yield when immunoblot data were compared to radio-ligand binding data, we attempted to alleviate this problem. Three vacuolar mutants did not provide a solution, although the *apm3Δ* strain did show potential as a recombinant host (Fig 3.14 and Fig 3.15). We therefore investigated *spt3Δ* in conjunction with the SA strategy as it had been shown to have improved folding properties (Bonander et al., 2009), preventing vacuolar internalisation.

For the *spt3Δ* strain with the SA strategy, we discovered that despite only yielding on average 60% of the A1 control total yield, as determined by immunoblot (Fig 3.18), single-point radio-ligand binding indicated a 3.3-fold increase in functional yield when compared to A1 (Fig 3.18). Confocal microscopy also confirmed no vacuolar internalisation of $hA_{2A}R$-Ura3p synthesised in SU1 membranes. This provided a solution to the low increase in functional yield and vacuolar accumulation issues exhibited by H1.

The vacuolar mutants, *apm3Δ*, *pep3Δ* and *vps1Δ*, were transformed using the SA vector pYX222-$A_{2A}R$-URA3 and pYX222-$A_{2A}R$ as the non-SA control. Subsequently confocal microscopy showed no vacuolar accumulation and radio-ligand binding suggested that *apm3Δ* and *vps1Δ* caused a reduction in yield of $hA_{2A}R$-Ura3p using the SA strategy. The

pep3Δ strain displayed vesicular internalisation of $hA_{2A}R$, while the SA counterpart *pep3Δ*:$hA_{2A}R$-Ura3p had very little expression. Interestingly the non-SA control of *apmΔ* had a yield of 1.9 pmol mg^{-1}, whereas the A1 control was only 1.3 pmol mg^{-1}, highlighting it as a possible expression host for $hA_{2A}R$ and other membrane proteins.

3.5.4 Effect of SA on $hA_{2A}R$ affinity

Homologous competition curves using antagonist [^{3}H] ZM241385 revealed that the affinity for recombinant $hA_{2A}R$ expressed in A1, H1 and SU1 had a pK_d of 8.6, 8.4 and 8.3 respectively, suggesting the SA strategy and/or expression in *spt3Δ* does not affect ligand binding (Fig 3.21). Initially we obtained B_{max} estimates for $hA_{2A}R$/$hA_{2A}R$-Ura3p yields using single-point saturation binding assays, but to arrive at more accurate values we performed saturation binding curves. We discovered that the estimates obtained were in-line with the single-point values from the curves for A1 and SU1 with no differences being seen, whereas in the case of H1 the single-point assay produced a value of 1.6 pmol mg^{-1} and the curves 2.3 pmol mg^{-1} (Fig 3.23B). This could have been due to the condition of the $hA_{2A}R$-Ura3p as a result of the vacuolar accumulation, or the event which caused the cell to undergo the accumulation. Although non-specific binding (NS) was taken into account during the binding assays to arrive at specific maximum binding, H1 had a larger proportion of NS (as a percentage of total binding) when compared to A1 and SU1 (~50% compared to ~30%), possibly caused by the misfolded and non-functional proportion of the receptor population that H1 exhibited. This suggested that single-point binding was not as accurate and subject to some error; H1 might have required a higher concentration of ligand, and a full curve for the trend to produce accurate B_{max} values.

3.5.5 Solubilisation of $hA_{2A}R$-Ura30p as a method to recover function

The membrane preparations for A1, H1 and SU1 were solubilised in an effort to establish whether misfolded $hA_{2A}R$-Ura3p could be recovered from H1 in a functional form, in effect increasing its functional yield by achieving a higher value from ligand binding post-solubilisation. The aim was to recover functional yield that is more in-line with the immunoblot values in the region of a 7-fold increase in $hA_{2A}R$-Ura3p yield over the A1

control. We also wanted to investigate whether solubilisation could improve the functional yield of $hA_{2A}R$-Ura3p from SU1 and possibly the A1 control.

Comparison of single-point binding assay data for the solubilised material with the previous single-point binding data for total membrane preparations, revealed an increase in B_{max} for H1 from 1.6 to 5.8 pmol mg^{-1} when solubilised (Fig 3.22). This represented an improvement from 1.3 to 5.2-fold relative increase in functional $hA_{2A}R$-Ura3p yield when compared with their respective A1 controls (A1 (-), A1(+)). This supported the earlier hypothesis that $hA_{2A}R$-Ura3p expressed in H1 was misfolded and that it could be recovered in detergent micelles.

Chapter 4: Employing knowledge of translational processes to improve $hA_{2A}R$ yield

4.1 Summary of experimental objectives

4.1.1 Rationale behind employing a translation slowdown strategy

The work presented in Chapter 3 illustrates that when *S. cerevisiae* is manipulated to synthesise $hA_{2A}R$-Ura3p in high yields by directly linking its expression to cellular survival (the SA strategy), the majority of the protein produced is non-functional, being localized to the vacuole (as seen in the case of H1, although $hA_{2A}R$-Ura3p could be recovered by extraction in DDM; Fig. 3.22). This was subsequently alleviated using the *spt3Δ* strain, which exhibits an initiation block (unpublished data by Dr Stephanie Cartwright, Aston University); Fig 4.6) causing translation to proceed at a reduced rate. When the *spt3Δ* strain was transformed with the SA plasmid, colony SU1 was generated. Immunoblot analysis showed SU1 synthesised 40% less total protein than the control (Fig. 3.18); however, the protein produced was functional as evidenced by the 3-fold increase in yield determined by radio-ligand binding (Fig. 3.19). In this Chapter, we therefore wished to investigate whether translation slowdown (TSD) can be used to increase the yield of functional yield $hA_{2A}R$ in *S. cerevisiae*.

A mild inhibition of translation, which reduces the rate of translation, has been shown to reduce protein misfolding (Bonander et al., 2005, Siller et al., 2010, Fredrick & Ibba, 2010, Tsaytler et al., 2011, Meriin et al., 2012, Sherman & Qian, 2013). This is presumably by reducing the rate of protein synthesis so that the introduction of newly-synthesised nascent peptides into the ER is manageable, reducing the load on ER chaperones to manageable levels (Tsaytler et al., 2011, Sherman & Qian, 2013). This strategy has recently been used successfully to increase the levels of correctly-folded and thus functional, recombinant mutant CFTR which was previously expressed in a non-functional form (Meriin et al., 2012). The slowing of translation elongation is thought to reduce the chance of errors in translation by allowing adequate time for the correct amino acid to be incorporated in the nascent polypeptide (Fredrick & Ibba, 2010). In bacteria, for instance, a slowdown in elongation resulted in a 20-fold increase in the accuracy of amino acid incorporation,

increased folding efficiency (Siller et al., 2010) and an increased proportion of functional protein (Meriin et al., 2012). Slowing the rate of translation at the initiation or elongation step is also thought to allow for favourable spacing between translating ribosomes, preventing ribosome congestion (Fig 4.1); the associated stalling of translation, which can result in the nascent polypeptide being degraded prematurely (Fredrick & Ibba, 2010), leading to reduced recombinant yields may then be minimised (Sherman & Qian, 2013) .

Fig 4.1 Diagram depicting the effects of translational slowdown on translating ribosomes. *An inhibition of translation at the initiation or elongation step allows for uniform spacing preventing ribosome congestion, allowing more efficient protein synthesis and reducing the rate at which newly synthesised polypeptides enter the ER. This is proposed to decrease the stress on the ER machinery, and increase the availability of chaperones (reproduced from Fredrick & Ibba, 2010).*

4.1.1.1 Mutant strain and drug choice

The potential benefits of TSD were investigated using two approaches. Examination of the literature supported the use of two drugs, guanabenz and emetine, known to inhibit translation in eukaryotes (Tsaytler et al., 2011, Sherman & Qian, 2013). We also selected BY4741 deletion strain, *tor1Δ*, as an expression host because it is known to exhibit translational inhibition (Kennedy & Kaeberlein, 2009).

4.1.1.1.1 Emetine

Emetine is an FDA-approved translational inhibitor which binds to the 40S ribosomal subunit inhibiting translation elongation, and is currently used to treat parasitic diseases such as amoebic dysentery (Kanner et al., 2003, Marie & Petri, 2013, Sherman 2013).

4.1.1.1.2 Guanabenz

Guanabenz is an FDA-approved hypertensive drug, and is also used to treat prion disease (Tribouillard-Tanvier et al., 2008), parasites such as *Toxoplasma gondii* (Konrad et al., 2013), metabolic disorders (Ye et al., 2013) in humans, and has been shown to be active in yeast (Tribouillard-Tanvier et al., 2008). The drug has been shown to bind to eIF2α inhibiting de-phosphorylation of the subunit brought about by stress, leading to an overall inhibition of translation (Tsaytler et al., 2011). Translation inhibition in this manner has been shown in cells under stress: it facilitates the rescue of proteins from misfolding, supposedly by reducing the work load on cellular chaperones (Tsaytler et al., 2011).

4.1.1.1.3 *tor1Δ*

The *TOR1* gene (target of rapamycin 1) is found in all eukaryotes and encodes an extremely important protein which forms the TORC1 complex. TORC1 regulates many cellular processes including protein synthesis, transcriptional activation, ribosome biogenesis, actin organisation and the cell cycle (Kennedy & Kaeberlein, 2009). *TOR1* has been a focus of much study in eukaryotes, particularly in yeast, on ageing, cancer, apoptosis and hypoxia (Bjornsti & Houghton, 2004, Martin & Hall, 2005, Kennedy & Kaeberlein, 2009). The *tor1Δ* strain has been shown to exhibit a phenotype with an inhibition of translation (Kennedy & Kaeberlein, 2009) and it was therefore selected for use in this study.

4.1.2 Rationale behind using Internal Ribosome Entry Sequences

It is well known that recombinant protein synthesis can cause stress in host cells whether it be through the cytotoxicity associated with the build-up of misfolded recombinant protein (a common occurrence during heterologous recombinant expression), or the metabolic stress caused through expression of heterologous protein (Mattanovich et al., 2004). This can lead to a multitude of cellular stress responses (Mattanovich et al., 2004), one important response being global translational inhibition at the initiation step, which halts all but essential protein synthesis (Hoffmann & Rinas, 2004, Gasser et al., 2008) via the phosphorylation of eIF2α regulated by eIF-2 kinase (Harding et al., 2000, Novoa et al., 2001).

We hypothesised that an alternative internal initiation site could be implemented to increase functional yields of recombinant protein using expression vectors that circumvent this block in translation initiation. As detailed in Section 1.2.1, IRES sequences allow internal initiation (Fig 1.11) independent of the canonical mechanism (Fig 1.6): IRES-augmented mRNA transcripts are processed independently of most if not all translation initiation factors depending on the IRES element chosen (Filbin & Kieft, 2009). Previous studies, discussed in Section 1.2.1.1, had suggested that the expression of reporter genes in *S. cerevisiae* could be increased by incorporation of IRES sequences into the expression plasmid (Chappell et al., 2000), setting a precedent for investigating the use of IRES sequences to produce increased yields of a functional, recombinant membrane protein, in this case $hA_{2A}R$.

4.1.2.1 IRES selection

4.1.2.1.1 *YAP1* IRES

Located in the 5′UTR of the mRNA of the yeast adapter protein 1 gene (*YAP1*), which is a transcription factor involved in regulating a variety of genes required for oxidative stress tolerance (Moye-Rowley et al., 1989, Delaunay et al., 2000), the *YAP1* IRES has been shown to drive expression of reported genes via internal initiation in *S. cerevisiae* (Zhou et al., 2001, Edwards & Wandless, 2010). It has been shown to increase translational efficiency 6-fold for the reporter, *Photinus* luciferase (Zhou et al., 2001).

4.1.2.1.2 p150 IRES

Located in the 5′UTR of the mRNA of the p150 gene (also called *TIF4631*; p150 is the yeast homologue of the cap-binding protein eIF4G), the p150 IRES has been shown to drive expression of a variety of reported genes via internal initiation in *S. cerevisiae* (Zhou et al., 2001, Edwards & Wandless, 2010), increasing translation efficiency 9-fold for the reporter gene chloramphenicol acetyltransferase (CAT) (Zhou et al., 2001).

4.1.2.1.3 CrPV IRES

The Cricket paralysis virus (CrPV) IRES, requires no initiation factors (IFs) at all to initiate translation (Deniz et al., 2009) in common with the Plautia stali intestine virus (PSIV) IRES (Kieft, 2008). Unlike the PSIV IRES, the CrPV IRES has been investigated in *S. cerevisiae* and is reportedly able to bind directly to the 40S ribosomal subunit and stimulate 60S subunit recruitment and subsequent polypeptide elongation (Deniz et al., 2009). It was also used to allow *S. cerevisiae* growth on plates deficient in uracil, when it was used to drive synthesis of Ura3p (Deniz et al., 2009). This finding suggested that the CrPV IRES has sufficient activity in yeast to drive expression of recombinant proteins.

4.1.3 Objectives

The specific objectives of Chapter 4 were therefore:

1. To examine the quality of $hA_{2A}R$ yield using TSD
2. To design and construct IRES vectors for the YAP1, p150 and CrPV IRESes
3. To examine the $hA_{2A}R$-Ura3p yield from the IRES vectors
4. To investigate methods to increase yields by increasing IRES activity

4.2 IRES vector construction

4.2.1 Overview

As our model membrane protein is $hA_{2A}R$ we decided to use pYX222-$A_{2A}R$, which was the same construct modified in Chapter 3 for the SA strategy. We planned to insert each IRES upstream of the *$A_{2A}R$* sequence construct (Fig 4.2), while using unmodified pYX222-$A_{2A}R$ as the control.

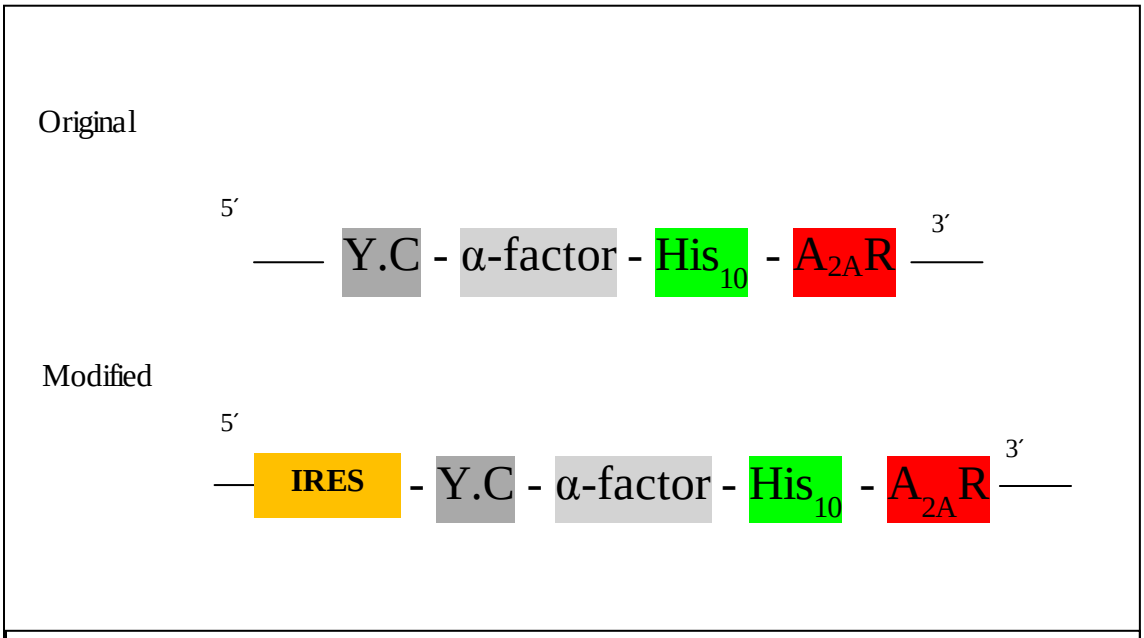


Fig 4.2 Schematic illustrating the modification of the previously-used pYX222-$A_{2A}R$ via the addition of a 5′ IRES. *The $A_{2A}R$ variant was devised by Niall Fraser and is a truncated form that has had the glycosylation site removed (red; Fraser, 2006). It is preceded by a yeast consensus sequence (Y.C) (dark grey) for enhanced translation initiation, α-factor secretion signal (light grey) for export of the recombinant protein to the cell membrane, and a sequence encoding a His_{10} tag (green) for purification. An IRES was placed upstream of the original construct (gold).*

The *YAP1* and p150 IRESes were from *S. cerevisiae* and were obtained by PCR from the genomic DNA with suitable restriction sites added at the 3′ and 5′ ends to allow insertion upstream of the $A_{2A}R$ sequence. Since we did not have access to CrPV genomic DNA, the relevant sequence was synthesised (GeneArt, Life Technologies) and cloned into a plasmid which we designated pCrPV(IRES). The cloning strategy for the three IRES plasmids is illustrated in detail in Section 4.2.2, Fig 4.3A and Fig 4.3B.

4.2.2 Cloning strategy for IRES plasmids, pYX222-CrPV(IRES)-$A_{2A}R$, pYX222-YAP1(IRES)-$A_{2A}R$ and pYX222-p150(IRES)-$A_{2A}R$

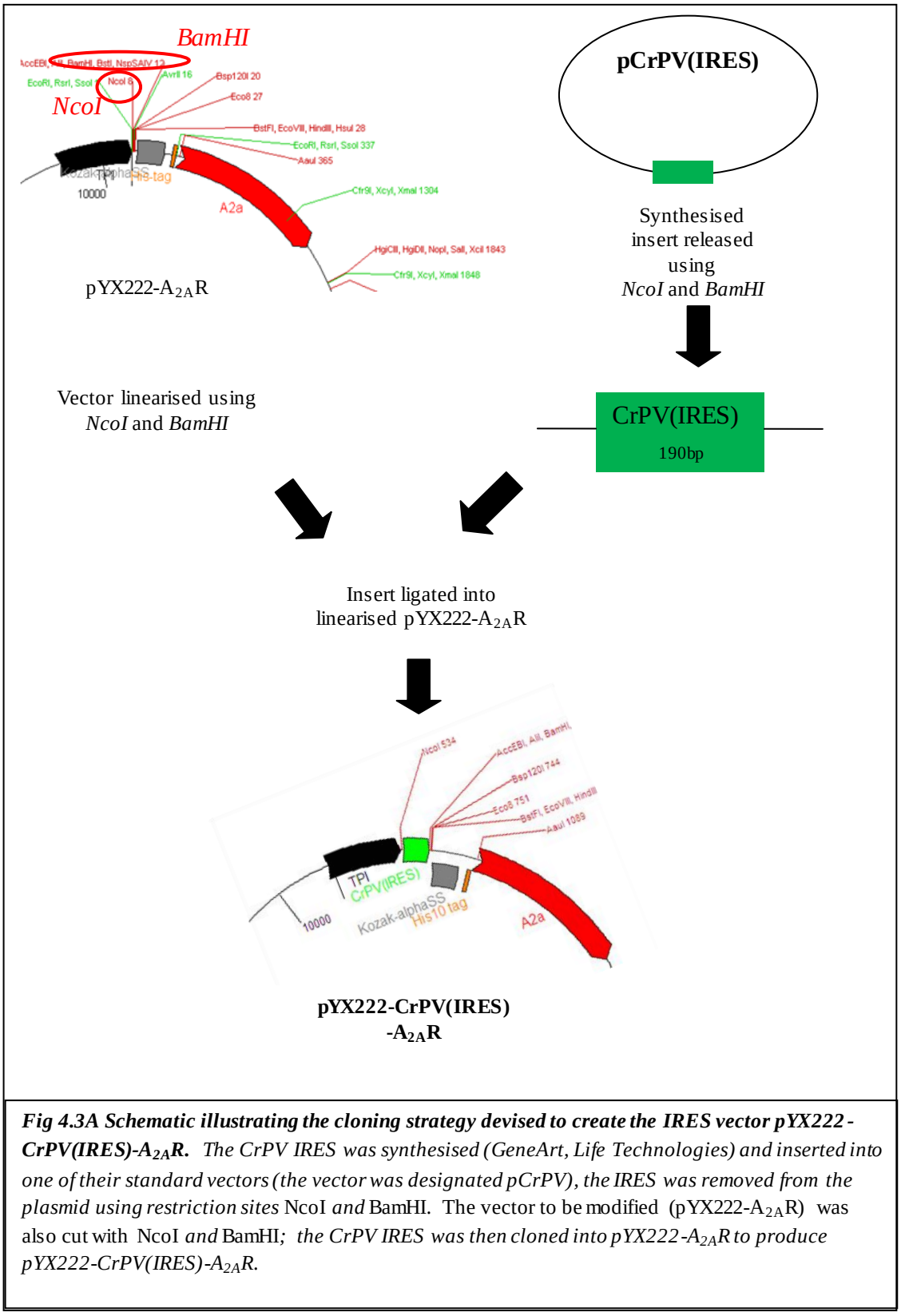


Fig 4.3A Schematic illustrating the cloning strategy devised to create the IRES vector pYX222-CrPV(IRES)-$A_{2A}R$. *The CrPV IRES was synthesised (GeneArt, Life Technologies) and inserted into one of their standard vectors (the vector was designated pCrPV), the IRES was removed from the plasmid using restriction sites* NcoI *and* BamHI. The vector to be modified (pYX222-$A_{2A}R$) was also cut with NcoI *and* BamHI; *the CrPV IRES was then cloned into pYX222-$A_{2A}R$ to produce pYX222-CrPV(IRES)-$A_{2A}R$.*

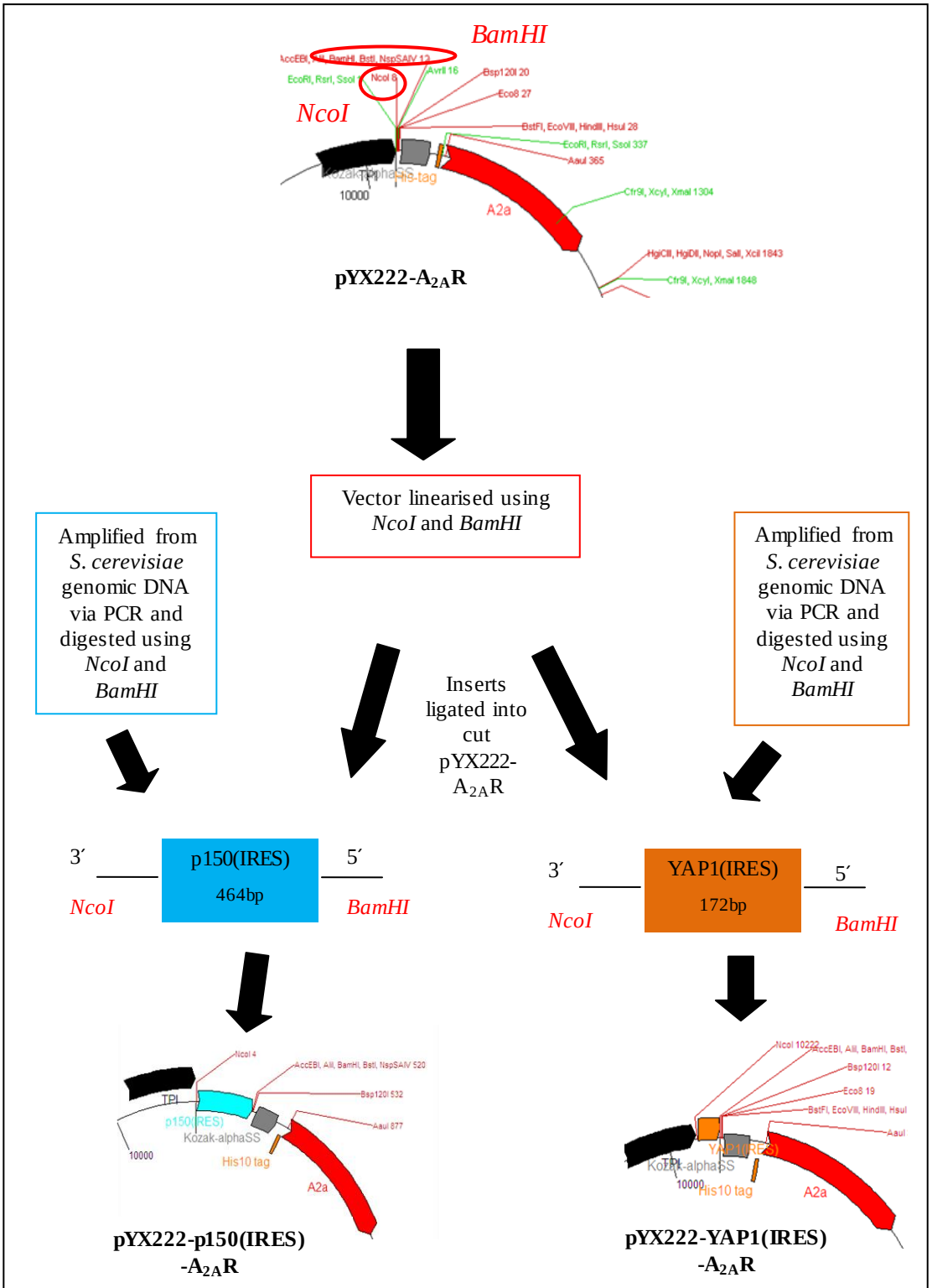


Fig 4.3B Schematic illustrating the cloning strategy devised to create the IRES vectors pYX222-YAP1(IRES)-A_{2A}R and pYX222-p150(IRES)-A_{2A}R . *The p150 and YAP1 IRESes were amplified using PCR from the genomic DNA of* S. cerevisiae *with the addition of restriction sites* NcoI *and* BamHI *to the 3′and 5′ ends respectively to allow insertion upstream of the* A_{2A}R *coding region in pYX222-A_{2A}R.The vector to be modified (pYX222-A_{2A}R) was also cut with* NcoI *and* BamHI, *with the IRESes then being cloned into pYX222-A_{2A}R to produce IRES vectors, pYX222-YAP1(IRES)-A_{2A}R and pYX222-p150(IRES)-A_{2A}R.*

4.2.3 Construction of IRES plasmids, pYX222-CrPV(IRES)-$A_{2A}R$, pYX222-YAP1(IRES)-$A_{2A}R$ and pYX222-p150(IRES)-$A_{2A}R$

Construction of sequence-verified pYX222-CrPV(IRES)-$A_{2A}R$, pYX222-YAP1(IRES)-$A_{2A}R$ and pYX222-p150(IRES)-$A_{2A}R$ is shown in Figure 4.4 and Appendix 1.

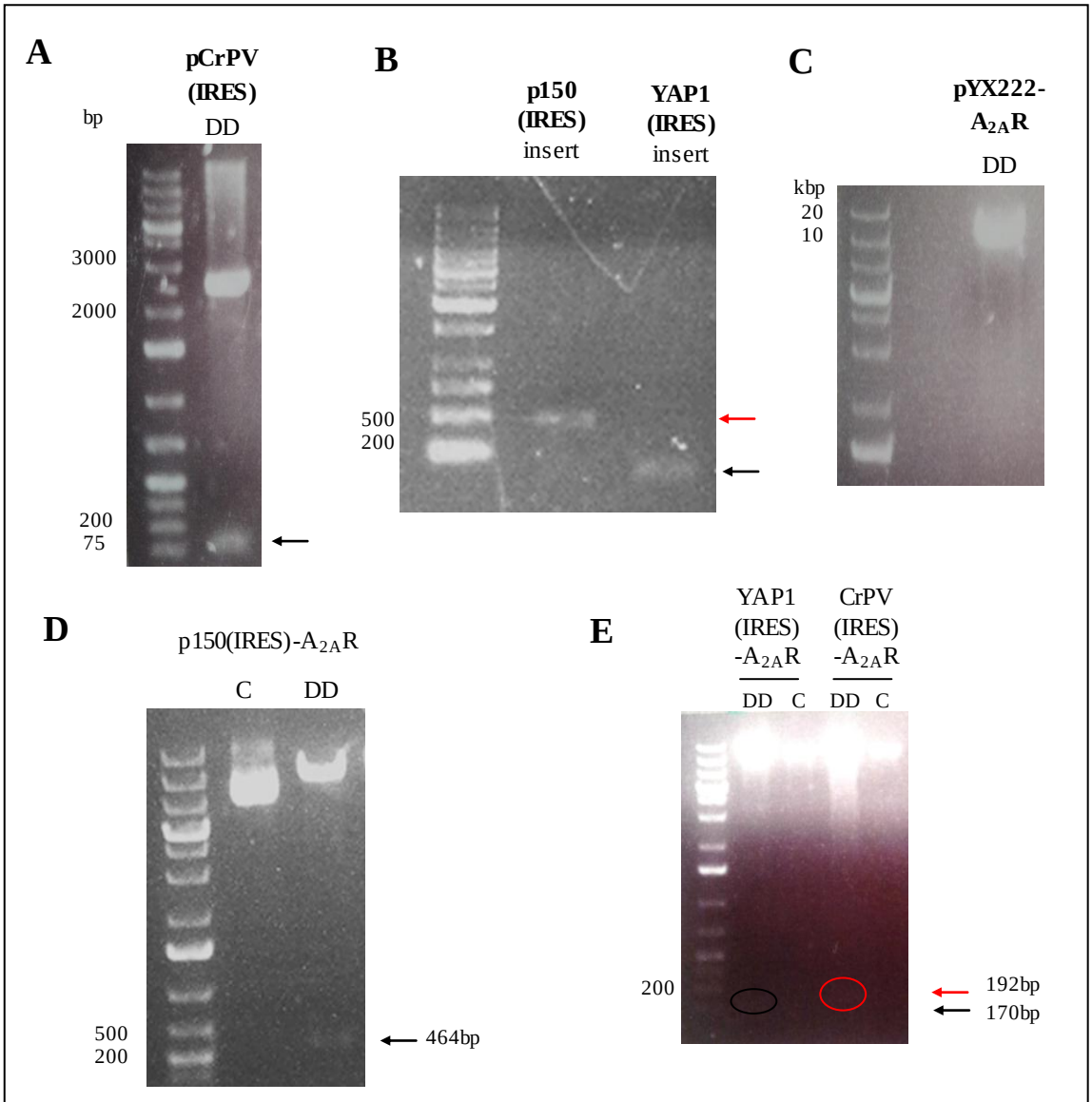


Fig 4.4 IRES vector construction. ***(A)*** *The pCrPV vector was transformed into XL2-Blue supercompetent* E. coli *and DNA retrieved by miniprep. A 1% agarose DNA electrophoresis gel was performed of the double digestion (DD,* NcoI *and* BamHI*) to confirm and retrieve the synthesised CrPV IRES sequence (C, control) from pCrPV. A band of approximately 192bp (arrow) representing the CrPV IRES insert was excised from the gel and purified.* ***(B)*** *A 1% agarose gel DNA electrophoresis gel showing PCR products at 464bp for p150 IRES (red arrow) and 170bp for YAP1 IRES (black arrow). These bands were excised and purified.* ***(C)*** *A 1% agarose gel DNA electrophoresis gel showing the double digested (DD,* NcoI *and* BamHI*) and linearised pYX222-$A_{2A}R$ at approximately 10kbp). The insert was excised and purified.* ***(D)*** *The purified CrPV IRES insert was ligated into pYX222-$A_{2A}R$ and transformed into XL2-Blue supercompetent* E. coli *and DNA retrieved by miniprep from successful transformants. A 1% agarose DNA electrophoresis gel was performed for a double digestion (DD,* NcoI *and* BamHI*) to confirm insert ligation a band of 464bp was released indicating success, which was followed up and confirmed by sequencing.* ***(E)*** *The purifed* YAP1 *and p150 IRES inserts were ligated into pYX222-$A_{2A}R$ and transformed into XL2-Blue supercompetent* E. coli *and DNA retrieved by miniprep from successful transformants. A 1% agarose DNA electrophoresis gel was performed of a double digestion (DD,* NcoI *and* BamHI*) to confirm insert ligation: a band of 192bp and 170bp was released indicating success for CrPV and YAP1 IRESes respectively (the inserts were visible by eye, red and black circles indicate their loaction), this was followed up and confirmed by sequencing.*

4.3 Radio-ligand binding analysis of $hA_{2A}R$ produced under conditions of translation inhibition

4.3.1 Radio-ligand binding analysis of $hA_{2A}R$ produced in the presence of guanabenz and emetine

We wanted to investigate whether imposing translation inhibition using the drugs guanabenz or emetine can lead to improved yields of $hA_{2A}R$. The *S. cerevisiae* parental wild-type strain, BY4741, was therefore transformed with the control $hA_{2A}R$ expression vector, pYX222-$A_{2A}R$, and cultures were grown in 100µM guanabenz (Sherman & Qian, 2013) or 2mM emetine (Kanner et al., 2003, Sherman & Qian, 2013); these concentrations had previously been established to cause sufficient translation inhibition in eukaryotes (Kanner et al., 2003, Sherman & Qian, 2013). The drugs were administered at the beginning of the culture, which was harvested at OD_{600} 4-5. Membranes were then prepared for single-point radio-ligand binding analysis with the antagonist [^{3}H] ZM241385. Fig 4.5 indicates that in the presence of guanabenz or emetine, $hA_{2A}R$ yields were 0.2 and 0.1 pmol mg^{-1}, respectively, compared with control values of 1.3 pmol mg^{-1}. Notably the growth rates of the cultures were 0.19 h^{-1} in the presence of guanabenz and 0.17 h^{-1} in the presence of emetine compared to 0.35 h^{-1} for control cultures suggesting that the drugs severely inhibited translation which significantly retarded growth which negatively affected recombinant yields. Since both drugs had a negative effect on $hA_{2A}R$ yield, and this approach was therefore not pursued further.

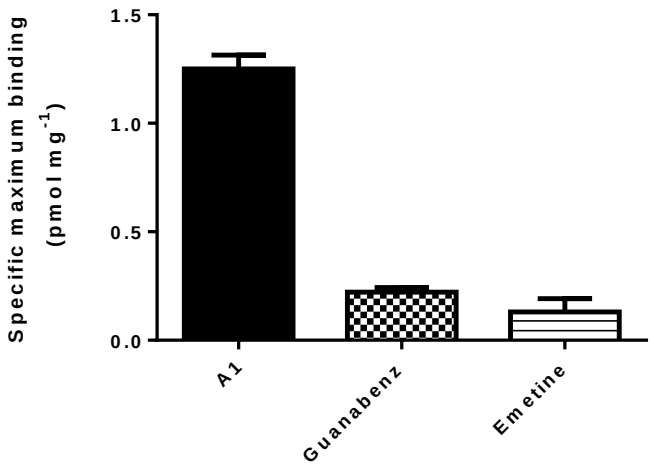


Fig 4.5 Single-point binding analysis of translationally inhibited yeast samples expressing $hA_{2A}R$ using the antagonist [^{3}H] ZM241385. *BY4741 expressing pYX222-$A_{2A}R$ was cultured in the presence of 2mM guanabenz) or 100µM emetine). Samples were grown to an OD_{600} 4-5 in 100ml cultures and 100µg total membrane preparations was used for each binding assay. Data are the mean of 3 experiments ± SEM.*

4.3.2 Radio-ligand binding analysis of $hA_{2A}R$ produced in the *tor1Δ* strain

The *tor1Δ* strain (together with the *spt3Δ* strain used in the SA strategy described in Chapter 3), are known to exhibit a translational block. This was confirmed by polysome profiling as shown in Fig 4.6, below.

Fig 4.6 The spt3Δ and tor1Δ strains are confirmed to have initiation blocks. *Polysome profiles, such as that shown for BY4741 in the upper panel, were determined for the BY4741 wild-type,* spt3Δ *and* tor1Δ *strains by Dr Stephanie Cartwright, Aston University. Profiles were determined in the replicates, as shown, and the ratio of peak areas of monosome (80S):polysome are reported. The corresponding standard error of the mean is shown in parentheses. The larger the value of the ratio, the greater the initiation block.*

Following this confirmation (Fig. 4.6), the *tor1Δ* strain was transformed with pYX222-$A_{2A}R$, the cells were cultured to an OD_{600} 4-5 and membranes were prepared. Fig 4.7 shows that there is no improvement in $hA_{2A}R$ yield (0.9 pmol mg^{-1}), compared to the control value of 1.3 pmol mg^{-1}. Notably the growth rates of the cultures were 0.24 h^{-1} for the *tor1Δ* transformant cultures and 0.35 h^{-1} for control cultures suggesting that perhaps the translational inhibition is too severe to increase $hA_{2A}R$ yield. Since there was no effect on $hA_{2A}R$ yield, despite an improvement in functional yield when the *spt3*Δ strain was used as a host cell for SA (Fig 3. 19), this approach was therefore not pursued further.

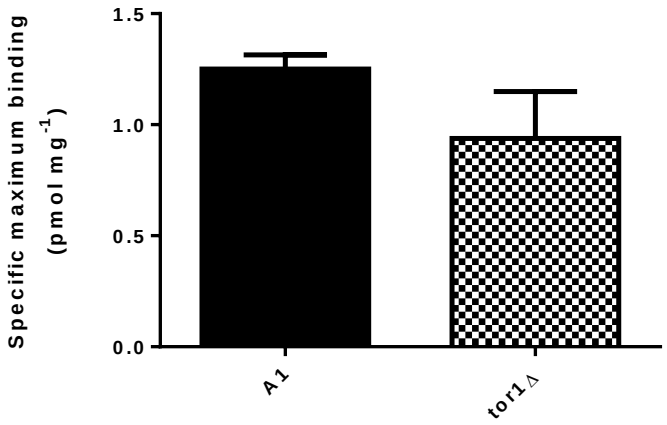


***Fig 4.7 Single-point binding analysis of the* tor1Δ *and BY4741 strains expressing $hA_{2A}R$ using the antagonist [^{3}H] ZM241385.** *Samples were grown to an OD_{600} 4-5 in 100ml cultures and 100µg total membrane preparations was used for each binding assay. Data are the mean of 3 experiments ± SEM.*

4.4 Employing IRES sequences to increase recombinant $hA_{2A}R$ yield

4.4.1 Radio-ligand binding analysis of $hA_{2A}R$ produced using IRES-A_{2A} vectors transformed into BY4741 WT and *spt3Δ*

IRES sequences are active when the non-canonical mechanism for translation initiation is inhibited (Komar & Hatzoglou, 2005). We therefore chose *S. cerevisiae* strain s*pt3Δ* for this study, since it is known to exhibit a mild initiation block (unpublished data by Dr Stephanie Cartwright, Aston University); and confirmed in Fig 4.6) its use with IRES-containing vectors should therefore boost translation initiation in this already effective strain (Fig. 3.19).

As controls, the BY4741 and s*pt3Δ* strains were both transformed with pYX222-$A_{2A}R$ to account for strain-related effects in cap-dependent translation; it was expected that the s*pt3Δ* strain would therefore give a lower total yield. The IRES augmented vectors, pYX222-YAP1(IRES)-$A_{2A}R$, pYX222-p150(IRES)-$A_{2A}R$ and pYX222-CrPV(IRES)-$A_{2A}R$, were also used to transform both *spt3Δ* and BY4741; it was expected that the IRES should have a bigger impact in the *spt3Δ* strain. Radio-ligand binding assays were performed using [^{3}H]ZM241385 on total cell membranes containing 100µg total membrane protein for each of the 3 IRES vector transformants and their respective controls (Fig 4.8), to investigate recombinant $hA_{2A}R$ yields.

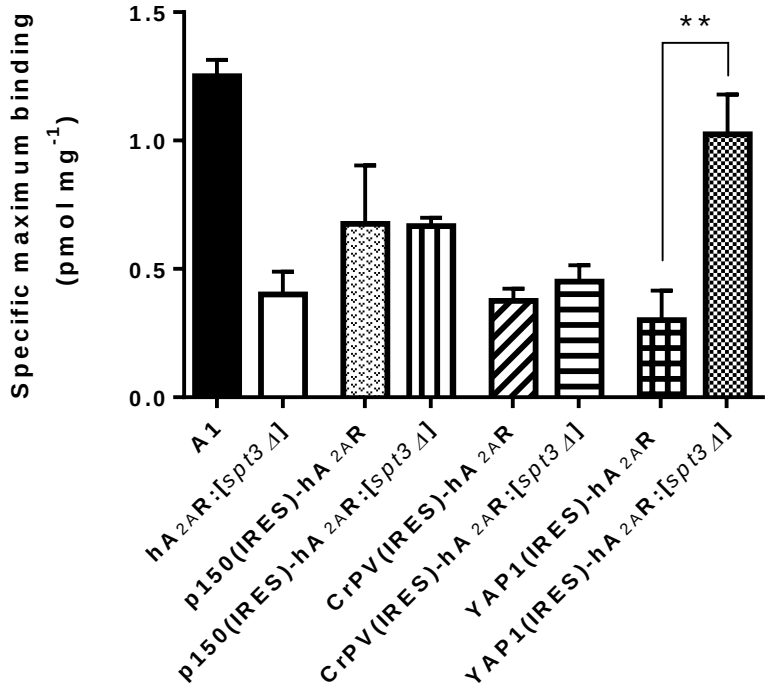


Fig 4.8 Single-point binding assay for $hA_{2A}R$ expressed by IRES-augmented vectors using antagonist [^{3}H]ZM241385*. Single-point saturation binding using the antagonist [^{3}H]ZM241385 was performed on total cell membranes containing 100µg total membrane protein harvested from each transformant sample; the samples were grown to an OD_{600} of 4-5. A 1-way ANOVA with a Holm-Sidak's multiple comparison test with samples grouped into 3 preselected groups p150(IRES), CrPV(IRES) and YAP1(IRES)gave significance with p = of 0.001 (***) for YAP1(IRES)-$hA_{2A}R$ versus YAP1(IRES)-$hA_{2A}R$[spt3Δ]. All data are the mean of at least 3 independent experiments ± SEM.*

4.4.1.1 p150 IRES

Expression under the control of the p150 IRES in both BY4741 (p150(IRES)-$hA_{2A}R$:[WT]) and s*pt3Δ* (p150(IRES)-$hA_{2A}R$:[s*pt3Δ*]) transformants appeared to be reduced compared to the A1 control; reporting a yield of 0.7 ± 0.2 and 0.7 ± 0.1 pmol mg^{-1}, suggesting that s*pt3Δ* did not activate the p150 IRES and increase the yield. The p150 IRES is located in the 5′ UTR of the yeast homolog of eIF4G, which is a vital protein for translation (Prévôt et al., 2003). It is therefore possible that since eIF4G is required for translation of essential housekeeping genes, especially during periods of global inhibition of initiation throughout the cell (Komar & Hatzoglou, 2005), that the regulation of p150 IRES is subject to tight regulation.

4.4.1.2 CrPV IRES

The CrPV IRES appeared to cause a general reduction in $hA_{2A}R$ expression in the s*pt3Δ* and BY4741 strains compared to the A1 control (1.3 pmol mg^{-1}), producing average yields of 0.4 ± 0.1 and 0.5 ± 0.1 pmol mg^{-1} respectively. Unlike the other two IRESes under study, the CrPV IRES is not endogenous to *S. cerevisiae*, instead being found as part of a virus that infects insect cells (Plus & Scotti, 1984). In order to enhance the IRES activity in yeast, another mutant of BY4741, *gcn3Δ*, was chosen because it had been suggested that as the CrPV IRES is inhibited in the presence of abundant ternary complexes (due to competition for the P-site of the 40S ribosomal subunit); therefore a reduction in ternary complexes would enhance IRES activity (Deniz et al., 2009). Since *GCN3* encodes the α subunit of eIF4B, which is involved in facilitating the formation of ternary complexes via guanine nucleotide exchange, a mutation leads to a reduction in ternary complexes (Gomez & Pavitt, 2000, Taylor et al., 2010).

The pYX222-CrPV(IRES)-$A_{2A}R$ plasmid and the control plasmid, pYX222-$A_{2A}R$, were therefore transformed into *gcn3Δ*. Fig 4.9 indicates that the CrPV IRES had improved activity in the *gcn3Δ* strain, evident by increased expression of $hA_{2A}R$ in the presence of the IRES when compared to the no-IRES vector control: CrPV(IRES)-$hA_{2A}R$:[*gcn3Δ*] gave a yield of 0.9 ± 0.1 pmol mg^{-1} compared to 0.3 ± 0.1 pmol mg^{-1} for $hA_{2A}R$:[*gcn3Δ*], representing a 3-fold increase that was attributed to the CrPV IRES. Additionally, the yield seen from CrPV(IRES)-$hA_{2A}R$:[*gcn3Δ*] was almost double the amount previously observed from the other mutant s*pt3Δ* using CrPV IRES (CrPV(IRES)-$hA_{2A}R$:[s*pt3Δ*] (0.5 ± 0.1 pmol mg^{-1}). This validated the hypothesis made by Deniz et al., 2009 and our decisions to choose a more suitable host for CrPV(IRES).

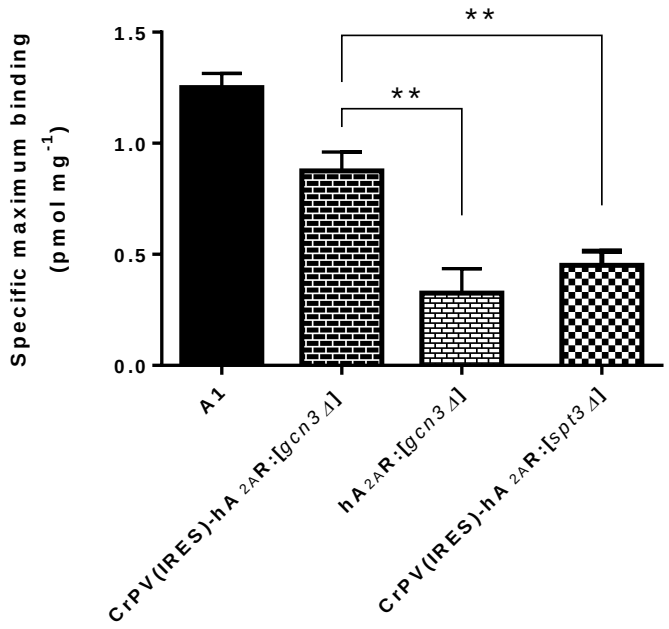


Fig 4.9 Single-point binding assay for $hA_{2A}R$ expressed in the CrPV(IRES) augmented vector. *Single-point saturation binding using the antagonist [^{3}H]ZM241385 was performed on total cell membranes containing 100µg total membrane protein harvested from CrPV(IRES)-$hA_{2A}R$:[*gcn3Δ*] and $hA_{2A}R$:[*gcn3Δ*]. Each transformant was grown to an OD of 4-5. All data are the mean of at least 3 independent experiments with error bars in SEM. A 1-way ANOVA with a Holm-Sidak's multiple comparison gave a p = 0.01 (**) for both $hA_{2A}R$ $hA_{2A}R$:[*gcn3Δ*] and CrPV(IRES)-$hA_{2A}R$:[*spt3Δ*] against CrPV(IRES)-$hA_{2A}R$:[*gcn3Δ*]. Data are representative of the mean of 3 independent experiments ± SEM.*

4.4.1.3 *YAP1* IRES

The binding data in Fig 4.8 suggest that the presence of the *YAP1* IRES enhanced $hA_{2A}R$ expression in the s*pt3Δ* strain (YAP1(IRES)-$hA_{2A}R$:[s*pt3Δ*] = 1.0 ± 0.1 pmol mg^{-1}) over the wild-type control (YAP1(IRES)-$hA_{2A}R$:[WT] = 0.3 ± 0.1 pmol mg^{-1}), by over 3-fold, and over the non-IRES control ($hA_{2A}R$:[*spt3Δ*] = 0.4 ± 0.1 pmol mg^{-1}) by over 2-fold. However compared to the A1 control ($hA_{2A}R$:[WT] = 1.3 ± 0.1 pmol mg^{-1}), the yields are comparable, suggesting that in the s*pt3Δ* strain, the *YAP1* IRES cannot exceed yields achieved by cap-dependent translation in BY4741.

The *YAP1* IRES is responsible for the transcription of vital oxidative genes during stress and is thought to be highly regulated, although as yet this is not fully understood (Delaunay et al., 2000). The data in Fig 4.8 might be therefore be interpreted to suggest that that the initiation block in the s*pt3Δ* strain was not sufficient to activate the *YAP1* IRES. We therefore attempted to activate the *YAP1* IRES using a stress related to its native function: the cap-independent initiation of translation of *YAP1* in response to oxidative stress.

We attempted to mimic oxidative stress conditions by culturing BY4741 in 100ml YPD cultures for 18h followed by a 1h treatment with varying concentration of H_2O_2 (0, 25, 50, 100, 200 and 300µM). These concentrations were the same concentration range that had been used previously in *S. cerevisiae* to induce and increase Yap1p expression (Delaunay et al., 2000). Immunoblots were performed (Fig 4.10A) and were analysed using ImageJ to compare band intensities relative to the control conditions (0µM H_2O_2; Fig 4.10B).

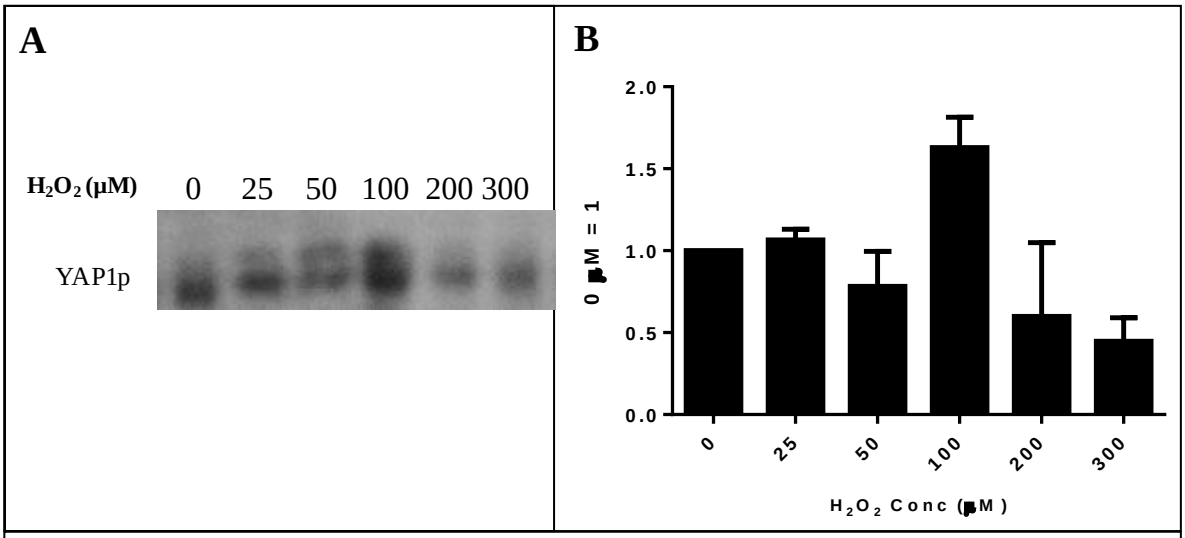


Fig 4.10(A) Immunoblot performed using anti-Yap1p antibody to measure YAP1 expression in BY4741 in varying concentrations of H_2O_2. *BY4741 cells were grown to an OD 4-5 (19h) in 100ml YPD cultures containing varying concentrations of H_2O_2. Cells were harvested and 25µg total cell lysates were loaded in each well (determined using a BCA assay), and Yap1p was visualised using anti-Yap1p antibody [Santa Cruz Biotechnology].* ***(B) Quantification of Yap1p using immunoblot data via ImageJ.*** *The Immunoblot data were used to quantify Yap1p expression relative to the 0µM control; data for each concentration are representative of the mean of 2 independent experiments with error bars in SEM.*

As seen in Fig 4.10B, it appears that 100µM H_2O_2 elicits the strongest response in terms of Yap1p expression. Although the experiment was only performed twice, this provides a basis on which trials for the optimisation of the YAP1(IRES)-hA_{2A}R plasmid could be started.

4.5 Summary

4.5.1 Increasing recombinant $hA_{2A}R$ yields through translation inhibition

We were able to modify the pYX222-$A_{2A}R$ vector with the *YAP1*, p150 and CrPV IRESes to create the pYX222-YAP1(IRES)-$A_{2A}R$, pYX222-p150(IRES)-$A_{2A}R$ and pYX222-CrPV(IRES)-$A_{2A}R$ IRES vectors (Fig 4.4). The *tor1Δ* strain is known to have an initiation block, which we hoped would activate the IRESes but did not lead to an increase in $hA_{2A}R$ yields; radio-ligand binding showed comparable yields to the BY4741 control (WT:$hA_{2A}R$ = 1.3 pmol mg^{-1}; Fig 4.7). Addition of emetine and guanabenz dramatically reduced yields compared to the control (0.2 and 0.1 pmol mg^{-1} respectively) (Fig 4.5). This could be followed up by harvesting at different times, concentrations and duration of dose. The treated samples took around 27-30h to reach an OD_{600} of 4-5, which is 8-11h more than *tor1Δ*:$hA_{2A}R$. This indicates that the drugs had too negative an impact on translation and growth to be useful.

4.5.2 Increasing $hA_{2A}R$ yield using IRES-activated translation

All IRES-containing vectors reduced yields of $hA_{2A}R$ compared with the yield from the no-IRES control, A1 (1.3 pmol mg^{-1}; Fig 4.8). However, some interesting trends were observed: the p150 IRES showed approximately no change in yield (0.7 pmol mg^{-1}) in either BY4741 or *spt3Δ* cells. Since the p150 gene encodes eIF4G (Zhou et al., 2001), it would not be surprising that the p150 IRES is tightly regulated. The *YAP1* IRES caused a 3-fold reduction in yield (0.3 pmol mg^{-1}) compared to the A1 control, yet when used with *spt3Δ* the yield was comparable to the control (1.0 pmol mg^{-1}) (Fig 4.8). The CrPV IRES also suffered reduced yields of $hA_{2A}R$ in BY4741 (0.4 pmol mg^{-1}) and *spt3Δ* (0.5 pmol mg^{-1}) compared the control, and did not seem more active in *spt3Δ* (Fig 4.8). Overall the *YAP1* IRES in *spt3Δ* was the most successful with a yield of 1.0 pmol mg^{-1} compared with 1.2 pmol mg^{-1} for the A1 control.

4.5.3 Activating the CrPV IRES in the *gcn3Δ* strain

The CrPV IRES vector was used to transform strain *gcn3Δ*, which has reduced ternary complexes known to increase CrPV IRES activity (Deniz et al., 2009). This transformant resulted in the highest $hA_{2A}R$ yields under the control of the CrPV IRES (0.9 pmol mg^{-1})

(Fig 4.9). Despite the yield being lower than the A1 control, it was still an approximate 2-fold increase over the *spt3Δ* and BY4741 transformants, suggesting there is potential and scope for the IRES in other mutant strains.

4.5.4 Activating *YAP1* IRES activity with oxidative stress

The *YAP1* IRES had the largest effect on yield of the three IRES sequences tested; this was achieved in the *spt3Δ* strain, with specific activity increasing from 0.3 pmol mg^{-1} in BY4741 to 1.0 pmol mg^{-1} in the *spt3Δ* strain. We therefore attempted to identify a H_2O_2 concentration that induced native Yap1p synthesis, which would be evidence of increased *YAP1* IRES activity. It was determined that 100µM H_2O_2 caused the highest levels of Yap1p of all the concentrations tested, with a 1.6-fold increase over the control (Fig 4.10).

Chapter 5: Discussion

A major finding of this thesis is that it is possible to use SA to select for transformants that produce an increased total and functional $hA_{2A}R$-Ura3p yield compared with controls (Fig 3.22). Importantly the strategy did not affect receptor affinity (Fig 3.21). Initially the strategy generated H1 which exhibited vacuolar internalisation of $hA_{2A}R$-Ura3p (Fig 3.12), and therefore low functional yield, despite high total yields (Fig 3.11 vs. Fig 3.10). The *spt3Δ* deletion strain, known to have improved folding capabilities, was therefore used with the SA strategy to alleviate this phenotype, leading to the generation of SU1 (Fig 3.16). SU1 had increased $hA_{2A}R$-Ura3p functional yield (3-fold) over controls (Fig 3.18) and did not suffer vacuolar internalisation (Fig 3.19). Additionally after solubilisation, $hA_{2A}R$-Ura3p could be recovered from H1 membranes following solubilisation with DDM and CHS; this increased functional yield recovery from H1 to 5.8-fold over the control (Fig 3.21).

Attempts to increase yield by manipulating translation processes were not as successful, with neither the TSD nor IRES strategies leading to an increased $hA_{2A}R$ yield. The data from the TSD strategy suggested that the translation-inhibiting drugs guanabenz and emetine caused a drastic reduction in yield compared to the control (Fig 4.5). The translation-deficient mutant *tor1Δ* gave a minor reduction in yield, suggesting its specific translation initiation block was not relevant to improve yield for this study (Fig 4.5). The data from the IRES strategy indicated that cap-independent translation should be explored further in combination with specific stress conditions (Fig 4.9).

5.1 Principle of SA as a strategy to increase yield

5.1.1 Generation of SA transformants

We were able confer a selective advantage on *S. cerevisiae* cells that expressed $hA_{2A}R$ by tagging the GPCR with Ura3p to create a fusion protein thereby generating H1, SU1 and U1 transformants (Fig 3.6 and 3.16). A two-step method was devised to allow adaptation on CSM-histidine plates before the colonies were spotted onto CSM-uracil. This generated

3 colonies (H1, H2 and H3) from a population of 60. Only H1 was viable, culturable and was discovered to be high yielding (Fig 3.6). The one-step method generated one colony from the BY4741 strain (U1, Fig 3.6), and one colony from the *spt3Δ* stain (SU1, Fig 3.16), suggesting a lower survival rate for the 1-step method.

U1 had a slow growth phenotype and negligible yield was observed via immunoblot (Fig 3.10) and radio-ligand binding assay (Fig 3.11). In contrast, SU1 had a minor growth defect, growing around 10% more slowly than BY4741; the *spt3Δ* strain already had this phenotype. Indeed, a phenotype that the one-step transformants have in common are slower growth (Fig 3.6 and 3.16), which raises the possibility that only cells with slower growth can deal with the stress associated with the one-step method because yeast transformation is already a stressful process (Kawai et al., 2010). Since the strain used in the one-step process to generate SU1 was a mutant (*spt3Δ*) that grew slower than BY4741, we expected a higher survival rate from *spt3Δ*-generated transformations and therefore more colonies to be generated. Since this was not observed, it might have been due to low transformability of the *spt3Δ* mutant, which is supported by evidence that mutant strains of *S. cerevisiae* and numerous other fungi have been found to have differing transformability dependent upon their mutation (Kawai et al., 2010). An experiment to assess the standard transformability under normal conditions (e.g. no SA), could have been conducted to discover if this was the case.

Post-transformation, cells have a damaged cell membrane, are severely stressed (Kawai et al., 2010) and have to survive long enough to reproduce. This might affect the cell's ability to produce and traffic a complex membrane protein such as the hA_{2A}R-Ura3p fusion. Any poorly folded recombinant product would be degraded before it could promote growth on CSM-uracil. In the two-step method, expression of hA_{2A}R-Ura3p is not linked to survival in the first step because CSM-histidine contains uracil, allowing the cells time to adapt before the second step. Therefore the decision to take into account the stresses imposed on the cells with regard to the selection process appeared to be validated.

5.1.2 Uracil requirement

The experiments in Table 3.1 showed that even when 100-fold less uracil was added to the culture medium than is typically found, A1 (non-SA control producing $hA_{2A}R$) grew at approximately one third of the biomass that it grew under standard uracil conditions. This is not surprising because Ura3p is the most efficient enzyme yet known (Houk et al., 2001) with even a small amount of uracil capable of promoting growth of auxotrophic strains (Metzger et al., 2008) and more uracil allowing higher biomass (Table 3.1). It is likely that this characteristic would prevent loss of the phenotype as long as H1 and SU1 were grown in medium lacking uracil, because the cells producing more $hA_{2A}R$-Ura3p within the population would out-compete the cells that were producing less $hA_{2A}R$-Ura3p. It is possible, however, that the H1 and SU1 phenotypes are conferred due to the acquisition of a genomic mutation, which has been reported previously using selective advantage in *L. lactis*, as discussed in Section 1.5 (Linares et al., 2010). We were able to rule out revertants because BY4741 is auxotrophic due to the deletion of the entire coding region for the *URA3* gene including the flanking 5′ and 3′ regions that do not affect neighbouring genes (Pronk, 2002). Attempts to promote plasmid loss were attempted, but were inconclusive; whether H1 or SU1 are therefore true transformants or mutant strains remains an open question. For the purposes of this study, and in the absence of other evidence, we have referred to them as transformants throughout the thesis.

The H1 transformant was grown in different selective media (that imposed different selective states): CSM-histidine, CSM-histidine -uracil and CSM-uracil to investigate the influence of different selective pressures on $hA_{2A}R$-Ura3p yield (Fig 3.10). In-line with our hypothesis, cells grown on CSM-uracil recorded the highest increase in yield (7-fold) when analysed via immunoblot, with both CSM-histidine -uracil (selecting for the plasmid and $hA_{2A}R$-Ura3p production) and CSM-histidine (selecting only for the plasmid) conditions only causing a 2-fold increase (Fig 3.10). As CSM-uracil had been shown to select a high-yielding phenotype, this was the selection also chosen for SU1. SU1 was found to have lower recombinant yields than A1 when assayed by immunoblot (Fig 3.17), but the functional yield was 3-fold higher (Fig 3.18), suggesting A1 must have a higher proportion of non-functional $hA_{2A}R$.

An interesting addition to the study would have been to perform a viability assay to determine the effect these different selection states imposed on the yeast. This would provide evidence to test the assumption that the double auxotrophy caused by CSM-histidine -uracil was responsible for the decreased yield observed compared to the A1 control (Fig 3.10). This assumption is supported by the literature which reports that a decreased growth rate and/or higher energy requirements imposed upon the cells results in lower recombinant protein yield (Hensing et al., 1995, Liu et al., 2013). Since CSM-histidine -uracil selects for high-expression of $hA_{2A}R$-Ura3p and plasmid maintenance, there is an additional metabolic load under these conditions. Despite this, all conditions caused an increase in $hA_{2A}R$ yield over A1 (Fig 3.10). However, there is evidence that tags (Ura3p as in this case) can have a positive effect on yield improving stability and function (Waugh, 2005) and even to some extent can render their fusion partner resistant to intracellular proteolysis (De Marco et al., 2004) perhaps decreasing the rate of turn-over.

5.1.3 Characterisation of $hA_{2A}R$-Ura3p

5.1.3.1 Sequence identification

From the immunoblot analysis of A1 and H1 expression (Fig 3.9) we expected two bands, a monomer and dimer for both A1-expressed $hA_{2A}R$ and H1-expressed $hA_{2A}R$-Ura3p. This has been reported previously for $hA_{2A}R$ expressed in yeast as an untagged protein (Niebauer & Robinson, 2006) and as a GFP fusion protein (O'Malley et al., 2007). This was the case for A1, with a 45kDa band denoting the monomer and a band of approximately 150kDa most likely representing a dimer. For H1, we saw a 45kDa band suggesting a $hA_{2A}R$ monomer, and a 170kDa band which most likely represents a dimer of the $hA_{2A}R$-Ura3p fusion, with no 75kDa monomer seen. This might suggest the $hA_{2A}R$-Ura3p monomer suffered C-terminal degradation; notably recombinant $hA_{2A}R$ synthesis has been reported to produce products of 35kDa in *E. coli* (Weiss & Grisshammer, 2002), *S. cerevisiae* (O'Malley et al., 2007) and *P. pastoris* (Singh et al., 2008) as seen for A1 and H1. Since Ura3p levels have been observed to be regulated according to required concentration, it is possible that some specific proteolysis occurred (Metzger et al., 2008). A likely explanation for the absence of a $hA_{2A}R$-Ura3p monomer could be the selection process itself. As Ura3p is only active as a dimer (Hu et al., 2008), it is possible that monomers of $hA_{2A}R$-Ura3p would not be active and therefore not confer the SA,

suggesting that perhaps the SA process caused transformant cells that express the monomer to be selected against (inactive Ura3p), and those that express dimers (active Ura3p) to be selected for. The 170kDa band was analysed using mass spectrometry (Table 3.4), subsequently confirming the presence of both the $hA_{2A}R$ and Ura3p. The analysis of the mass spectrometry data cannot definitively confirm fusion proteins because the software identifies unique peptide sequences of protein fragments cross-referenced against known proteins from a database. The fact that the two proteins are found at the correct molecular weight for a $hA_{2A}R$-Ura3p dimer, and the $hA_{2A}R$ and Ura3p are of different molecular weights individually suggests strongly the fusions' presence.

5.1.3.2 Functional characterisation of $hA_{2A}R$-Ura3p

Single-point saturation binding analysis was used to measure functional yield (i.e. the amount of protein that can bind ligand in a native-like manner) of the $hA_{2A}R$ moiety synthesised by A1, H1 and SU1. These transformants yielded B_{max} values of 1.3 ± 0.1 pmol mg^{-1}, 1.6 ± 0.1 pmol mg^{-1} and 3.3 ± 0.2 pmol mg^{-1}, respectively (Fig 3.11 and 3.18). This represented only a minor functional yield increase for H1, but a 3-fold increase for SU1. Notably there was a 420% yield decrease for H1 (7-fold to 1.3-fold; Fig 3.10 and 3.11) and an 82% yield increase for SU1 (0.5-fold to 2.8-fold; Fig 3.17 and 3.18) when comparing functional yield values to total yield values (from the immunoblots, when A1 =1 ; the immunoblot data measure total recombinant protein that has an accessible His_{10} epitope). The decrease in functional yield for H1 can be attributed to vacuolar internalisation; as we discussed earlier, in 2006 Niebauer and Robinson also observed vacuolar internalisation and reported that only 60% of their $hA_{2A}R$-GFP was functional, which they surmised might be due to the receptor being in a low affinity state. They speculated that this might be due to the presence of the sterol, ergosterol, rather than mammalian cholesterol in yeast membranes (Niebauer & Robinson, 2006). This is in line with our findings in that a large proportion of $hA_{2A}R$-Ura3p from H1 was non-functional. However, it is unlikely that ergosterol is an issue because when $hA_{2A}R$ was synthesised in A1, it was not internalised to the vacuole. The common factor in the Niebauer and Robinson study and this study is that $hA_{2A}R$-GFP and $hA_{2A}R$-Ura3p have tags, so it might be speculated that these were the cause of the internalisation.

Despite quantification via immunoblot indicating a 7-fold increase in recombinant expression over the A1 control (Fig 3.10), when a radio-ligand binding assay was performed on the same samples, there was only a slight yield increase for H1 over A1 (Fig 3.11). SU1 had comparable levels of recombinant expression compared to A1 from immunoblot data (Fig 3.17), yet radio-ligand binding showed a 3-fold increase over A1 (Fig 3.18). As none of the previous studies that have used selective advantage to increase membrane protein yield have supplemented their methods with a functional assay, we cannot make a direct comparison to the proportion of unfolded to folded recombinant expression in previous studies. Our overall conclusion is that SU1 makes higher quality protein than A1; as such, our data are consistent with a higher proportion of misfolded $hA_{2A}R$ being produced by the A1 transformant, and a lower proportion of misfolded $hA_{2A}R$-Ura3p being produced by SU1.

In a study focused on directed evolution of *E. coli* transformants to increase yields of membrane proteins fused to GFP, Gul et al,.2014 used a temperature decrease to ensure that the GFP fusion partner was folded correctly; this was because a normal growth temperature of 37°C yielded no fluorescence signal. This decreased the *E. coli* growth rate and might therefore alleviate misfolding and subsequent degradation. Notably, a reduction in growth rate has already been shown to improve yields in yeast (Hackel et al., 2006, Gasser et al., 2007). Lowering growth temperature is likely to have a similar beneficial effect as the use of *spt3Δ* in our study; *spt3Δ* has a reduced growth rate compared to wild-type cells. In contrast, H1 was generated and cultured at the standard growth temperature for *S. cerevisiae* of 30°C; it is perhaps therefore not surprising that we encountered misfolding and that a similar decrease in temperature to 25°C might have been usefully employed for generating improved transformants.

Confocal imagery was performed to assess any differences in recombinant protein localisation between the A1 and H1 transformants (Fig 3.12). The images revealed that H1 had a concentrated accumulation of $hA_{2A}R$-Ura3p in the vacuole, whereas the A1 control exhibited evenly distributed expression throughout the cell with a vacuole devoid of $hA_{2A}R$. It is possible that H2 and H3, the viable but non-culturable (VBNC) transformants from the two-step selection (Fig 3.6), exhibited their phenotype due to a high abundance of

misfolded recombinant protein in their vacuoles. This is supported in the literature for *S. cerevisiae*, where increases in misfolded recombinant proteins have been shown to decreases fitness and retard growth dramatically due to cytotoxicity (Geiler-Samerotte et al., 2011). No confocal microscopy was performed on H2 and H3, which would have been useful in their analysis to confirm this speculation.

Three factors needed to be addressed with respect to their effect on the affinity of the $hA_{2A}R$ moiety of $hA_{2A}R$-Ura3p: the influence of the Ura3p tag; the effect of expression as part of SA strategy; and the influence of the *spt3Δ* strain. Homologous competition binding experiments were therefore conducted using ZM241385 (Fig 3.20), with pK_d values for A1, H1 and SU1 being 8.6 ± 0.2, 8.4 ± 0.2 and 8.3 ± 0.2 respectively. This suggests $hA_{2A}R$ from all sources has values in line with those reported for membrane-bound $hA_{2a}R$ using various constructs in the literature (Singh et al., 2010). Therefore it was determined that neither the Ura3p tag, expression in an SA manner or *spt3Δ* had any effect on $hA_{2A}R$-Ura3p affinity. Additionally, as discussed earlier in this section, our data suggest that there is a significant heterogeneous receptor population in membranes tested from H1 comprising of non-functional and functional $hA_{2A}R$-Urap3.

5.1.4 Comparison with previous studies

To our knowledge, using SA as a tool to boost eukaryotic recombinant protein yields has not been previously reported in yeast, but has been performed in bacteria. The first study was carried out in 1999 by Maxwell and colleagues, who discovered that *E. coli* cells that expressed soluble forms of a fusion of HIV integrase with chloramphenicol acetyltransferase (CAT) had higher resistance than cells that expressed insoluble forms when cultured on plates containing high levels of chloramphenicol (Maxwell et al., 1999). Strains of *E. coli* could therefore be selected and isolated that produced more soluble protein.

In 2009, Massey-Gendel and colleagues created a fusion protein of the membrane protein, rhomboid-Rv1337 (from *Mycobacterium tuberculosis*, responsible for most cases of tuberculosis), fused to chloramphenicol acetyltransferase (CAT), an enzyme conferring

antibiotic resistance. They reported a 75-fold increase in yield (Massey-Gendel et al., 2009), which was quantified by immunoblot. As shown in Chapter 3, this method is not able to assess the functional or folded state of membrane proteins as demonstrated by our results and literature reports (Niebauer & Robinson, 2006).

In 2010, Linares and colleagues used *L. lactis* strains to increase the expression of recombinant membrane proteins by fusing the membrane protein with a protein that conferred resistance to the antibiotic erythromycin (the erythromycin-resistance protein (ErmC) together with a GFP tag) (Linares et al., 2010). By gradually increasing the erythromycin concentration, cell viability decreased, with the surviving population being higher yielding (2-8 fold) than controls. The authors claim that the resulting strains possessed an improved folding state, although no functional assay was performed to assess true functionality. They also discovered that when selected strains had their genome sequenced, that they had acquired a mutation in the *nisK* gene which encodes a sensor histidine kinase, allowing increased transcription due to their plasmid having a nisin promoter (Linares et al., 2010). By analogy with this study, we do not know whether our selected transformants have acquired mutations in their genome, which will be the subject of future work.

The most recent SA study reported in the literature, from the *L. lactis* research group, used the same selective advantage fusion strategy but with *E. coli*. This also generated strains that had increased expression of functional recombinant target membrane proteins when they were fused to ErmC and GFP and cultured in increasing concentrations of erythromycin (Gul et al., 2014). Selected strains that exhibited improved yield characteristics had acquired genomic mutations; notably no functional assays were performed on the proteins produced. The addition of toxic antibiotics to the growth medium for selection might appear beneficial in the short-term, but if the experiment is scaled up, the antibiotics are still needed to prevent revertants, which could negate the benefits (M A Romanos et al., 1992, Hensing et al., 1995). The traditional manner for selection in yeast is through auxotrophy of compounds needed for synthesis, and is well established and understood and therefore was the most logical choice for our study (Pronk, 2002). This does perhaps leave open the option of an anti-fungal resistance protein being

used in the place for Ura3p. However, through the authors' own admission, wild-type *E. coli* cells are highly resistant to their chosen antibiotic, erythromycin, forcing the use of a triple drug/antibiotic export null strain (Gul et al., 2014). The selection of bacteria based on drug resistance could possibly lead to health and safety issues (Joakim Larsson & Fick, 2009), which would not be an issue when selecting using auxotrophy in yeast. It is therefore likely that the strategy explored in this thesis would be more readily received in the biopharmaceutical industry.

None of the studies described above attempted to use detergents to increase functional yield. For example Linares and colleagues and Gul and colleagues performed no functional assays and relied on the folding of GFP as an indicator of protein quality (Linares et al., 2010, Gul et al., 2014). Niebauer and Robinson expressed a $hA_{2A}R$-GFP fusion in *S. cerevisiae* and used FACS to sort high-yielding transformants, which were less than 3.5% of the total population. This approach was initially successful, yet expression decreased on a per cell basis over the course of their study (Niebauer et al., 2004). Despite this, there might be potential for the use of a GFP tag for screening yeast SA transformants. However, due to the very low number of colonies in our study (Table 3.3), a more beneficial improvement might be to select in liquid medium cultures (instead if plate medium) with decreasing concentrations of uracil in the growth medium, which could then be sorted using FACS.

5.1.5 Conclusions

The SA strategy allows selection of host cells for production of a functional target protein. Yeast is increasingly relevant for the production of human membrane proteins due to its closer evolutionary links than bacteria (Grisshammer & Tate, 1995), while being cheaper than more authentic host cells such as mammalian and insect cells (Bill et al., 2011). Importantly, the strategy used in Chapter 3 did not appear to have an effect on receptor affinity, which we confirmed by homologous competition binding assays. In this work, we were able to link yeast cell survival to high yields of functional recombinant membrane protein in the first example of the application of SA in this organism.

5.2 Impact on yeast host cells of the SA strategy: vacuolar internalisation of non-functional protein

5.2.1 Total recombinant protein vs. functional recombinant protein

The conflicting data from immunoblot and ligand binding assays for H1 hinted that the SA strategy had achieved more recombinant $hA_{2A}R$-Ura3p over the control, yet ligand binding indicated much was non-functional and potentially misfolded bringing the actual level of functional $hA_{2A}R$-Ura3p in line with values seen from the control (Fig 3.10 and 3.11).

$hA_{2A}R$ has been expressed previously in *S. cerevisiae* as a $hA_{2A}R$-GFP fusion and a similar vacuolar internalisation was observed by the authors' also using confocal microscopy (Niebauer et al., 2004, Niebauer & Robinson, 2006), with the vacuole appearing as a solid block similar to our findings (Fig 3.12). The authors' chosen *S. cerevisiae* strain was BJ5464, which lacks the genes for the vacuolar proteases Pep4p and Prb1, thought to reduce proteolyic artifacts during the preparation of cell extracts (Jones, 1991, Kowalski et al., 1998). This might be the reason why they observed this internalisation in the vacuole; after sequestering $hA_{2A}R$, the vacuole perhaps lacks the ability to recycle or degrade it. This also might have answered some potential questions raised during the study, namely whether or not the internalisation we observed was caused by the SA strategy or whether the SA strategy has selected for mutants which are amenable to the SA stress, as perhaps vacuolar mutant are. The evidence leaned towards the latter proposition in that H1 might have been a mutant lacking one or more vacuolar protease, thus conferring an advantage over wild-type cells that are able to degrade the $hA_{2A}R$-Ura3p. Niebauer and colleagues used whole cell fluorescence for their $hA_{2A}R$-GFP construct, and ligand binding using whole cells lysates as a measure of functional expression (as opposed to total cell membranes as in our study), and also observed significant discrepancy when comparing to total expression from immunoblots (Niebauer et al., 2004, Niebauer & Robinson, 2006). They were of the opinion that the $hA_{2A}R$ that was internalised was functional because the GFP was correctly folded and was able to fluoresce even when inside the vacuole. If this was the case, it would indicate that perhaps the $hA_{2A}R$-Ura3p from H1 was misfolded prior to being internalised. However, it is possible that despite GFP being folded correctly in their $hA_{2A}R$ fusion, the $hA_{2A}R$ was not, which is the conclusion we have drawn from the data.

Niebauer and colleagues selected for the highest fluorescing cells through flow assisted cell sorting (FACS) (Niebauer et al., 2004), which selected for those cells that expressed the most GFP (further compounded through using anti-GFP antibody for the immunoblot), rather than correct folding of its $hA_{2A}R$ partner. GFP screening was essential as they used the pITY plasmid vector capable of integrating 1-30 times at different positions throughout the cells genome (Niebauer et al., 2004, Niebauer & Robinson, 2006), allowing for a massive variation in expression potential. The study reported an estimated potential yield of 6 ± 2 mg/L (Niebauer & Robinson, 2006), using this system.

Gul and colleagues observed no fluorescence of their GFP-tagged target when *E. coli* was grown at 37°C, instead needing to culture at 20-25°C (Gul et al., 2014), indicating that their SA strategy caused undue stress which results in misfolded or inadequately-processed products. This correlates with our SA finding that showed the majority of our recombinant product in H1 was unable to bind ZM241385, which perhaps could be remedied by a reduction in culture temperature. Reductions in temperature have been reported in *S. cerevisiae* and *P. pastoris* to improve protein yield, quality and function (Hackel et al., 2006, Gasser et al., 2007), by alleviating the metabolic burden of overexpression due slower and more efficient cell translation (Bawa et al., 2011). It has also been suggested to prevent saturation of the membrane protein insertion machinery (Loll, 2003, Wagner et al., 2006) allowing the appropriate amount of chaperones and foldases (Tate et al., 1999, Higgins et al., 2003). This is in line with our findings as when *spt3Δ* was used in conjunction with the SA strategy, as mentioned above.

The quality control mechanisms in *S. cerevisiae* to deal with misfolded cytosolic proteins are not entirely understood, but it is thought that Ura3p has a very short half-life and is ubiquitinated and transported to proteasomes for degradation (Gilon et al., 1998, Metzger et al., 2008). It is not clear whether or not this caused Ura3p to be cleaved from $hA_{2A}R$ or whether the entire fusion is degraded during the process, but the western data showing $hA_{2A}R$ monomers from H1 (Fig 3.9) could support that theory. There is the potential to use the mutant strain *ydj1pΔ* which is a deletion of the cytosol/ER membrane-localized chaperone Ydj1p, which has been shown to impair the ubiquitination and degradation of a

Ura3p-fusion (a fusion with destabilising sequences used to discover degradation pathways for Ura3p), which was shown to allow the majority of the Ura3p-fusions to escape degradation (Metzger et al., 2008).

The yeast secretion pathway can respond to misfolded proteins by sending them back to Golgi/ER to be refolded and if this fails they are sent to the vacuole for storage or degradation (Nielsen, 2013). If indeed H1 is vacuolar protease deficient this would allow an accumulation of misfolded $hA_{2A}R$-Ura3p (resulting in the internalisation observed, Fig 3.12) which might have been an important factor for cell survival, thus providing a selective advantage; Ura3p must be correctly folded or the cells will not survive. Assays could be conducted in an effort to judge whether any kind of unfolded protein response (UPR) or ER-associated degradation (ERAD) is taking place, although as discussed earlier this vacuolar accumulation had been observed before although the authors did not elaborate further (Niebauer et al., 2004).

5.2.2 Vacuolar mutants

Vacuolar deletion strains were employed in an attempt to prevent vacuolar accumulation, through the deletion of genes vital for the CPY (*vps1Δ*), ALP (*apm3Δ*) vacuolar transport pathways and PEP3 (*pep3Δ*) responsible for vacuolar biogenesis (Fig 3.13). The vacuolar mutant controls were selected in the same fashion as A1, and SA vacuolar transformants were generated using the two-step method because the one-step process yielded no colonies. Interestingly for the vacuolar mutants, fewer colonies arose during the first step on CSM-histidine (around 7-15 as opposed to 60 for H1) which was expected as different strains have different rates of transformability (Kawai et al., 2010). The colonies took longer to form during the second step on CSM-uracil (approximately 14-16 days as opposed to 7-10 for H1 and SU1), yet they had a higher survival rate with at least 50% surviving the spotting procedure from CSM-histidine to CSM-uracil. this possibly indicates that vacuolar mutant strains have a distinct advantage over BY4741 in the SA method, although this could also be due to the longer growth time afforded to the vacuolar mutants. The higher survival rate of the vacuolar mutants further supported the idea that perhaps the H1 transformant is a vacuolar mutant.

None of the vacuolar SA transformants had an increase in yield over the control (Fig 3.15); they did however not suffer the same vacuolar internalisation because *apm3Δ*:$hA_{2A}R$-Ura3p had a vacuole devoid of recombinant protein similar to the control A1 WT:$hA_{2A}R$ (Fig 3.12) and the mutant control *apm3Δ:*$hA_{2A}R$ (Fig 3.14). Similarly the *vps1Δ and pep3Δ* transformants did not have any vacuole formation (Fig 3.14), which was not surprising for *pep3Δ* transformants as the gene for the vacuole formation is deleted. However, for the no-SA control *pep3Δ:*$hA_{2A}R$ there appeared to be vesicular accumulation of $hA_{2A}R$ and in contrast the SA transformant *pep3Δ*:$hA_{2A}R$-Ura3p had very low expression throughout (Fig 3.14). The *vps1Δ*:$hA_{2A}R$-Ura3p transformant appeared to have vesicular accumulation of $hA_{2A}R$-Ura3p with its control *vps1Δ*:$hA_{2A}R$ having uniform expression throughout the cell. Since there was no vacuole present in either transformant, it might be possible that there is disruption of vesicular budding from the Golgi and other organelle fission events, which is a property of the *vps1Δ* strain (Ekena et al., 1993, Nothwehr et al., 1995).

The vacuolar mutant SA transformants and their controls all had decreased yield of $hA_{2A}R$ determined by ligand binding compared to the A1 control, except *apm3Δ:*$hA_{2A}R$ (Fig 3.15), which surprisingly was the non-SA control of *apmΔ* and had an increased yield of 1.9 pmol mg^{-1}, compared to the A1 control (1.3 pmol mg^{-1}). This result highlighted it as a potential host for $hA_{2A}R$ and other membrane proteins, and also indicated that the ALP pathway might be responsible for low yields of $hA_{2A}R$ and possibly other membrane proteins. Additionally, immunoblot analysis could have been conducted to supplement the confocal and binding data, allowing for a more insightful and thorough comparison against H1 in terms of recombinant protein localisation.

The *apm3Δ* gene is involved in the targeting and transport of membrane proteins in the ALP pathway from the Golgi to the vacuole (Odorizzi et al., 1998, Yeung et al., 1999). A deletion of any of its subunits results in the mislocalisation of cargo vesicles (Cowles et al., 1997). Our data suggest that this mislocalisation must either be beneficial in some way, perhaps reducing the rate of $hA_{2A}R$ turnover within the cell allowing for more $hA_{2A}R$ per cell than would usually be permitted compared to the A1 control. $hA_{2A}R$ is not trafficked via the ALP pathway at all and might be diverted to the CPY or endocytic pathways which

traffics cargo to the plasma membrane in most cases, and not necessarily the vacuole which is the case for cargo regulated via the ALP pathway (Conibear & Stevens, 1998). Interestingly earlier attempts at $hA_{2A}R$ expression in *S. cerevisiae* attributed reduced expression to translational or post-translational events (Niebauer et al., 2004), fitting with the idea that the ALP pathways is somehow involved. In future work, specific proteins essential to certain steps in the trafficking pathway of membrane proteins from the plasma membrane to the vacuole could be inhibited using mutant strains. Another avenue that could be pursued to prevent vacuolar internalisation of $hA_{2A}R$-Ura3p, might have been to use a deletion strain of the heat-shock protein 70 (Hsp70p), as the *hsp70Δ* strain has been shown to be unable to turn over Ura3p within the cell, suggesting that Hsp70p is essential for Ura3p proteolysis (Horst et al., 1999).

5.2.3 Strategies to recover non-functional protein

A large proportion of $hA_{2A}R$-Ura3p from H1 seemed to be non-functional. Factoring together the immunoblot and ligand binding values, it was calculated that only approximately 24% of total recombinant $hA_{2A}R$-Ura3p was functionally active as determined by radio-ligand binding, meaning a potential further 76% could be recovered, increasing the overall total functional yield. Solubilisation of $hA_{2A}R$-Ura3p was therefore attempted using DDM, which has been used in structural characterisation of many membrane proteins deposited in the Protein Data Bank (O'Malley et al., 2007). Most importantly it has been used successfully for solubilisation of the $hA_{2A}R$ receptor expressed in *S. cerevisiae* (O'Malley et al., 2007, Singh et al., 2010). DDM was supplemented with CHS, a soluble form of cholesterol which had been shown to promote GCPR activity *in vivo* (Weiss & Grisshammer, 2002, O'Malley et al., 2007, Singh et al., 2010); the equivalent membrane sterol in yeast is ergosterol. This allows the solubilisation environment and subsequent micelles to more closely resemble the properties that $hA_{2A}R$ would have in its native plasma membrane. Additionally it has been reported that solubilisation attempts on $hA_{2A}R$ in *S. cerevisiae* membranes with DDM but not CHS resulted in loss of protein function assessed through ligand binding (O'Malley et al., 2007). CHS has also been shown to be essential for maximal recovery of functional $hA_{2A}R$ from solubilisation (Singh et al., 2010).

We also solubilised A1 and SU1 as controls (Fig 3.21). The $hA_{2A}R$-Ura3p from H1 reported a significant increase in the amount of functional receptors (increase in B_{max}) after solubilisation (1.6 to 5.8 pmol mg^{-1}), while the A1 and SU1 had no change (Fig 3.21).

5.2.4 Conclusion

The SA strategy initially lead to a transformant, H1, that suffered vacuolar internalisation of $hA_{2A}R$-Ura3p. Attempts to circumvent this through the use of vacuolar mutants were only partly successful because the transformants had a decrease in total $hA_{2A}R$/$hA_{2A}R$-Ura3p yield compared to the A1 control. The *apm3Δ* strain might prove to be a new membrane protein over-expression host because it had a 1.6-fold increase in $hA_{2A}R$ yield over the A1 control. Use of s*pt3Δ* in the SA strategy generated SU1, which did not suffer internalisation and had a reported 2.5-fold functional yield increase. A large proportion of $hA_{2A}R$-Ura3p in H1was non-functional; solubilisation with DDM and CHS was used to recover functionality and increase overall functional yield from H1.

The new B_{max} value for solubilised $hA_{2A}R$-Ura3p from H1 represented a 4.5-fold increase over the Al control, making it the new highest yielding SA transformant; SU1 had a 2.5-fold increase pre and post-solubilisation (fig 3.21). For H1, this brought its yield more in line with the immunoblot values (Fig 3.10). We note, however, that the binding data from the solubilisation did not take into account the insoluble fraction, so we are likely to be underestimating the yield. Overall the data support the earlier hypothesis that a large proportion of $hA_{2A}R$-Ura3p expressed in H1 was misfolded causing it to be non-functional in a radio-ligand binding assay. Of the 74% misfolded $hA_{2A}R$-Ura3p available for recovery, 36% was successfully solubilised.

5.3 Manipulation of translational processes as a strategy to increase yield

5.3.1 Translation slowdown

The attempt to increase yields of $hA_{2A}R$ using drugs by translational inhibition did not result in increased yields. In fact the drugs caused a significant reduction, with guanabenz and emetine having yields of 0.2 and 0.1 pmol mg^{-1} respectively compared to the $hA_{2A}R$

control A1 (1.3 pmol mg^{-1})(Fig 4.6). The extremely slow grow rates in the presence of the drugs indicated they caused a severe growth defect due to a much more severe translational inhibition than intended which appeared to negatively affect yield. Use of the *tor1Δ* strain had a slight reduction (0.9 pmol mg^{-1}; Fig 4.7), indicating that the specific translational inhibition exhibited by *tor1Δ* was not suitable to improve hA2AR yield.

A larger ranging screen using smaller culture volumes could have been used allowing for more concentrations to be assayed, and possibly using immunoblots as an initial screen. Additionally polysome profiling could be performed to detect increases in monosomes which would confirm any translational inhibition (Bonander et al., 2009) after emetine or guanabenz treatment. An immunoblot for phosphorylated eIF2A (which causes inhibition of translation initiation specifically) would also be an indicator of translational inhibition (Sherman & Qian, 2013).

5.3.2 IRES

Overall it was not possible to increase $hA_{2A}R$ expression using IRES sequences over our non-IRES control and, in fact, the IRESes caused an overall repression in yield. This is not unexpected for IRESes in wild-type yeast cells because IRESes have very low activity if cap-dependent initiation is fully active (Paz et al., 1999). We expected that translation-deficient *spt3Δ* cells would have sufficiently inhibited the cap-dependent mechanism thus allowing the IRESes to increase $hA_{2A}R$ yields over the no-IRES control, which it did not (Fig 4.8). However, the *spt3Δ* strain IRES transformants did not have increased yield over the WT IRES transformants apart from the *spt3Δ YAP1* IRES (*YAP1*(IRES)-$hA_{2A}R$:[*spt3Δ*]) which had a 3-fold increase in yield compared to the of WT *YAP1* IRES (*YAP1*(IRES)-$hA_{2A}R$) (Fig 4.8).

For the CrPV IRES, both the WT and *spt3Δ* transformants had comparable yields (Fig 4.8), therefore another deletion strain was chosen as that was more amenable to the IRES. It had been speculated according to results reported in the literature that as the CrPV IRES is inhibited in the presence of abundant ternary complexes (due to competition for the P-site of the 40S ribosomal subunit), a reduction in ternary complexes should enhance IRES

activity (Deniz et al., 2009). The deletion strain *gcn3Δ* was chosen, as *GCN3* encodes the α subunit of eIF4B which is involved in facilitating the formation of ternary complexes via the guanine nucleotide exchange, a mutation leads to a reduction in ternary complexes (Gomez & Pavitt, 2000, Taylor et al., 2010). This resulted in approximately a 2-fold (0.9 pmol mg^{-1}) increase in $hA_{2A}R$ yield over *spt3Δ* and BY4741 CrPV IRES transformants (CrPV(IRES)-$hA_{2A}R$:[*spt3Δ*] and CrPV(IRES)-$hA_{2A}R$), and not far off the A1 control (1.3 pmol mg^{-1}) (Fig 4.9).

We also searched for optimal conditions for the *YAP1* IRES by manipulating the oxidative stress response, performing an immunoblot for Yap1p expression in BY4741 cells that had been stressed using varying concentrations of H_2O_2 which were added for the last 1h of growth of a 19h culture (Fig 4.10). The data suggested that 100µM was the concentration that elicited the strongest Yap1p response (1.6-fold higher Yap1p expression than the 0µM control;Fig 4.10).

As the YAP1 IRES is not fully understood, it is unknown which and how many factors are required for it to facilitate internal initiation; this is coupled with the fact that the control mechanisms for IRESes harboured in the 5´UTR of cellular mRNAs is not entirely understood and speculated to possess multiple pathways that mediate internal initiation (Komar & Hatzoglou, 2005, Komar & Hatzoglou, 2011). Additionally, unlike the CrPV IRES, the *YAP1* IRES originates in the 5´UTR of a cellular mRNA and most likely also needs IRES-transacting factors (ITAFs) as it is reported that ITAFs are responsible for sensing changes in cellular metabolism and are thought to be responsible for regulating IRES activity (Komar & Hatzoglou, 2005, Lewis & Holcik, 2007). A possible solution to investigate what initiation factors and ITAFs are required for *YAP1*, and even p150, has been reported using purified factors and translation apparatus in a reconstituted *in vitro* translation system (Kolupaeva et al., 2007, Pisarev et al., 2007). A more high-throughput alternative could use GFP in the place of $hA_{2A}R$ to determine the highest yielding deletion strain for every given IRES, additionally using a 96-well format would maximise the amount of translation-deficient deletion strains that could be trailed using the IRESes. If the plate was recorded in real time, the data might also indicate the highest peak in expression/IRES activity that the cells should be harvested. It is thought that starved cells

or cells in stationary phase have a higher capacity to support internal initiation in yeast (Paz et al., 1999), as cap-dependent translation activity is dramatically reduced, yet cells maintain excess translational capacity (Dickson & Brown, 1998). Using these data the same conditions could be applied to the IRES $hA_{2A}R$ constructs and then binding performed to see if the relationship between the condition and high GFP expression correlates to high $hA_{2A}R$ functional yield.

5.3.3 Conclusions

In a preliminary screen, using concentrations that had been reported in the literature, guanabenz and emetine had a negative effect on protein yield. The *tor1Δ* strain did not have as significant a decrease in $hA_{2A}R$ yield with a B_{max} 0.9 pmol mg^{-1}, compared to the A1 control 1.2 pmol mg^{-1}. The *TOR1* gene (target of rapamycin 1) encodes an extremely important protein, which forms the TORC1 complex. TORC1 regulates many cellular processes including protein synthesis, transcriptional activation, ribosome biogenesis, actin organisation and the cell cycle (Bjornsti & Houghton, 2004, Martin & Hall, 2005, Kennedy & Kaeberlein, 2009); it is therefore conceivable that the reduction in translation that is exhibited in *tor1Δ* does not result in an increased yield of $hA_{2A}R$ as other factors are involved in such a complex set of pathways.

Despite none of the IRESes having an increase in yield over the A1 control, through the use of deletion strains it was possible to increase the activity of the *YAP1* IRES (using *spt3Δ*) and the CrPV IRES (using *gcn3Δ*). A screen of BY4741 deletion strains using the IRES vectors could be done if $hA_{2A}R$ was to be replaced with GFP and a 96-well plate format incorporated. This would allow for a high-throughput screen to individually identify optimal–IRES activity strains particular to each IRES. Also preliminary *YAP1* IRES optimisation efforts suggested relevant concentrations of H_2O_2 that might be used to boost *YAP1* IRES activity.

5.4 Further work

5.4.1 SA

- Sequence the H1 and SU1 genomes to discover if a mutant was indeed selected for by the SA as in previous attempts at SA in bacteria.
- Performed further pharmacology using other $hA_{2A}R$ ligands such as NECA and XAC
- Mutate *URA3* causing the Ura3p of the hA2aR-Ura3p fusion to be deficient with the aim of forcing higher yield from SA transformants
- Apply strategy to other membrane proteins such as CGRP receptor
- Apply strategy to industrially relevant soluble proteins such as HRP

5.4.2 Knowledge of translational processes

- 96-well screen with GFP-reporter to assay the culture time that correlates with maximal expression/IRES activity

5.4.2.1 TSD

- Use other translationally-deficient strains in 96-well screen with GFP-reporter vectors (replace $hA_{2A}R$ with GFP in the pYX222-$hA_{2A}R$)
- Use varying concentrations of emetine and guanabenz in a in 96-well screen with GFP-reporter vectors (replace $hA_{2A}R$ with GFP in the pYX222-$hA_{2A}R$)

5.4.2.2 IRES

- Use other translationally-deficient strains, perhaps in 96-well screen with IRES-GFP-reporter vectors (replace $hA_{2A}R$ with GFP in the pYX222-(IRES)-$hA_{2A}R$).
- Test 100µM H_2O_2 to investigate the hypothesis that an induction more tailored to *YAP1* can increase the yield further
- Use other translationally-deficient strains, in 96-well screen against all the 3 IRESes
- Apply strategy to other membrane proteins such as CGRP receptor

- Apply strategy to industrially relevant soluble proteins such as HRP
- No native *P. pastoris* IRES has yet been shown to work *in vivo* in *P. pastoris* and there is high conservation between *S. cerevisiae* and *P. pastoris* regarding IRES-dependent translation. The *S. cerevisiae GRP1* IRES has been shown to internally initiate translation in *P. pastoris* (Liang, et al., 2012a) allowing the possibility for adapting the strategy to *P. pastoris*, using the *YAP1*, p150 and even the CrPV IRESes.

6. References

Alani, E. & Kleckner, N. (1987) A new type of fusion analysis applicable to many organisms: protein fusions to the URA3 gene of yeast. *Genetics*, 117 (1), pp.5–12.

Almén, M.S., Nordström, K.J.V., Fredriksson, R. & Schiöth, H.B. (2009) Mapping the human membrane proteome: a majority of the human membrane proteins can be classified according to function and evolutionary origin. *BMC biology*, 7, p.50.

André, N., Cherouati, N., Prual, C., Steffan, T., Zeder-Lutz, G., Magnin, T., Pattus, F., Michel, H., Wagner, R. & Reinhart, C. (2006) Enhancing functional production of G protein-coupled receptors in Pichia pastoris to levels required for structural studies via a single expression screen. *Protein Science : A Publication of the Protein Society*, 15 (5), pp.1115–1126.

Attwood, T.K. & Findlay, J.B. (1994) Fingerprinting G-protein-coupled receptors. *Protein engineering*, 7 (2), pp.195–203.

Bartfai, T., Lu, X., Badie-Mahdavi, H., Barr, A.M., Mazarati, A., Hua, X.-Y., Yaksh, T., Haberhauer, G., Ceide, S.C., Trembleau, L., Somogyi, L., Kröck, L. & Rebek, J. (2004) Galmic, a nonpeptide galanin receptor agonist, affects behaviors in seizure, pain, and forced-swim tests. *Proceedings of the National Academy of Sciences of the United States of America*, 101 (28), pp.10470–10475.

Bassett, D.E., Jr, Basrai, M.A., Connelly, C., Hyland, K.M., Kitagawa, K., Mayer, M.L., Morrow, D.M., Page, A.M., Resto, V.A., Skibbens, R.V. & Hieter, P. (1996) Exploiting the complete yeast genome sequence. *Current Opinion in Genetics & Development*, 6 (6), pp.763–766.

Bawa, Z., Bland, C.E., Bonander, N., Bora, N., Cartwright, S.P., Clare, M., Conner, M.T., Darby, R.A.J., Dilworth, M.V., Holmes, W.J., Jamshad, M., Routledge, S.J., Gross, S.R. & Bill, R.M. (2011) Understanding the yeast host cell response to recombinant membrane protein production. *Biochemical Society Transactions*, 39 (3), pp.719–723.

Berlec, A. & Strukelj, B. (2013) Current state and recent advances in biopharmaceutical production in Escherichia coli, yeasts and mammalian cells. *Journal of industrial microbiology & biotechnology*, 40 (3-4), pp.257–274.

Bernaudat, F., Frelet-Barrand, A., Pochon, N., Dementin, S., Hivin, P., Boutigny, S., Rioux, J.-B., Salvi, D., Seigneurin-Berny, D., Richaud, P., Joyard, J., Pignol, D., Sabaty, M., Desnos, T., Pebay-Peyroula, E., Darrouzet, E., Vernet, T. & Rolland, N. (2011) Heterologous expression of membrane proteins: choosing the appropriate host. *PloS one*, 6 (12), p.e29191.

Bill, R.M. (2014) Playing catch-up with Escherichia coli: using yeast to increase success rates in recombinant protein production experiments. *Microbiotechnology, Ecotoxicology and Bioremediation*, 5.

Bill, R.M. (2001) Yeast--a panacea for the structure-function analysis of membrane proteins? *Current genetics*, 40 (3), pp.157–171.

Bill, R.M., Henderson, P.J.F., Iwata, S., Kunji, E.R.S., Michel, H., Neutze, R., Newstead, S., Poolman, B., Tate, C.G. & Vogel, H. (2011) Overcoming barriers to membrane protein structure determination. *Nat Biotech*, 29 (4), pp.335–340.

Bitter, G.A., Chen, K.K., Banks, A.R. & Lai, P.H. (1984) Secretion of foreign proteins from Saccharomyces cerevisiae directed by alpha-factor gene fusions. *Proceedings of the National Academy of Sciences of the United States of America*, 81 (17), pp.5330–5334.

Bjornsti, M.-A. & Houghton, P.J. (2004) The TOR pathway: a target for cancer therapy. *Nature reviews. Cancer*, 4 (5), pp.335–348.

Böer, E., Steinborn, G., Kunze, G. & Gellissen, G. (2007) Yeast expression platforms. *Applied Microbiology and Biotechnology*, 77 (3), pp.513–523.

Böhme, I. & Beck-Sickinger, A.G. (2009) Illuminating the life of GPCRs. *Cell Communication and Signaling: CCS*, 7, p.16.

Bonander, N. & Bill, R.M. (2012) Optimising Yeast as a Host for Recombinant Protein Production (Review). In: R. M. Bill ed. *Recombinant Protein Production in Yeast*. Methods in Molecular Biology. Humana Press, pp.1–9.

Bonander, N., Darby, R.A., Grgic, L., Bora, N., Wen, J., Brogna, S., Poyner, D.R., O'Neill, M.A. & Bill, R.M. (2009) Altering the ribosomal subunit ratio in yeast maximizes recombinant protein yield. *Microbial Cell Factories*, 8 (1), p.10.

Bonander, N., Hedfalk, K., Larsson, C., Mostad, P., Chang, C., Gustafsson, L. & Bill, R.M. (2005) Design of improved membrane protein production experiments: Quantitation of the host response. *Protein Science : A Publication of the Protein Society*, 14 (7), pp.1729–1740.

Brachmann, C.B., Davies, A., Cost, G.J., Caputo, E., Li, J., Hieter, P. & Boeke, J.D. (1998) Designer deletion strains derived from Saccharomyces cerevisiae S288C: a useful set of strains and plasmids for PCR-mediated gene disruption and other applications. *Yeast (Chichester, England)*, 14 (2), pp.115–132.

Brake, A.J., Merryweather, J.P., Coit, D.G., Heberlein, U.A., Masiarz, F.R., Mullenbach, G.T., Urdea, M.S., Valenzuela, P. & Barr, P.J. (1984) Alpha-factor-directed synthesis and secretion of mature foreign proteins in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences of the United States of America*, 81 (15), pp.4642–4646.

Canals, M., Burgueño, J., Marcellino, D., Cabello, N., Canela, E.I., Mallol, J., Agnati, L., Ferré, S., Bouvier, M., Fuxe, K., Ciruela, F., Lluis, C. & Franco, R. (2004) Homodimerization of adenosine A2A receptors: qualitative and quantitative assessment by fluorescence and bioluminescence energy transfer. *Journal of neurochemistry*, 88 (3), pp.726–734.

Celik, E. & Calık, P. (2012) Production of recombinant proteins by yeast cells. *Biotechnology advances*, 30 (5), pp.1108–1118.

Chappell, S.A., Edelman, G.M. & Mauro, V.P. (2000) A 9-nt segment of a cellular mRNA can function as an internal ribosome entry site (IRES) and when present in linked multiple copies greatly enhances IRES activity. *Proceedings of the National Academy of Sciences of the United States of America*, 97 (4), pp.1536–1541.

Cheung, T.T. & Cheung, B.M.Y. (2014) Managing blood pressure control in Asian patients: safety and efficacy of losartan. *Clinical interventions in aging*, 9, pp.443–450.

Clemens, M.J. & Bommer, U.A. (1999) Translational control: the cancer connection. *The International Journal of Biochemistry & Cell Biology*, 31 (1), pp.1–23.

Conibear, E. & Stevens, T.H. (1998) Multiple sorting pathways between the late Golgi and the vacuole in yeast. *Biochimica et biophysica acta*, 1404 (1-2), pp.211–230.

Cowles, C.R., Odorizzi, G., Payne, G.S. & Emr, S.D. (1997) The AP-3 adaptor complex is essential for cargo-selective transport to the yeast vacuole. *Cell*, 91 (1), pp.109–118.

Deisenhofer, J., Epp, O., Miki, K., Huber, R. & Michel, H. (1985) Structure of the protein subunits in the photosynthetic reaction centre of Rhodopseudomonas viridis at 3Å resolution. *Nature*, 318 (6047), pp.618–624.

Delaunay, A., Isnard, A.D. & Toledano, M.B. (2000) H2O2 sensing through oxidation of the Yap1 transcription factor. *The EMBO journal*, 19 (19), pp.5157–5166.

Demain, A.L. & Vaishnav, P. (2009) Production of recombinant proteins by microbes and higher organisms. *Biotechnology advances*, 27 (3), pp.297–306.

Deniz, N., Lenarcic, E.M., Landry, D.M. & Thompson, S.R. (2009a) Translation initiation factors are not required for Dicistroviridae IRES function in vivo. *RNA*.

Deniz, N., Lenarcic, E.M., Landry, D.M. & Thompson, S.R. (2009b) Translation initiation factors are not required for Dicistroviridae IRES function in vivo. *RNA*.

Dibrov, S.M., Ding, K., Brunn, N.D., Parker, M.A., Bergdahl, B.M., Wyles, D.L. & Hermann, T. (2012) Structure of a hepatitis C virus RNA domain in complex with a translation inhibitor reveals a binding mode reminiscent of riboswitches. *Proceedings of the National Academy of Sciences*.

Dickson, L.M. & Brown, A.J. (1998) mRNA translation in yeast during entry into stationary phase. *Molecular & general genetics: MGG*, 259 (3), pp.282–293.

Drew, D., Newstead, S., Sonoda, Y., Kim, H., von Heijne, G. & Iwata, S. (2008) GFP-based optimization scheme for the overexpression and purification of eukaryotic membrane proteins in Saccharomyces cerevisiae. *Nature protocols*, 3 (5), pp.784–798.

Edwards, S.R. & Wandless, T.J. (2010) Dicistronic regulation of fluorescent proteins in the budding yeast Saccharomyces cerevisiae. *Yeast (Chichester, England)*, 27 (4), pp.229–236.

Eisenstein, M. (2009) GPCRs: insane in the membrane. *Nat Meth*, 6 (12), pp.929–933.

Ekena, K., Vater, C.A., Raymond, C.K. & Stevens, T.H. (1993) The VPS1 protein is a dynamin-like GTPase required for sorting proteins to the yeast vacuole. *Ciba Foundation symposium*, 176, pp.198–211; discussion 211–214.

Entzeroth, M. (2003) Emerging trends in high-throughput screening. *Current Opinion in Pharmacology*, 3 (5), pp.522–529.

Fernández, I.S., Bai, X.-C., Murshudov, G., Scheres, S.H.W. & Ramakrishnan, V. (2014) Initiation of Translation by Cricket Paralysis Virus IRES Requires Its Translocation in the Ribosome. *Cell*, 157 (4), pp.823–831.

Ferrer-Miralles, N., Domingo-Espin, J., Corchero, J.L., Vazquez, E. & Villaverde, A. (2009) Microbial factories for recombinant pharmaceuticals. *Microbial Cell Factories*, 8.

Filbin, M.E. & Kieft, J.S. (2009a) Toward a structural understanding of IRES RNA function. *Current Opinion in Structural Biology*, 19 (3), pp.267–276.

Filbin, M.E. & Kieft, J.S. (2009b) Toward a structural understanding of IRES RNA function. *Current Opinion in Structural Biology*, 19 (3), pp.267–276.

Fraser, N.J. (2006a) Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies. *Protein expression and purification*, 49 (1), pp.129–137.

Fraser, N.J. (2006b) Expression and functional purification of a glycosylation deficient version of the human adenosine 2a receptor for structural studies. *Protein expression and purification*, 49 (1), pp.129–137.

Fredrick, K. & Ibba, M. (2010) How the sequence of a gene can tune its translation. *Cell*, 141 (2), pp.227–229.

Fredriksson, R., Lagerström, M.C., Lundin, L.-G. & Schiöth, H.B. (2003) The G-Protein-Coupled Receptors in the Human Genome Form Five Main Families. Phylogenetic Analysis, Paralogon Groups, and Fingerprints. *Molecular Pharmacology*, 63 (6), pp.1256–1272.

Fredriksson, R. & Schiöth, H.B. (2005) The Repertoire of G-Protein–Coupled Receptors in Fully Sequenced Genomes. *Molecular Pharmacology*, 67 (5), pp.1414–1425.

Garcia-Perez, J., Rueda, P., Alcami, J., Rognan, D., Arenzana-Seisdedos, F., Lagane, B. & Kellenberger, E. (2011) An allosteric model of maraviroc binding to CCR5. *The Journal of Biological Chemistry*.

Gasser, B., Maurer, M., Rautio, J., Sauer, M., Bhattacharyya, A., Saloheimo, M., Penttilä, M. & Mattanovich, D. (2007) Monitoring of transcriptional regulation in Pichia pastoris under protein production conditions. *BMC genomics*, 8, p.179.

Gasser, B., Saloheimo, M., Rinas, U., Dragosits, M., Rodriguez-Carmona, E., Baumann, K., Giuliani, M., Parrilli, E., Branduardi, P., Lang, C., Porro, D., Ferrer, P., Tutino,

M.L., Mattanovich, D. & Villaverde, A. (2008) Protein folding and conformational stress in microbial cells producing recombinant proteins: a host comparative overview. *Microbial Cell Factories*, 7.

Geiler-Samerotte, K.A., Dion, M.F., Budnik, B.A., Wang, S.M., Hartl, D.L. & Drummond, D.A. (2011) Misfolded proteins impose a dosage-dependent fitness cost and trigger a cytosolic unfolded protein response in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, 108 (2), pp.680–685.

Geoffrey M Cooper (2000) *The Cell: A Molecular Approach*. 2nd ed. Sunderland, Massachusetts: Sinauer Associates.

Gilchrist, A. & Mazzoni, M.R. (2010) Traditional GPCR Pharmacology and Beyond. In: A. Sitaramayya ed. *Signal Transduction: Pathways, Mechanisms and Diseases*. Berlin, Heidelberg, Springer Berlin Heidelberg, pp.3–24.

Gilon, T., Chomsky, O. & Kulka, R.G. (1998a) Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. *The EMBO Journal*, 17 (10), pp.2759–2766.

Gilon, T., Chomsky, O. & Kulka, R.G. (1998b) Degradation signals for ubiquitin system proteolysis in Saccharomyces cerevisiae. *The EMBO Journal*, 17 (10), pp.2759–2766.

Gomez, E. & Pavitt, G.D. (2000) Identification of Domains and Residues within the ε Subunit of Eukaryotic Translation Initiation Factor 2B (eIF2Bε) Required for Guanine Nucleotide Exchange Reveals a Novel Activation Function Promoted by eIF2B Complex Formation. *Molecular and Cellular Biology*, 20 (11), pp.3965–3976.

Grisshammer, R. (2009) Purification of recombinant G-protein-coupled receptors. *Methods in enzymology*, 463, pp.631–645.

Grisshammer, R. & Tate, C.G. (1995) Overexpression of integral membrane proteins for structural studies. *Quarterly reviews of biophysics*, 28 (3), pp.315–422.

Gul, N., Linares, D.M., Ho, F.Y. & Poolman, B. (2014) Evolved Escherichia coli strains for amplified, functional expression of membrane proteins. *Journal of molecular biology*, 426 (1), pp.136–149.

Hackel, B.J., Huang, D., Bubolz, J.C., Wang, X.X. & Shusta, E.V. (2006) Production of soluble and active transferrin receptor-targeting single-chain antibody using Saccharomyces cerevisiae. *Pharmaceutical Research*, 23 (4), pp.790–797.

Haga, K., Kruse, A.C., Asada, H., Yurugi-Kobayashi, T., Shiroishi, M., Zhang, C., Weis, W.I., Okada, T., Kobilka, B.K., Haga, T. & Kobayashi, T. (2012) Structure of the human M2 muscarinic acetylcholine receptor bound to an antagonist. *Nature*, 482 (7386), pp.547–551.

Han, B., Liu, N., Yang, X., Sun, H.B. & Yang, Y.C. (2001) MRG1 expression in fibroblasts is regulated by Sp1/Sp3 and an Ets transcription factor. *The Journal of Biological Chemistry*, 276 (11), pp.7937–7942.

Harding, H.P., Zhang, Y., Bertolotti, A., Zeng, H. & Ron, D. (2000) Perk is essential for translational regulation and cell survival during the unfolded protein response. *Molecular cell*, 5 (5), pp.897–904.

Harris, P., Navarro Poulsen, J.C., Jensen, K.F. & Larsen, S. (2000) Structural basis for the catalytic mechanism of a proficient enzyme: orotidine 5'-monophosphate decarboxylase. *Biochemistry*, 39 (15), pp.4217–4224.

Hensing, M.C., Rouwenhorst, R.J., Heijnen, J.J., van Dijken, J.P. & Pronk, J.T. (1995a) Physiological and technological aspects of large-scale heterologous-protein production with yeasts. *Antonie van Leeuwenhoek*, 67 (3), pp.261–279.

Hensing, M.C., Rouwenhorst, R.J., Heijnen, J.J., van Dijken, J.P. & Pronk, J.T. (1995b) Physiological and technological aspects of large-scale heterologous-protein production with yeasts. *Antonie van Leeuwenhoek*, 67 (3), pp.261–279.

Hershey, J. & Cold Spring Harbor Laboratory. (1996) *Translational control*. Plainview N.Y., Cold Spring Harbor Laboratory Press.

Higgins, M.K., Demir, M. & Tate, C.G. (2003) Calnexin co-expression and the use of weaker promoters increase the expression of correctly assembled Shaker potassium channel in insect cells. *Biochimica et biophysica acta*, 1610 (1), pp.124–132.

Hinnebusch, A.G. (2001) Unleashing yeast genetics on a factor-independent mechanism of internal translation initiation. *Proceedings of the National Academy of Sciences*, 98 (23), pp.12866 –12868.

Hoffmann, F. & Rinas, U. (2004) Stress induced by recombinant protein production in Escherichia coli. *Advances in biochemical engineering/biotechnology*, 89, pp.73–92.

Holcík, M. (2004) Targeting translation for treatment of cancer--a novel role for IRES? *Current cancer drug targets*, 4 (3), pp.299–311.

Holcik, M. & Sonenberg, N. (2005) Translational control in stress and apoptosis. *Nat Rev Mol Cell Biol*, 6 (4), pp.318–327.

Horst, M., Knecht, E.C. & Schu, P.V. (1999) Import into and Degradation of Cytosolic Proteins by Isolated Yeast Vacuoles. *Molecular Biology of the Cell*, 10 (9), pp.2879–2889.

Houk, K.N., Lee, J.K., Tantillo, D.J., Bahmanyar, S. & Hietbrink, B.N. (2001) Crystal Structures of Orotidine Monophosphate Decarboxylase: Does the Structure Reveal the Mechanism of Nature's Most Proficient Enzyme? *ChemBioChem*, 2 (2), pp.113–118.

Hu, H., Boone, A. & Yang, W. (2008) Mechanism of OMP Decarboxylation in Orotidine 5?-Monophosphate Decarboxylase. *Journal of the American Chemical Society*, 130 (44).

Hulme, E.C. & Trevethick, M.A. (2010) Ligand binding assays at equilibrium: validation and interpretation. *British journal of pharmacology*, 161 (6), pp.1219–1237.

Ismaila, A., Corriveau, D., Vaillancourt, J., Parsons, D., Stanford, R., Su, Z. & Sampalis, J. (2014) Impact of adherence to treatment with fluticasone propionate / salmeterol in asthma patients. *Current medical research and opinion*.

Jaakola, V.-P., Griffith, M.T., Hanson, M.A., Cherezov, V., Chien, E.Y.T., Lane, J.R., Ijzerman, A.P. & Stevens, R.C. (2008) The 2.6 angstrom crystal structure of a human A2A adenosine receptor bound to an antagonist. *Science (New York, N.Y.)*, 322 (5905), pp.1211–1217.

Jacobson, S. & Pillus, L. (2009) The SAGA subunit Ada2 functions in transcriptional silencing. *Molecular and cellular biology*, 29 (22), pp.6033–6045.

Jang, S.K., Davies, M.V., Kaufman, R.J. & Wimmer, E. (1989) Initiation of protein synthesis by internal entry of ribosomes into the 5' nontranslated region of encephalomyocarditis virus RNA in vivo. *Journal of Virology*, 63 (4), pp.1651–1660.

Jang, S.K., Kräusslich, H.G., Nicklin, M.J., Duke, G.M., Palmenberg, A.C. & Wimmer, E. (1988) A segment of the 5' nontranslated region of encephalomyocarditis virus RNA directs internal entry of ribosomes during in vitro translation. *Journal of Virology*, 62 (8), pp.2636–2643.

Jin, S. & Fredholm, B.B. (1996) Adenosine A2A receptor stimulation increases release of acetylcholine from rat hippocampus but not striatum, and does not affect catecholamine release. *Naunyn-Schmiedeberg's Archives of Pharmacology*, 355, pp.48–56.

Joakim Larsson, D.G. & Fick, J. (2009) Transparency throughout the production chain—a way to reduce pollution from the manufacturing of pharmaceuticals? *Regulatory Toxicology and Pharmacology*, 53 (3), pp.161–163.

Jones, E.W. (1991) Tackling the protease problem in Saccharomyces cerevisiae. *Methods in enzymology*, 194, pp.428–453.

Kanner, E.M., Friedlander, M. & Simon, S.M. (2003) Co-translational Targeting and Translocation of the Amino Terminus of Opsin across the Endoplasmic Membrane Requires GTP but Not ATP. *Journal of Biological Chemistry*, 278 (10), pp.7920–7926.

Kawai, S., Hashimoto, W. & Murata, K. (2010a) Transformation of Saccharomyces cerevisiae and other fungi. *Bioengineered Bugs*, 1 (6), pp.395–403.

Kawai, S., Hashimoto, W. & Murata, K. (2010b) Transformation of Saccharomyces cerevisiae and other fungi. *Bioengineered Bugs*, 1 (6), pp.395–403.

Kennedy, B.K. & Kaeberlein, M. (2009) Hot Topics in Aging Research: Protein Translation, 2009. *Aging cell*, 8 (6), pp.617–623.

Kieft, J.S. (2008) Viral IRES RNA structures and ribosome interactions. *Trends in Biochemical Sciences*, 33 (6), pp.274–283.

Kolakowski, L.F., Jr (1994) GCRDb: a G-protein-coupled receptor database. *Receptors & channels*, 2 (1), pp.1–7.

Kolupaeva, V.G., de Breyne, S., Pestova, T.V. & Hellen, C.U.T. (2007) In vitro reconstitution and biochemical characterization of translation initiation by internal ribosomal entry. *Methods in enzymology*, 430, pp.409–439.

Komar, A.A. & Hatzoglou, M. (2011) Cellular IRES-mediated translation: the war of ITAFs in pathophysiological states. *Cell Cycle (Georgetown, Tex.)*, 10 (2), pp.229–240.

Komar, A.A. & Hatzoglou, M. (2005) Internal ribosome entry sites in cellular mRNAs: mystery of their existence. *The Journal of Biological Chemistry*, 280 (25), pp.23425–23428.

Konrad, C., Queener, S.F., Wek, R.C. & Sullivan, W.J., Jr (2013) Inhibitors of eIF2α dephosphorylation slow replication and stabilize latency in Toxoplasma gondii. *Antimicrobial agents and chemotherapy*, 57 (4), pp.1815–1822.

Koppitz, M. & Eis, K. (2006) Automated medicinal chemistry. *Drug Discovery Today*, 11 (11-12), pp.561–568.

Kowalski, J.M., Parekh, R.N., Mao, J. & Wittrup, K.D. (1998) Protein Folding Stability Can Determine the Efficiency of Escape from Endoplasmic Reticulum Quality Control. *Journal of Biological Chemistry*, 273 (31), pp.19453–19458.

Leach, K., Valant, C., Sexton, P.M. & Christopoulos, A. (2010) Measurement of Ligand–G Protein-Coupled Receptor Interactions. In: D. R. Poyner & rk Wheatley eds. *G Protein-Coupled Receptors*. Wiley-Blackwell, pp.1–29.

Lebon, G., Bennett, K., Jazayeri, A. & Tate, C.G. (2011) Thermostabilisation of an agonist-bound conformation of the human adenosine A(2A) receptor. *Journal of Molecular Biology*, 409 (3), pp.298–310.

Lee, S., Bhattacharya, S., Grisshammer, R., Tate, C. & Vaidehi, N. (2014) Dynamic Behavior of the Active and Inactive States of the Adenosine A2A Receptor. *The journal of physical chemistry. B*, 118 (12), pp.3355–3365.

Lewis, S.M. & Holcik, M. (2007) For IRES trans-acting factors, it is all about location. *Oncogene*, 27 (8), pp.1033–1035.

Liang, S., Lin, Y., Li, C. & Ye, Y. (2012) Internal ribosome entry site mediates protein synthesis in yeast Pichia pastoris. *Biotechnology letters*, 34 (5), pp.957–964.

Liang, S., Wang, B., Pan, L., Ye, Y., He, M., Han, S., Zheng, S., Wang, X. & Lin, Y. (2012) Comprehensive structural annotation of Pichia pastoris transcriptome and the response to various carbon sources using deep paired-end RNA sequencing. *BMC Genomics*, 13 (1), p.738.

Linares, D.M., Geertsma, E.R. & Poolman, B. (2010a) Evolved Lactococcus lactis Strains for Enhanced Expression of Recombinant Membrane Proteins. *Journal of Molecular Biology*, 401 (1), pp.45–55.

Linares, D.M., Geertsma, E.R. & Poolman, B. (2010b) Evolved Lactococcus lactis Strains for Enhanced Expression of Recombinant Membrane Proteins. *Journal of Molecular Biology*, 401 (1), pp.45–55.

Liu, Z., Hou, J., Martínez, J.L., Petranovic, D. & Nielsen, J. (2013) Correlation of cell growth and heterologous protein production by Saccharomyces cerevisiae. *Applied Microbiology and Biotechnology*, 97 (20), pp.8955–8962.

Loll, P.J. (2003) Membrane protein structural biology: the high throughput challenge. *Journal of structural biology*, 142 (1), pp.144–153.

Lucas, L.L. & Unterweger, M.P. (2000) *Comprehensive review and critical evaluation of the half-life of tritium*. National Institute of Standards and Technology.

Lundstrom, K., Wagner, R., Reinhart, C., Desmyter, A., Cherouati, N., Magnin, T., Zeder-Lutz, G., Courtot, M., Prual, C., André, N., Hassaine, G., Michel, H., Cambillau, C. & Pattus, F. (2006) Structural genomics on membrane proteins: comparison of more than 100 GPCRs in 3 expression systems. *Journal of Structural and Functional Genomics*, 7 (2), pp.77–91.

Lyseng-Williamson, K.A. (2011) Levetiracetam: a review of its use in epilepsy. *Drugs*, 71 (4), pp.489–514.

De Marco, V., Stier, G., Blandin, S. & de Marco, A. (2004) The solubility and stability of recombinant proteins are increased by their fusion to NusA. *Biochemical and biophysical research communications*, 322 (3), pp.766–771.

Marie, C. & Petri, W.A., Jr (2013) Amoebic dysentery. *Clinical evidence*, 2013.

Marrs, T., Anagnostou, K., Fitzsimons, R. & Fox, A.T. (2013) Optimising treatment of allergic rhinitis in children. *The Practitioner*, 257 (1762), pp.13–18, 2.

Martin, D.E. & Hall, M.N. (2005) The expanding TOR signaling network. *Current opinion in cell biology*, 17 (2), pp.158–166.

Massey-Gendel, E., Zhao, A., Boulting, G., Kim, H.-Y., Balamotis, M.A., Seligman, L.M., Nakamoto, R.K. & Bowie, J.U. (2009) Genetic selection system for improving recombinant membrane protein expression in E. coli. *Protein Science : A Publication of the Protein Society*, 18 (2), pp.372–383.

Mattanovich, D., Branduardi, P., Dato, L., Gasser, B., Sauer, M. & Porro, D. (2012) Recombinant Protein Production in Yeasts. In: A. Lorence ed. *Recombinant Gene Expression*. Methods in Molecular Biology. Humana Press, pp.329–358.

Mattanovich, D., Gasser, B., Hohenblum, H. & Sauer, M. (2004) Stress in recombinant protein producing yeasts. *Journal of biotechnology*, 113 (1-3), pp.121–135.

Maxwell, K.L., Mittermaier, A.K., Forman-Kay, J.D. & Davidson, A.R. (1999) A simple in vivo assay for increased protein solubility. *Protein Science : A Publication of the Protein Society*, 8 (9), pp.1908–1911.

McCudden, C.R., Majewski, A., Chakrabarti, S. & Wagner, G.F. (2004) Co-localization of stanniocalcin-1 ligand and receptor in human breast carcinomas. *Molecular and Cellular Endocrinology*, 213 (2), pp.167–172.

McKinney, M. & Raddatz, R. (2006) Practical aspects of radioligand binding. *Current protocols in pharmacology / editorial board, S.J. Enna (editor-in-chief) ... [et al.]*, Chapter 1, p.Unit1.3.

Meriin, A.B., Mense, M., Colbert, J.D., Liang, F., Bihler, H., Zaarur, N., Rock, K.L. & Sherman, M.Y. (2012a) A Novel Approach to Recovery of Function of Mutant Proteins by Slowing Down Translation. *Journal of Biological Chemistry*, 287 (41), pp.34264–34272.

Meriin, A.B., Mense, M., Colbert, J.D., Liang, F., Bihler, H., Zaarur, N., Rock, K.L. & Sherman, M.Y. (2012b) A Novel Approach to Recovery of Function of Mutant Proteins by Slowing Down Translation. *Journal of Biological Chemistry*, 287 (41), pp.34264–34272.

Metzger, M.B., Maurer, M.J., Dancy, B.M. & Michaelis, S. (2008) Degradation of a Cytosolic Protein Requires Endoplasmic Reticulum-associated Degradation Machinery. *Journal of Biological Chemistry*, 283 (47), pp.32302–32316.

Miller, B.G. & Wolfenden, R. (2002) Catalytic proficiency: the unusual case of OMP decarboxylase. *Annual review of biochemistry*, 71, pp.847–885.

Moraes, I., Evans, G., Sanchez-Weatherby, J., Newstead, S. & Stewart, P.D.S. (2014) Membrane protein structure determination — The next generation. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, 1838 (1, Part A), pp.78–87.

Morris, D.R. (1995) Growth control of translation in mammalian cells. *Progress in Nucleic Acid Research and Molecular Biology*, 51, pp.339–363.

Moye-Rowley, W.S., Harshman, K.D. & Parker, C.S. (1989) Yeast YAP1 encodes a novel form of the jun family of transcriptional activator proteins. *Genes & development*, 3 (3), pp.283–292.

Nettleship, J.E., Assenberg, R., Diprose, J.M., Rahman-Huq, N. & Owens, R.J. (2010) Recent advances in the production of proteins in insect and mammalian cells for structural biology. *Journal of structural biology*, 172 (1), pp.55–65.

Newstead, S., Kim, H., von Heijne, G., Iwata, S. & Drew, D. (2007) High-throughput fluorescent-based optimization of eukaryotic membrane protein overexpression and purification in Saccharomyces cerevisiae. *Proceedings of the National Academy of Sciences*, 104 (35), pp.13936 –13941.

Niebauer, R.T. & Robinson, A.S. (2006) Exceptional total and functional yields of the human adenosine (A2a) receptor expressed in the yeast Saccharomyces cerevisiae. *Protein expression and purification*, 46 (2), pp.204–211.

Niebauer, R.T., Wedekind, A. & Robinson, A.S. (2004) Decreases in yeast expression yields of the human adenosine A2a receptor are a result of translational or post-translational events. *Protein expression and purification*, 37 (1), pp.134–143.

Nielsen, J. (2013) Production of biopharmaceutical proteins by yeast: advances through metabolic engineering. *Bioengineered*, 4 (4), pp.207–211.

Nothwehr, S.F., Conibear, E. & Stevens, T.H. (1995) Golgi and vacuolar membrane proteins reach the vacuole in vps1 mutant yeast cells via the plasma membrane. *The Journal of cell biology*, 129 (1), pp.35–46.

Novoa, I., Zeng, H., Harding, H.P. & Ron, D. (2001) Feedback Inhibition of the Unfolded Protein Response by GADD34-Mediated Dephosphorylation of eIF2? *The Journal of Cell Biology*, 153 (5), pp.1011–1022.

Noyd, R., Krueger, J. & Hill, K. (2013) *Biology: Organisms and Adaptations*. Cengage Learning.

O'Malley, M.A., Lazarova, T., Britton, Z.T. & Robinson, A.S. (2007) High-level expression in Saccharomyces cerevisiae enables isolation and spectroscopic characterization of functional human adenosine A2a receptor. *Journal of structural biology*, 159 (2), pp.166–178.

Odorizzi, G., Cowles, C.R. & Emr, S.D. (1998) The AP-3 complex: a coat of many colours. *Trends in cell biology*, 8 (7), pp.282–288.

Otterstedt, K., Larsson, C., Bill, R.M., Ståhlberg, A., Boles, E., Hohmann, S. & Gustafsson, L. (2004) Switching the mode of metabolism in the yeast Saccharomyces cerevisiae. *EMBO Reports*, 5 (5), pp.532–537.

Pain, V.M. (1996) Initiation of Protein Synthesis in Eukaryotic Cells. *European Journal of Biochemistry*, 236 (3), pp.747–771.

Palczewski, K., Kumasaka, T., Hori, T., Behnke, C.A., Motoshima, H., Fox, B.A., Le Trong, I., Teller, D.C., Okada, T., Stenkamp, R.E., Yamamoto, M. & Miyano, M. (2000) Crystal structure of rhodopsin: A G protein-coupled receptor. *Science (New York, N.Y.)*, 289 (5480), pp.739–745.

Paz, I., Abramovitz, L. & Choder, M. (1999) Starved Saccharomyces cerevisiae cells have the capacity to support internal initiation of translation. *The Journal of Biological Chemistry*, 274 (31), pp.21741–21745.

Peeters, M.C., Li, Q., van Westen, G.J.P. & Ijzerman, A.P. (2011) Three 'hotspots' important for adenosine A(2B) receptor activation: a mutational analysis of transmembrane domains 4 and 5 and the second extracellular loop. *Purinergic Signalling*.

Pelletier, J. & Sonenberg, N. (1988) Internal initiation of translation of eukaryotic mRNA directed by a sequence derived from poliovirus RNA. *Nature*, 334 (6180), pp.320–325.

Pérez-Martínez, L., Pérez-Matute, P., Aguilera-Lizarraga, J., Rubio-Mediavilla, S., Narro, J., Recio, E., Ochoa-Callejero, L., Oteo, J.-A. & Blanco, J.-R. (2014) Maraviroc, a CCR5 antagonist, ameliorates the development of hepatic steatosis in a mouse model of non-alcoholic fatty liver disease (NAFLD). *The Journal of antimicrobial chemotherapy*.

Pisarev, A.V., Unbehaun, A., Hellen, C.U.T. & Pestova, T.V. (2007) Assembly and analysis of eukaryotic translation initiation complexes. *Methods in enzymology*, 430, pp.147–177.

Plus, N. & Scotti, P.D. (1984) The biological properties of eight different isolates of cricket paralysis virus. *Annales de l'Institut Pasteur / Virologie*, 135 (3), pp.257–268.

Prévôt, D., Darlix, J.-L. & Ohlmann, T. (2003) Conducting the initiation of protein synthesis: the role of eIF4G. *Biology of the Cell*, 95 (3-4), pp.141–156.

Pronk, J.T. (2002) Auxotrophic Yeast Strains in Fundamental and Applied Research. *Applied and Environmental Microbiology*, 68 (5), pp.2095–2100.

Radzicka, A. & Wolfenden, R. (1995) A proficient enzyme. *Science (New York, N.Y.)*, 267 (5194), pp.90–93.

Rasmussen, S.G.F., Choi, H.-J., Rosenbaum, D.M., Kobilka, T.S., Thian, F.S., Edwards, P.C., Burghammer, M., Ratnala, V.R.P., Sanishvili, R., Fischetti, R.F., Schertler, G.F.X., Weis, W.I. & Kobilka, B.K. (2007) Crystal structure of the human beta2 adrenergic G-protein-coupled receptor. *Nature*, 450 (7168), pp.383–387.

Raugei, S., Cascella, M. & Carloni, P. (2004) A proficient enzyme: insights on the mechanism of orotidine monophosphate decarboxylase from computer simulations. *Journal of the American Chemical Society*, 126 (48), pp.15730–15737.

Robbins-Pianka, A., Rice, M.D. & Weir, M.P. (2010) The mRNA landscape at yeast translation initiation sites. *Bioinformatics*, 26 (21), pp.2651–2655.

Rogawski, M.A. (2006) Diverse Mechanisms of Antiepileptic Drugs in the Development Pipeline. *Epilepsy research*, 69 (3), pp.273–294.

Romanos, M.A., Scorer, C.A. & Clare, J.J. (1992) Foreign gene expression in yeast: a review. *Yeast*, 8 (6), pp.423–488.

Romanos, M.A., Scorer, C.A. & Clare, J.J. (1992) Foreign gene expression in yeast: a review. *Yeast (Chichester, England)*, 8 (6), pp.423–488.

Sabbadin, D., Ciancetta, A. & Moro, S. (2014) Bridging Molecular Docking to Membrane Molecular Dynamics To Investigate GPCR–Ligand Recognition: The Human A2A Adenosine Receptor as a Key Study. *Journal of Chemical Information and Modeling*, 54 (1), pp.169–183.

Sherman, M.Y. & Qian, S.-B. (2013) Less is more: improving proteostasis by translation slow down. *Trends in biochemical sciences*, 38 (12), pp.585–591.

Shimamura, T., Shiroishi, M., Weyand, S., Tsujimoto, H., Winter, G., Katritch, V., Abagyan, R., Cherezov, V., Liu, W., Han, G.W., Kobayashi, T., Stevens, R.C. & Iwata, S. (2011) Structure of the human histamine H1 receptor complex with doxepin. *Nature*, 475 (7354), pp.65–70.

Siller, E., DeZwaan, D.C., Anderson, J.F., Freeman, B.C. & Barral, J.M. (2010) Slowing Bacterial Translation Speed Enhances Eukaryotic Protein Folding Efficiency. *Journal of Molecular Biology*, 396 (5), pp.1310–1318.

Singh, S., Gras, A., Fiez-Vandal, C., Ruprecht, J., Rana, R., Martinez, M., Strange, P.G., Wagner, R. & Byrne, B. (2008) Large-scale functional expression of WT and truncated human adenosine A2A receptor in Pichia pastoris bioreactor cultures. *Microbial Cell Factories*, 7, p.28.

Singh, S., Hedley, D., Kara, E., Gras, A., Iwata, S., Ruprecht, J., Strange, P.G. & Byrne, B. (2010a) A purified C-terminally truncated human adenosine A(2A) receptor construct is functionally stable and degradation resistant. *Protein expression and purification*, 74 (1), pp.80–87.

Singh, S., Hedley, D., Kara, E., Gras, A., Iwata, S., Ruprecht, J., Strange, P.G. & Byrne, B. (2010b) A purified C-terminally truncated human adenosine A(2A) receptor construct is functionally stable and degradation resistant. *Protein expression and purification*, 74 (1), pp.80–87.

Singh, S., Zhang, M., Bertheleme, N., Kara, E., Strange, P.G. & Byrne, B. (2012) Radioligand binding analysis as a tool for quality control of GPCR production for structural characterization: adenosine A(2a)R as a template for study. *Current protocols in protein science / editorial board, John E. Coligan ... [et al.]*, Chapter 29, p.Unit 29.3.

Siu, F.Y., He, M., de Graaf, C., Han, G.W., Yang, D., Zhang, Z., Zhou, C., Xu, Q., Wacker, D., Joseph, J.S., Liu, W., Lau, J., Cherezov, V., Katritch, V., Wang, M.-W. & Stevens, R.C. (2013) Structure of the human glucagon class B G-protein-coupled receptor. *Nature*, advance online publication.

Sonenberg, N. (1994a) mRNA translation: influence of the 5' and 3' untranslated regions. *Current Opinion in Genetics & Development*, 4 (2), pp.310–315.

Sonenberg, N. (1994b) Regulation of translation and cell growth by eIF-4E. *Biochimie*, 76 (9), pp.839–846.

Sørensen, H.P. (2010) Towards universal systems for recombinant gene expression. *Microbial cell factories*, 9, p.27.

Srivastava, A., Woolford, C.A. & Jones, E.W. (2000) Pep3p/Pep5p complex: a putative docking factor at multiple steps of vesicular transport to the vacuole of Saccharomyces cerevisiae. *Genetics*, 156 (1), pp.105–122.

Stoneley, M. & Willis, A.E. (2004a) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene*, 23 (18), pp.3200–3207.

Stoneley, M. & Willis, A.E. (2004b) Cellular internal ribosome entry segments: structures, trans-acting factors and regulation of gene expression. *Oncogene*, 23 (18), pp.3200–3207.

Sugimoto, K., Nukada, T., Tanabe, T., Takahashi, H., Noda, M., Minamino, N., Kangawa, K., Matsuo, H., Hirose, T. & Inayama, S. (1985) Primary structure of the beta-subunit of bovine transducin deduced from the cDNA sequence. *FEBS Letters*, 191 (2), pp.235–240.

Takeda, S., Kadowaki, S., Haga, T., Takaesu, H. & Mitaku, S. (2002) Identification of G protein-coupled receptor genes from the human genome sequence. *FEBS letters*, 520 (1-3), pp.97–101.

Tan, Q., Zhu, Y., Li, J., Chen, Z., Han, G.W., Kufareva, I., Li, T., Ma, L., Fenalti, G., Li, J., Zhang, W., Xie, X., Yang, H., Jiang, H., Cherezov, V., Liu, H., Stevens, R.C., Zhao, Q. & Wu, B. (2013) Structure of the CCR5 chemokine receptor-HIV entry inhibitor maraviroc complex. *Science (New York, N.Y.)*, 341 (6152), pp.1387–1390.

Tate, C.G. (2001) Overexpression of mammalian integral membrane proteins for structural studies. *FEBS Letters*, 504 (3), pp.94–98.

Tate, C.G., Whiteley, E. & Betenbaugh, M.J. (1999) Molecular chaperones stimulate the functional expression of the cocaine-sensitive serotonin transporter. *The Journal of biological chemistry*, 274 (25), pp.17551–17558.

Taylor, E.J., Campbell, S.G., Griffiths, C.D., Reid, P.J., Slaven, J.W., Harrison, R.J., Sims, P.F.G., Pavitt, G.D., Delneri, D. & Ashe, M.P. (2010) Fusel Alcohols Regulate Translation Initiation by Inhibiting eIF2B to Reduce Ternary Complex in a Mechanism That May Involve Altering the Integrity and Dynamics of the eIF2B Body. *Molecular Biology of the Cell*, 21 (13), pp.2202–2216.

Thim, L., Hansen, M.T., Norris, K., Hoegh, I., Boel, E., Forstrom, J., Ammerer, G. & Fiil, N.P. (1986) Secretion and processing of insulin precursors in yeast. *Proceedings of the National Academy of Sciences of the United States of America*, 83 (18), pp.6766–6770.

Tribouillard-Tanvier, D., Béringue, V., Desban, N., Gug, F., Bach, S., Voisset, C., Galons, H., Laude, H., Vilette, D. & Blondel, M. (2008) Antihypertensive drug guanabenz is active in vivo against both yeast and mammalian prions. *PloS one*, 3 (4), p.e1981.

Tsaytler, P., Harding, H.P., Ron, D. & Bertolotti, A. (2011) Selective inhibition of a regulatory subunit of protein phosphatase 1 restores proteostasis. *Science (New York, N.Y.)*, 332 (6025), pp.91–94.

Verduyn, C., Zomerdijk, T.P.L., Dijken, J.P. & Scheffers, W.A. (1984) Continuous measurement of ethanol production by aerobic yeast suspensions with an enzyme electrode. *Applied Microbiology and Biotechnology*, 19 (3), pp.181–185.

Wagner, S., Bader, M.L., Drew, D. & de Gier, J.-W. (2006) Rationalizing membrane protein overexpression. *Trends in biotechnology*, 24 (8), pp.364–371.

Wang, C., Jiang, Y., Ma, J., Wu, H., Wacker, D., Katritch, V., Han, G.W., Liu, W., Huang, X.-P., Vardy, E., McCorvy, J.D., Gao, X., Zhou, X.E., Melcher, K., Zhang, C., Bai, F., Yang, H., Yang, L., Jiang, H., Roth, B.L., Cherezov, V., Stevens, R.C. & Xu,

H.E. (2013) Structural Basis for Molecular Recognition at Serotonin Receptors. *Science*, 340 (6132), pp.610–614.

Wang, C., Wu, H., Katritch, V., Han, G.W., Huang, X.-P., Liu, W., Siu, F.Y., Roth, B.L., Cherezov, V. & Stevens, R.C. (2013) Structure of the human smoothened receptor bound to an antitumour agent. *Nature*, 497 (7449), pp.338–343.

Wang, S.-H., Shih, Y.-H. & Lin, L.-Y. (1998) Yeast consensus initiator sequence markedly enhances the synthesis of metallothionein III in Saccharomyces cerevisiae. *Biotechnology Letters*, 20 (1), pp.9–13.

Warne, T., Serrano-Vega, M.J., Baker, J.G., Moukhametzianov, R., Edwards, P.C., Henderson, R., Leslie, A.G.W., Tate, C.G. & Schertler, G.F.X. (2008) Structure of a beta1-adrenergic G-protein-coupled receptor. *Nature*, 454 (7203), pp.486–491.

Waugh, D.S. (2005) Making the most of affinity tags. *Trends in biotechnology*, 23 (6), pp.316–320.

Weiss, H.M. & Grisshammer, R. (2002) Purification and characterization of the human adenosine A(2a) receptor functionally expressed in Escherichia coli. *European journal of biochemistry / FEBS*, 269 (1), pp.82–92.

Werner-Washburne, M., Braun, E., Johnston, G.C. & Singer, R.A. (1993) Stationary phase in the yeast Saccharomyces cerevisiae. *Microbiological Reviews*, 57 (2), pp.383–401.

Wildt, S. & Gerngross, T.U. (2005) The humanization of N-glycosylation pathways in yeast. *Nature reviews. Microbiology*, 3 (2), pp.119–128.

Willis, A.E. (1999) Translational control of growth factor and proto-oncogene expression. *The International Journal of Biochemistry & Cell Biology*, 31 (1), pp.73–86.

Wilson, J.E., Pestova, T.V., Hellen, C.U. & Sarnow, P. (2000) Initiation of protein synthesis from the A site of the ribosome. *Cell*, 102 (4), pp.511–520.

Wu, G. (2010) *Assay Development: Fundamentals and Practices*. John Wiley and Sons.

Wu, H., Wang, C., Gregory, K.J., Han, G.W., Cho, H.P., Xia, Y., Niswender, C.M., Katritch, V., Meiler, J., Cherezov, V., Conn, P.J. & Stevens, R.C. (2014) Structure of a class C GPCR metabotropic glutamate receptor 1 bound to an allosteric modulator. *Science (New York, N.Y.)*, 344 (6179), pp.58–64.

Wu, N., Mo, Y., Gao, J. & Pai, E.F. (2000) Electrostatic stress in catalysis: Structure and mechanism of the enzyme orotidine monophosphate decarboxylase. *Proceedings of the National Academy of Sciences of the United States of America*, 97 (5), pp.2017–2022.

Xia, X. & Holcik, M. (2009) Strong Eukaryotic IRESs Have Weak Secondary Structure. *PLoS ONE*, 4 (1), p.e4136.

Xu, F., Wu, H., Katritch, V., Han, G.W., Jacobson, K.A., Gao, Z.-G., Cherezov, V. & Stevens, R.C. (2011) Structure of an Agonist-Bound Human A2A Adenosine Receptor. *Science*, 332 (6027), pp.322 –327.

Yang, J., Wang, Z., Song, L., Tian, F., Yu, Z., Li, F. & Tang, L. (2014) Clinical Research of Tashinone IIA Combined with Endocrine Therapy in Treating Advanced-Stage Prostate Cancer. *Cell biochemistry and biophysics*.

Ye, H., Charpin-El Hamri, G., Zwicky, K., Christen, M., Folcher, M. & Fussenegger, M. (2013) Pharmaceutically controlled designer circuit for the treatment of the metabolic syndrome. *Proceedings of the National Academy of Sciences of the United States of America*, 110 (1), pp.141–146.

Yeung, B.G., Phan, H.L. & Payne, G.S. (1999) Adaptor Complex-independent Clathrin Function in Yeast. *Molecular Biology of the Cell*, 10 (11), pp.3643–3659.

Zhang, K., Zhang, J., Gao, Z.-G., Zhang, D., Zhu, L., Han, G.W., Moss, S.M., Paoletta, S., Kiselev, E., Lu, W., Fenalti, G., Zhang, W., Müller, C.E., Yang, H., Jiang, H., Cherezov, V., Katritch, V., Jacobson, K.A., Stevens, R.C., Wu, B. & Zhao, Q. (2014) Structure of the human P2Y12 receptor in complex with an antithrombotic drug. *Nature*, advance online publication.

Zhang, Y., Devries, M.E. & Skolnick, J. (2006) Structure modeling of all identified G protein-coupled receptors in the human genome. *PLoS Computational Biology*, 2 (2), p.e13.

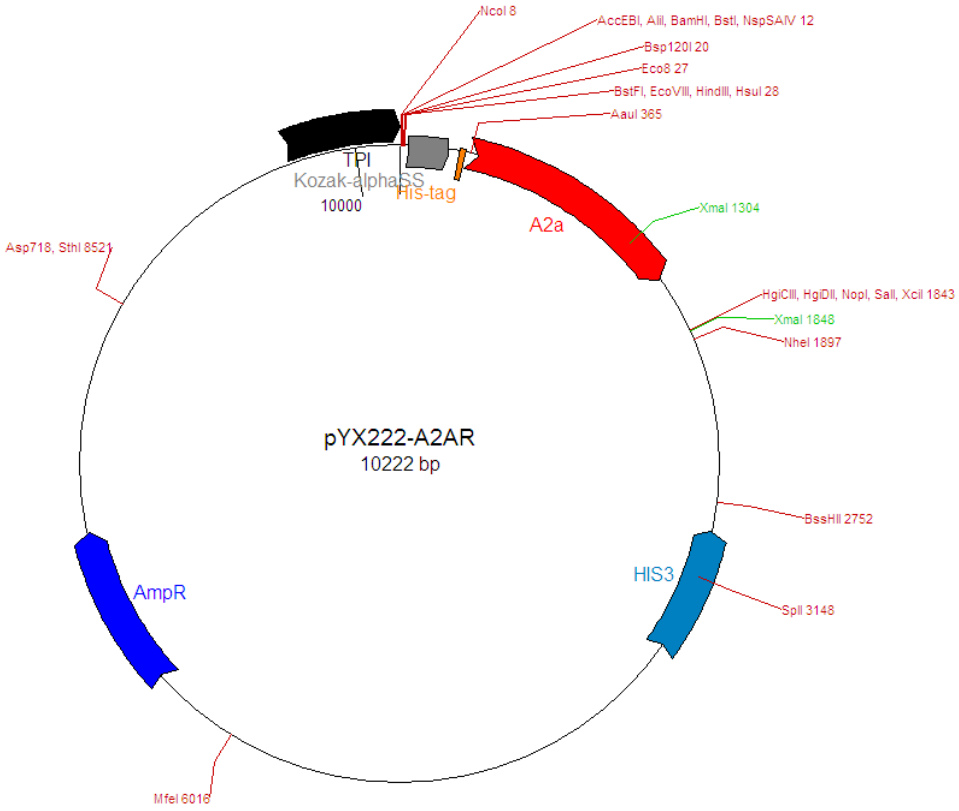
Zhou, W., Edelman, G.M. & Mauro, V.P. (2001) Transcript leader regions of two Saccharomyces cerevisiae mRNAs contain internal ribosome entry sites that function in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98 (4), pp.1531–1536.

Zhou, W., Edelman, G.M. & Mauro, V.P. (2001) Transcript leader regions of two Saccharomyces cerevisiae mRNAs contain internal ribosome entry sites that function in living cells. *Proceedings of the National Academy of Sciences of the United States of America*, 98 (4), pp.1531–1536.

7. Appendices

A.1. Vectors

A.1.1. pYX222-$A_{2A}R$



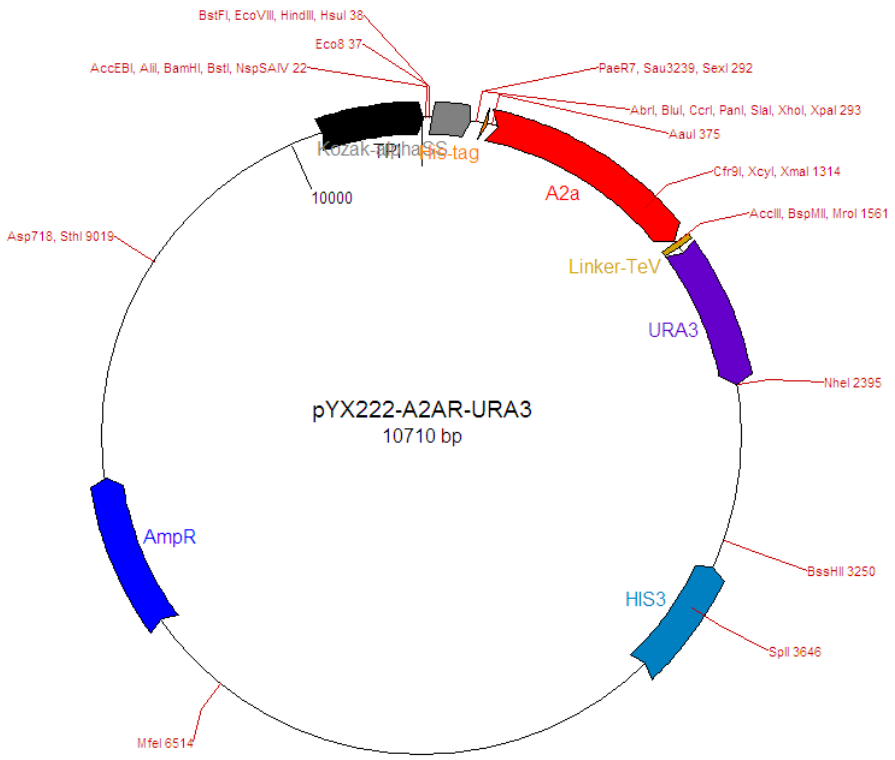
GAATTCACCATGGATCCTAGGGCCCACAAGCTTAACAAAATGAGATTTCCTTCAATTTTTA
CTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGA
TGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTC
GATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTA
CTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGC
TCATCATCATCATCATCATCATCATCATCATGAATTCATGCCCATCATGGGCTCCTCGGTG
TACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTGCTGGTGTGCT
GGGCCGTGTGGCTCAACAGCAACCTGCAGAACGTCACCAACTACTTTGTGGTGTCACTGGC
GGCGGCCGACATCGCAGTGGGTGTGCTCGCCATCCCCTTTGCCATCACCATCAGCACCGGG
TTCTGCGCTGCCTGCCACGGCTGCCTCTTCATTGCCTGCTTCGTCCTGGTCCTCACGCAGA
GCTCCATCTTCAGTCTCCTGGCCATCGCCATTGACCGCTACATTGCCATCCGCATCCCGCT
CCGGTACAATGGCTTGGTGACCGGCACGAGGGCTAAGGGCATCATTGCCATCTGCTGGGTG
CTGTCGTTTGCCATCGGCCTGACTCCCATGCTAGGTTGGAACAACTGCGGTCAGCCAAAGG
AGGGCAAGCAGCACTCCCAGGGCTGCGGGGAGGGCCAAGTGGCCTGTCTCTTTGAGGATGT
GGTCCCCATGAACTACATGGTGTACTTCAACTTCTTTGCCTGTGTGCTGGTGCCCCTGCTG

CTCATGCTGGGTGTCTATTTGCGGATCTTCCTGGCGGCGCGACGACAGCTGAAGCAGATGG
AGAGCCAGCCTCTGCCGGGGGAGCGGGCACGGTCCACACTGCAGAAGGAGGTCCATGCTGC
CAAGTCACTGGCCATCATTGTGGGGCTCTTTGCCCTCTGCTGGCTGCCCCTACACATCATC
AACTGCTTCACTTTCTTCTGCCCCGACTGCAGCCACGCCCCTCTCTGGCTCATGTACCTGG
CCATCGTCCTCTCCCACACCAATTCGGTTGTGAATCCCTTCATCTACGCCTACCGTATCCG
CGAGTTCCGCCAGACCTTCCGCAAGATCATTCGCAGCCACGTCCTGAGGCAGCAAGAACCT
TTCAAGGCAGCTGGCACCAGTGCCCGGGTCTTGGCAGCTCATGGCAGTGACGGAGAGCAGG
TCAGCCTCCGTCTCAACGGCCACCCGCCAGGAGTGTGGGCCAACGGCAGTGCTCCCCACCC
TGAGCGGAGGCCCAATGGCTACGCCCTGGGGCTGGTGAGTGGAGGGAGTGCCCAAGAGTCC
CAGGGGAACACGGGCCTCCCAGACGTGGAGCTCCTTAGCCATGAGCTCAAGGGAGTGTGCC
CAGAGCCCCCTGGCCTAGATGACCCCCTGGCCCAGGATGGAGCAGGAGTGTCCGCGGCCGC
TGAAAATCTGTATTTCCAGAGTGCCGGTAAGGCCGGAGAGGGCGAGATTCCCGCTCCGCTG
GCCGGCACCGTCTCCAAGATCCTCGTGAAGGAGGGTGACACGGTCAAGGCTGGTCAGACCG
TGCTCGTTCTCGAGGCCATGAAGATGGAGACCGAGATCAACGCTCCCACCGACGGCAAGGT
CGAGAAGGTCCTGGTCAAGGAGCGTGACGCGGTGCAGGGCGGTCAGGGTCTCATCAAGATC
GGGTGATCTAGAGTCGACCCGGGTATCCGTATGATGTGCCTGACTACGCATGATATCTCGA
GCTCAGCTAGCTAACTGAATAAGGAACAATGAACGTTTTTCCTTTCTCTTGTTCCTAGTAT
TAATGACTGACCGATACATCCCTTTTTTTTTTTGTCTTTGTCTAGCTCCAATTCGCCCTAT
AGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTG
GCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGA
AGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGC
CCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACT
TGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCC
GGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTAC
GGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTG
ATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTC
CAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGC
CGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAA
CAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTA
TTTCACACCGCATAGATCCGTCGAGTTCAAGAGAAAAAAAAAGAAAAAGCAAAAAGAAAAA
AGGAAAGCGCGCCTCGTTCAGAATGACACGTATAGAATGATGCATTACCTTGTCATCTTCA
GTATCATACTGTTCGTATACATACTTACTGACATTCATAGGTATACATATATACACATGTA
TATATATCGTATGCTGCAGCTTTAAATAATCGGTGTCACTACATAAGAACACCTTTGGTGG
AGGGAACATCGTTGGTTCCATTGGGCGAGGTGGCTTCTCTTATGGCAACCGCAAGAGCCTT
GAACGCACTCTCACTACGGTGATGATCATTCTTGCCTCGCAGACAATCAACGTGGAGGGTA
ATTCTGCTTGCCTCTGCAAAACTTTCAAGAAAATGCGGGATCATCTCGCAAGAGAGATCTC
CTACTTTCTCCCTCTGCAAACCAAGTTCGACAACTGCGTACGGCCTGTTCGAAAGATCTAC
CACCGCTCTGGAAAGTGCCTCATCCAAAGGCGCAAATCCTGATCCAAACCTTTTTACTCCA
CGCACGGCCCCTAGGGCCTCTTTAAATGCTTGACCGAGAGCAATCCCGCAGTCTTCAGTGG
TGTGATGGTCGTCTATGTGTAAGTCACCAATGCACTCAACGATTAGCGACCAGCCGGAATG
CTTGGCCAGAGCATGTATCATATGGTCCAGAAACCCTATACCTGTGTGGACGTTAATCACT
TGCGATTGTGTGGCCTGTTCTGCTACTGCTTCTGCCTCTTTTTCTGGGAAGATCGAGTGCT
CTATCGCTAGGGGACCACCCTTTAAAGAGATCGCAATCTGAATCTTGGTTTCATTTGTAAT
ACGCTTTACTAGGGCTTTCTGCTCTGTCATCTTTGCCTTCGTTTATCTTGCCTGCTCATTT
TTTAGTATATTCTTCGAAGAAATCACATTACTTTATATAATGTATAATTCATTATGTGATA
ATGCCAATCGCTAAGAAAAAAAAAGAGTCATCCGCTAGGGGAAAAAAAAAAATGAAAATCA
TTACCGAGGCATAAAAAAATATAGAGTGTACTAGAGGAGGCCAAGAGTAATAGAAAAAGAA
AATTGCGGGAAAGGACTGTGTTATGACTTCCCTGACTAATGCCGTGTTCAAACGATACCTG
GCAGTGACTCCTAGCGCTCACCAAGCTCTTAAAACGGGAATTTATGGTGCACTCTCAGTAC
AATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCG
CCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGA

GCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGT
GATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTGCGGCCGC
TCTAGAACTAGTGGATCAATTCCACGGACTATAGACTATACTAGTATACTCCGTCTACTGT
ACGATACACTTCCGCTCAGGTCCTTGTCCTTTAACGAGGCCTTACCACTCTTTTGTTACTC
TATTGATCCAGCTCAGCAAAGGCAGTGTGATCTAAGATTCTATCTTCGCGATGTAGTAAAA
CTAGCTAGACCGAGAAAGAGACTAGAAATGCAAAAGGCACTTCTACAATGGCTGCCATCAT
TATTATCCGATGTGACGCTGCAGCTTCTCAATGATATTCGAATACGCTTTGAGGAGATACA
GCCTAATATCCGACAAACTGTTTTACAGATTTACGATCGTACTTGTTACCCATCATTGAAT
TTTGAACATCCGAACCTGGGAGTTTTCCCTGAAACAGATAGTATATTTGAACCTGTATAAT
AATATATAGTCTAGCGCTTTACGGAAGACAATGTATGTATTTCGGTTCCTGGAGAAACTAT
TGCATCTATTGCATAGGTAATCTTGCACGTCGCATCCCCGGTTCATTTTCTGCGTTTCCAT
CTTGCACTTCAATAGCATATCTTTGTTAACGAAGCATCTGTGCTTCATTTTGTAGAACAAA
AATGCAACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGAACA
GAAATGCAACGCGAAAGCGCTATTTTACCAACGAAGAATCTGTGCTTCATTTTTGTAAAAC
AAAAATGCAACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGA
ACAGAAATGCAACGCGAGAGCGCTATTTTACCAACAAAGAATCTATACTTCTTTTTTGTTC
TACAAAAATGCATCCCGAGAGCGCTATTTTTCTAACAAAGCATCTTAGATTACTTTTTTTC
TCCTTTGTGCGCTCTATAATGCAGTCTCTTGATAACTTTTTGCACTGTAGGTCCGTTAAGG
TTAGAAGAAGGCTACTTTGGTGTCTATTTTCTCTTCCATAAAAAAAGCCTGACTCCACTTC
CCGCGTTTACTGATTACTAGCGAAGCTGCGGGTGCATTTTTTCAAGATAAAGGCATCCCCG
ATTATATTCTATACCGATGTGGATTGCGCATACTTTGTGAACAGAAAGTGATAGCGTTGAT
GATTCTTCATTGGTCAGAAAATTATGAACGGTTTCTTCTATTTTGTCTCTATATACTACGT
ATAGGAAATGTTTACATTTTCGTATTGTTTTCGATTCACTCTATGAATAGTTCTTACTACA
ATTTTTTTGTCTAAAGAGTAATACTAGAGATAAACATAAAAAATGTAGAGGTCGAGTTTAG
ATGCAAGTTCAAGGAGCGAAAGGTGGATGGGTAGGTTATATAGGGATATAGCACAGAGATA
TATAGCAAAGAGATACTTTTGAGCAATGTTTGTGGAAGCGGTATTCGCAATATTTTAGTAG
CTCGTTACAGTCCGGTGCGTTTTTGGTTTTTTGAAAGTGCGTCTTCAGAGCGCTTTTGGTT
TTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTT
CAAAGCGTTTCCGAAAACGAGCGCTTCCGAAAATGCAACGCGAGCTGCGCACATACAGCTC
ACTGTTCACGTCGCACCTATATCTGCGTGTTGCCTGTATATATATATACATGAGAAGAACG
GCATAGTGCGTGTTTATGCTTAAATGCGTACTTATATGCGTCTATTTATGTAGGATGAAAG
GTAGTCTAGTACCTCCTGTGATATTATCCCATTCCATGCGGGGTATCGTATGCTTCCTTCA
GCACTACCCTTTAGCTGTTCTATATGCTGCCACTCCTCAATTGGATTAGTCTCATCCTTCA
ATGCTATCATTTCCTTTGATATTGGATCATATGCATAGTACCGAGAAACTAGTGCGAAGTA
GTGATCAGGTATTGCTGTTATCTGATGAGTATACGTTGTCCTGGCCACGGCAGAAGCACGC
TTATCGCTCCAATTTCCCACAACATTAGTCAACTCCGTTAGGCCCTTCATTGAAAGAAATG
AGGTCATCAAATGTCTTCCAATGTGAGATTTTGGGCCATTTTTTATAGCAAAGATTGAATA
AGGCGCATTTTTCTTCAAAGCTGCGGCCGCACGTCAGGTGGCACTTTTCGGGGAAATGTGC
GCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACA
ATAACCGTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTC
CGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAA
CGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACT
GGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATG
AGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGC
AACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGA
AAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGT
GATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTT
TTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGA
AGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGC
AAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGG
AGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGC

TGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGAT
GGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAAC
GAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCA
AGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAG
GTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACT
GAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGT
AATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAA
GAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTG
TTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATA
CCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACC
GGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTT
CGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGA
GCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGC
AGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATA
GTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGG
GCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGG
CCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCG
CCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAG
CGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCAT
TAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTA
ATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTAT
GTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTAC
GCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGTACCGGGCCGGCCGTCGG
GCCGTCGAGCTTGATGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCT
CCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAG
CTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTT
ATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTG
GTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCC
GGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGA
AAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGT
AACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTG
AGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGA
ATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGA
GCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCC
CCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAAT
AGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTGGGGATCTACGTATGGTCATTTCTTC
TTCAGATTCCCTCATGGAGAAAGTGCGGCAGATGTATATGACAGAGTCGCCAGTTTCCAAG
AGACTTTATTCAGGCACTTCCATGATAGGCAAGAGAGAAGACCCAGAGATGTTGTTGTCCT
AGTTACACATGGTATTTATTCCAGAGTATTCCTGATGAAATGGTTTAGATGGACATACGAA
GAGTTTGAATCGTTTACCAATGTTCCTAACGGGAGCGTAATGGTGATGGAACTGGACGAAT
CCATCAATAGATACGTCCTGAGGACCGTGCTACCCAAATGGACTGATTGTGAGGGAGACCT
AACTACATAGTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGCCATTTTTTTG
AGTTATAATAATCCTACGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCTTAATACATTC
AGACACTTCTGCGGTATCACCCTACTTATTCCCTTCGAGATTATATCTAGGAACCCATCAG
GTTGGTGGAAGATTACCCGTTCTAAGACTTTTCAGCTTCCTCTATTGATGTTACACCTGGA
CACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTGGCATGTGAGATTCTCCGAAATTAA
TTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGACAGGTGGTTTGTT
ACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAACAGGGAATATA
AAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTTCTTACG
TAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTC
TTGCTTAAATCTATAACTACAAAAAACACATACAG

A.1.2. pYX222-A_{2A}R-URA3



ACACATACAGGAATTCACCATGGATCCTAGGGCCCACAAGCTTAACAAAATGAGATTTCCT
TCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTA
CAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGA
AGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTT
ATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAG
AGGCTGAAGCTCATCATCATCATCATCATCATCATCATCATGAATTCATGCCCATCATGGG
CTCCTCGGTGTACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTG
CTGGTGTGCTGGGCCGTGTGGCTCAACAGCAACCTGCAGAACGTCACCAACTACTTTGTGG
TGTCACTGGCGGCGGCCGACATCGCAGTGGGTGTGCTCGCCATCCCCTTTGCCATCACCAT
CAGCACCGGGTTCTGCGCTGCCTGCCACGGCTGCCTCTTCATTGCCTGCTTCGTCCTGGTC
CTCACGCAGAGCTCCATCTTCAGTCTCCTGGCCATCGCCATTGACCGCTACATTGCCATCC
GCATCCCGCTCCGGTACAATGGCTTGGTGACCGGCACGAGGGCTAAGGGCATCATTGCCAT
CTGCTGGGTGCTGTCGTTTGCCATCGGCCTGACTCCCATGCTAGGTTGGAACAACTGCGGT
CAGCCAAAGGAGGGCAAGCAGCACTCCCAGGGCTGCGGGGAGGGCCAAGTGGCCTGTCTCT
TTGAGGATGTGGTCCCCATGAACTACATGGTGTACTTCAACTTCTTTGCCTGTGTGCTGGT
GCCCCTGCTGCTCATGCTGGGTGTCTATTTGCGGATCTTCCTGGCGGCGCGACGACAGCTG
AAGCAGATGGAGAGCCAGCCTCTGCCGGGGGAGCGGGCACGGTCCACACTGCAGAAGGAGG
TCCATGCTGCCAAGTCACTGGCCATCATTGTGGGGCTCTTTGCCCTCTGCTGGCTGCCCCT

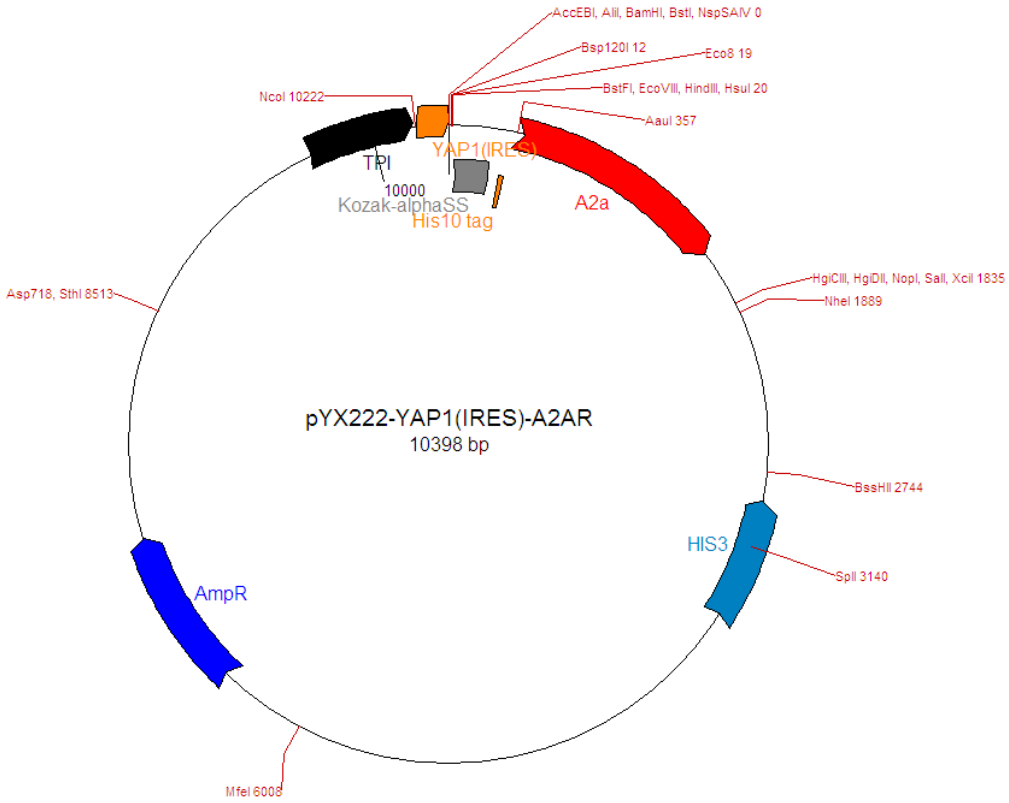
ACACATCATCAACTGCTTCACTTTCTTCTGCCCCGACTGCAGCCACGCCCCTCTCTGGCTC
ATGTACCTGGCCATCGTCCTCTCCCACACCAATTCGGTTGTGAATCCCTTCATCTACGCCT
ACCGTATCCGCGAGTTCCGCCAGACCTTCCGCAAGATCATTCGCAGCCACGTCCTGAGGCA
GCAAGAACCTTTCAAGGCAGCTGGCACCAGTGCCCGGGTCTTGGCAGCTCATGGCAGTGAC
GGAGAGCAGGTCAGCCTCCGTCTCAACGGCCACCCGCCAGGAGTGTGGGCCAACGGCAGTG
CTCCCCACCCTGAGCGGAGGCCCAATGGCTACGCCCTGGGGCTGGTGAGTGGAGGGAGTGC
CCAAGAGTCCCAGGGGAACACGGGCCTCCCAGACGTGGAGCTCCTTAGCCATGAGCTCAAG
GGAGTGTGCCCAGAGCCCCCTGGCCTAGAGGGGGGTCCGGAGAGAACCTGTACTTCCAGGG
GAGTATGTCGAAAGCTACATATAAGGAACGTGCTGCTACTCATCCTAGTCCTGTTGCTGCC
AAGCTATTTAATATCATGCACGAAAAGCAAACAAACTTGTGTGCTTCATTGGATGTTCGTA
CCACCAAGGAATTACTGGAGTTAGTTGAAGCATTAGGTCCCAAAATTTGTTTACTAAAAAC
ACATGTGGATATCTTGACTGATTTTTCCATGGAGGGCACAGTTAAGCCGCTAAAGGCATTA
TCCGCCAAGTACAATTTTTTACTCTTCGAAGACAGAAAATTTGCTGACATTGGTAATACAG
TCAAATTGCAGTACTCTGCGGGTGTATACAGAATAGCAGAATGGGCAGACATTACGAATGC
ACACGGTGTGGTGGGCCCAGGTATTGTTAGCGGTTTGAAGCAGGCGGCGGAAGAAGTAACA
AAGGAACCTAGAGGCCTTTTGATGTTAGCAGAATTGTCATGCAAGGGCTCCCTAGCTACTG
GAGAATATACTAAGGGTACTGTTGACATTGCGAAGAGCGACAAAGATTTTGTTATCGGCTT
TATTGCTCAAAGAGACATGGGTGGAAGAGATGAAGGTTACGATTGGTTGATTATGACACCC
GGTGTGGGTTTAGATGACAAGGGAGACGCATTGGGTCAACAGTATAGAACCGTGGATGATG
TGGTCTCTACAGGATCTGACATTATTATTGTTGGAAGAGGACTATTTGCAAAGGGAAGGGA
TGCTAAGGTAGAGGGTGAACGTTACAGAAAAGCAGGCTGGGAAGCATATTTGAGAAGATGC
GGCCAGCAAAACTAAGCTAGCTAACTGAATAAGGAACAATGAACGTTTTTCCTTTCTCTTG
TTCCTAGTATTAATGACTGACCGATACATCCCTTTTTTTTTTTGTCTTTGTCTAGCTCCAA
TTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGG
GAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGC
GTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGA
ATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGA
CCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGC
CACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTT
AGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGC
CATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGG
ACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAA
GGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACG
CGAATTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCAT
CTGTGCGGTATTTCACACCGCATAGATCCGTCGAGTTCAAGAGAAAAAAAAAGAAAAAGCA
AAAAGAAAAAAGGAAAGCGCGCCTCGTTCAGAATGACACGTATAGAATGATGCATTACCTT
GTCATCTTCAGTATCATACTGTTCGTATACATACTTACTGACATTCATAGGTATACATATA
TACACATGTATATATATCGTATGCTGCAGCTTTAAATAATCGGTGTCACTACATAAGAACA
CCTTTGGTGGAGGGAACATCGTTGGTTCCATTGGGCGAGGTGGCTTCTCTTATGGCAACCG
CAAGAGCCTTGAACGCACTCTCACTACGGTGATGATCATTCTTGCCTCGCAGACAATCAAC
GTGGAGGGTAATTCTGCTTGCCTCTGCAAAACTTTCAAGAAAATGCGGGATCATCTCGCAA
GAGAGATCTCCTACTTTCTCCCTCTGCAAACCAAGTTCGACAACTGCGTACGGCCTGTTCG
AAAGATCTACCACCGCTCTGGAAAGTGCCTCATCCAAAGGCGCAAATCCTGATCCAAACCT
TTTTACTCCACGCACGGCCCCTAGGGCCTCTTTAAATGCTTGACCGAGAGCAATCCCGCAG
TCTTCAGTGGTGTGATGGTCGTCTATGTGTAAGTCACCAATGCACTCAACGATTAGCGACC
AGCCGGAATGCTTGGCCAGAGCATGTATCATATGGTCCAGAAACCCTATACCTGTGTGGAC
GTTAATCACTTGCGATTGTGTGGCCTGTTCTGCTACTGCTTCTGCCTCTTTTTCTGGGAAG
ATCGAGTGCTCTATCGCTAGGGGACCACCCTTTAAAGAGATCGCAATCTGAATCTTGGTTT
CATTTGTAATACGCTTTACTAGGGCTTTCTGCTCTGTCATCTTTGCCTTCGTTTATCTTGC
CTGCTCATTTTTTAGTATATTCTTCGAAGAAATCACATTACTTTATATAATGTATAATTCA
TTATGTGATAATGCCAATCGCTAAGAAAAAAAAAGAGTCATCCGCTAGGGGAAAAAAAAA

ATGAAAATCATTACCGAGGCATAAAAAAATATAGAGTGTACTAGAGGAGGCCAAGAGTAAT
AGAAAAAGAAAATTGCGGGAAAGGACTGTGTTATGACTTCCCTGACTAATGCCGTGTTCAA
ACGATACCTGGCAGTGACTCCTAGCGCTCACCAAGCTCTTAAAACGGGAATTTATGGTGCA
CTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACC
CGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACC
GTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAA
AGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGAC
GTGCGGCCGCTCTAGAACTAGTGGATCAATTCCACGGACTATAGACTATACTAGTATACTC
CGTCTACTGTACGATACACTTCCGCTCAGGTCCTTGTCCTTTAACGAGGCCTTACCACTCT
TTTGTTACTCTATTGATCCAGCTCAGCAAAGGCAGTGTGATCTAAGATTCTATCTTCGCGA
TGTAGTAAAACTAGCTAGACCGAGAAAGAGACTAGAAATGCAAAAGGCACTTCTACAATGG
CTGCCATCATTATTATCCGATGTGACGCTGCAGCTTCTCAATGATATTCGAATACGCTTTG
AGGAGATACAGCCTAATATCCGACAAACTGTTTTACAGATTTACGATCGTACTTGTTACCC
ATCATTGAATTTTGAACATCCGAACCTGGGAGTTTTCCCTGAAACAGATAGTATATTTGAA
CCTGTATAATAATATATAGTCTAGCGCTTTACGGAAGACAATGTATGTATTTCGGTTCCTG
GAGAAACTATTGCATCTATTGCATAGGTAATCTTGCACGTCGCATCCCCGGTTCATTTTCT
GCGTTTCCATCTTGCACTTCAATAGCATATCTTTGTTAACGAAGCATCTGTGCTTCATTTT
GTAGAACAAAAATGCAACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTT
TTACAGAACAGAAATGCAACGCGAAAGCGCTATTTTACCAACGAAGAATCTGTGCTTCATT
TTTGTAAAACAAAAATGCAACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCA
TTTTTACAGAACAGAAATGCAACGCGAGAGCGCTATTTTACCAACAAAGAATCTATACTTC
TTTTTTGTTCTACAAAAATGCATCCCGAGAGCGCTATTTTTCTAACAAAGCATCTTAGATT
ACTTTTTTTCTCCTTTGTGCGCTCTATAATGCAGTCTCTTGATAACTTTTTGCACTGTAGG
TCCGTTAAGGTTAGAAGAAGGCTACTTTGGTGTCTATTTTCTCTTCCATAAAAAAAGCCTG
ACTCCACTTCCCGCGTTTACTGATTACTAGCGAAGCTGCGGGTGCATTTTTTCAAGATAAA
GGCATCCCCGATTATATTCTATACCGATGTGGATTGCGCATACTTTGTGAACAGAAAGTGA
TAGCGTTGATGATTCTTCATTGGTCAGAAAATTATGAACGGTTTCTTCTATTTTGTCTCTA
TATACTACGTATAGGAAATGTTTACATTTTCGTATTGTTTTCGATTCACTCTATGAATAGT
TCTTACTACAATTTTTTTGTCTAAAGAGTAATACTAGAGATAAACATAAAAAATGTAGAGG
TCGAGTTTAGATGCAAGTTCAAGGAGCGAAAGGTGGATGGGTAGGTTATATAGGGATATAG
CACAGAGATATATAGCAAAGAGATACTTTTGAGCAATGTTTGTGGAAGCGGTATTCGCAAT
ATTTTAGTAGCTCGTTACAGTCCGGTGCGTTTTTGGTTTTTTGAAAGTGCGTCTTCAGAGC
GCTTTTGGTTTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGA
ATAGGAACTTCAAAGCGTTTCCGAAAACGAGCGCTTCCGAAAATGCAACGCGAGCTGCGCA
CATACAGCTCACTGTTCACGTCGCACCTATATCTGCGTGTTGCCTGTATATATATATACAT
GAGAAGAACGGCATAGTGCGTGTTTATGCTTAAATGCGTACTTATATGCGTCTATTTATGT
AGGATGAAAGGTAGTCTAGTACCTCCTGTGATATTATCCCATTCCATGCGGGGTATCGTAT
GCTTCCTTCAGCACTACCCTTTAGCTGTTCTATATGCTGCCACTCCTCAATTGGATTAGTC
TCATCCTTCAATGCTATCATTTCCTTTGATATTGGATCATATGCATAGTACCGAGAAACTA
GTGCGAAGTAGTGATCAGGTATTGCTGTTATCTGATGAGTATACGTTGTCCTGGCCACGGC
AGAAGCACGCTTATCGCTCCAATTTCCCACAACATTAGTCAACTCCGTTAGGCCCTTCATT
GAAAGAAATGAGGTCATCAAATGTCTTCCAATGTGAGATTTTGGGCCATTTTTTATAGCAA
AGATTGAATAAGGCGCATTTTTCTTCAAAGCTGCGGCCGCACGTCAGGTGGCACTTTTCGG
GGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGC
TCATGAGACAATAACCGTGATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTAT
TCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCT
CACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTT
ACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTT
TCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCC
GGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCAC
CAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCAT

AACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAG
CTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGG
AGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAAC
AACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATA
GACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCT
GGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACT
GGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACT
ATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAAC
TGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAA
AAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTT
TCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTT
TTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTT
GCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATA
CCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCAC
CGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTC
GTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGA
ACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACC
TACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCC
GGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGG
TATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCT
CGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGC
CTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAAC
CGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCG
AGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTG
GCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGC
AACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTC
CGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGA
CCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGTACCGGGC
CGGCCGTCGGGCCGTCGAGCTTGATGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCT
TCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAA
AAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATC
ACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTT
TCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTT
GCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCT
CATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCC
AGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCG
TTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACG
GAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTAT
TGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGC
GCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAAC
CTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTGGGGATCTACGTATGG
TCATTTCTTCTTCAGATTCCCTCATGGAGAAAGTGCGGCAGATGTATATGACAGAGTCGCC
AGTTTCCAAGAGACTTTATTCAGGCACTTCCATGATAGGCAAGAGAGAAGACCCAGAGATG
TTGTTGTCCTAGTTACACATGGTATTTATTCCAGAGTATTCCTGATGAAATGGTTTAGATG
GACATACGAAGAGTTTGAATCGTTTACCAATGTTCCTAACGGGAGCGTAATGGTGATGGAA
CTGGACGAATCCATCAATAGATACGTCCTGAGGACCGTGCTACCCAAATGGACTGATTGTG
AGGGAGACCTAACTACATAGTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGC
CATTTTTTTGAGTTATAATAATCCTACGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCT
TAATACATTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTCGAGATTATATCTAGG
AACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTCAGCTTCCTCTATTGATGT
TACACCTGGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTGGCATGTGAGATTCT

CCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGACAG
GTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAAC
AGGGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTC
CCTTCTTACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTG
TATTCTTTTCTTGCTTAAATCTATAACTACAAAAA

A.1.3. pYX222-YAP1(IRES)-$A_{2A}R$



GATCCATCCTAGGGCCCACAAGCTTAACAAAATGAGATTTCCTTCAATTTTTACTGCAGTT
TTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGG
CACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGC
TGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCC
AGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTCATCATC
ATCATCATCATCATCATCATCATGAATTCATGCCCATCATGGGCTCCTCGGTGTACATCAC
GGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTGCTGGTGTGCTGGGCCGTG
TGGCTCAACAGCAACCTGCAGAACGTCACCAACTACTTTGTGGTGTCACTGGCGGCGGCCG
ACATCGCAGTGGGTGTGCTCGCCATCCCCTTTGCCATCACCATCAGCACCGGGTTCTGCGC
TGCCTGCCACGGCTGCCTCTTCATTGCCTGCTTCGTCCTGGTCCTCACGCAGAGCTCCATC
TTCAGTCTCCTGGCCATCGCCATTGACCGCTACATTGCCATCCGCATCCCGCTCCGGTACA
ATGGCTTGGTGACCGGCACGAGGGCTAAGGGCATCATTGCCATCTGCTGGGTGCTGTCGTT
TGCCATCGGCCTGACTCCCATGCTAGGTTGGAACAACTGCGGTCAGCCAAAGGAGGGCAAG

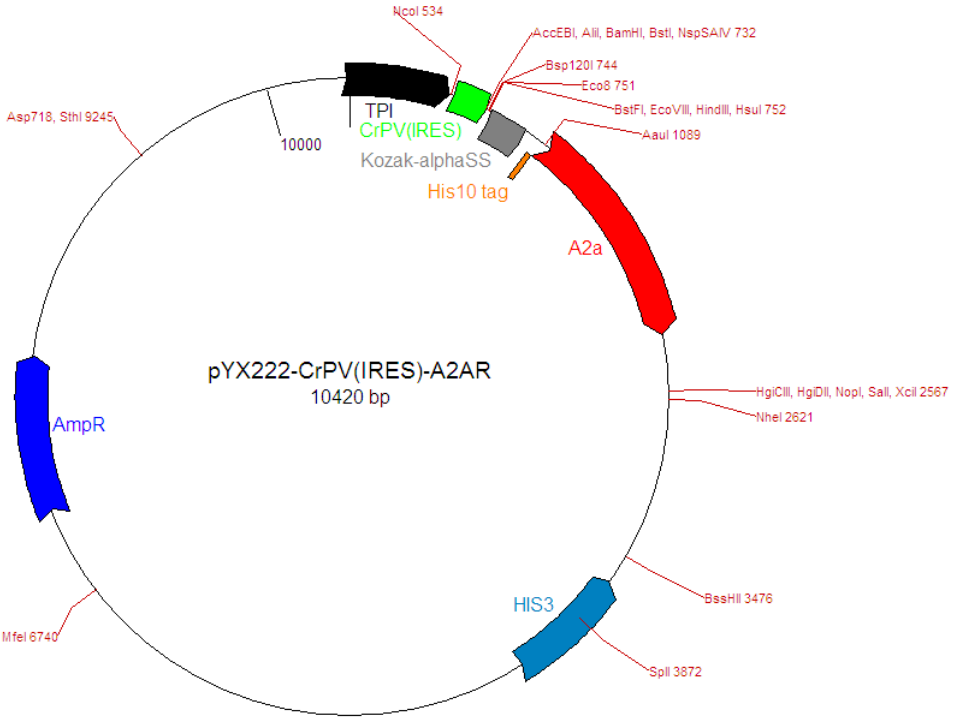
CAGCACTCCCAGGGCTGCGGGGAGGGCCAAGTGGCCTGTCTCTTTGAGGATGTGGTCCCCA
TGAACTACATGGTGTACTTCAACTTCTTTGCCTGTGTGCTGGTGCCCCTGCTGCTCATGCT
GGGTGTCTATTTGCGGATCTTCCTGGCGGCGCGACGACAGCTGAAGCAGATGGAGAGCCAG
CCTCTGCCGGGGGAGCGGGCACGGTCCACACTGCAGAAGGAGGTCCATGCTGCCAAGTCAC
TGGCCATCATTGTGGGGCTCTTTGCCCTCTGCTGGCTGCCCCTACACATCATCAACTGCTT
CACTTTCTTCTGCCCCGACTGCAGCCACGCCCCTCTCTGGCTCATGTACCTGGCCATCGTC
CTCTCCCACACCAATTCGGTTGTGAATCCCTTCATCTACGCCTACCGTATCCGCGAGTTCC
GCCAGACCTTCCGCAAGATCATTCGCAGCCACGTCCTGAGGCAGCAAGAACCTTTCAAGGC
AGCTGGCACCAGTGCCCGGGTCTTGGCAGCTCATGGCAGTGACGGAGAGCAGGTCAGCCTC
CGTCTCAACGGCCACCCGCCAGGAGTGTGGGCCAACGGCAGTGCTCCCCACCCTGAGCGGA
GGCCCAATGGCTACGCCCTGGGGCTGGTGAGTGGAGGGAGTGCCCAAGAGTCCCAGGGGAA
CACGGGCCTCCCAGACGTGGAGCTCCTTAGCCATGAGCTCAAGGGAGTGTGCCCAGAGCCC
CCTGGCCTAGATGACCCCCTGGCCCAGGATGGAGCAGGAGTGTCCGCGGCCGCTGAAAATC
TGTATTTCCAGAGTGCCGGTAAGGCCGGAGAGGGCGAGATTCCCGCTCCGCTGGCCGGCAC
CGTCTCCAAGATCCTCGTGAAGGAGGGTGACACGGTCAAGGCTGGTCAGACCGTGCTCGTT
CTCGAGGCCATGAAGATGGAGACCGAGATCAACGCTCCCACCGACGGCAAGGTCGAGAAGG
TCCTGGTCAAGGAGCGTGACGCGGTGCAGGGCGGTCAGGGTCTCATCAAGATCGGGTGATC
TAGAGTCGACCCGGGTATCCGTATGATGTGCCTGACTACGCATGATATCTCGAGCTCAGCT
AGCTAACTGAATAAGGAACAATGAACGTTTTTCCTTTCTCTTGTTCCTAGTATTAATGACT
GACCGATACATCCCTTTTTTTTTTTGTCTTTGTCTAGCTCCAATTCGCCCTATAGTGAGTC
GTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCC
GCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGC
GGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCG
CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCC
CCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTC
GACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGG
TTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGG
AACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG
GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATAT
TAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACAC
CGCATAGATCCGTCGAGTTCAAGAGAAAAAAAAAGAAAAAGCAAAAAGAAAAAAGGAAAGC
GCGCCTCGTTCAGAATGACACGTATAGAATGATGCATTACCTTGTCATCTTCAGTATCATA
CTGTTCGTATACATACTTACTGACATTCATAGGTATACATATATACACATGTATATATATC
GTATGCTGCAGCTTTAAATAATCGGTGTCACTACATAAGAACACCTTTGGTGGAGGGAACA
TCGTTGGTTCCATTGGGCGAGGTGGCTTCTCTTATGGCAACCGCAAGAGCCTTGAACGCAC
TCTCACTACGGTGATGATCATTCTTGCCTCGCAGACAATCAACGTGGAGGGTAATTCTGCT
TGCCTCTGCAAAACTTTCAAGAAAATGCGGGATCATCTCGCAAGAGAGATCTCCTACTTTC
TCCCTCTGCAAACCAAGTTCGACAACTGCGTACGGCCTGTTCGAAAGATCTACCACCGCTC
TGGAAAGTGCCTCATCCAAAGGCGCAAATCCTGATCCAAACCTTTTTACTCCACGCACGGC
CCCTAGGGCCTCTTTAAATGCTTGACCGAGAGCAATCCCGCAGTCTTCAGTGGTGTGATGG
TCGTCTATGTGTAAGTCACCAATGCACTCAACGATTAGCGACCAGCCGGAATGCTTGGCCA
GAGCATGTATCATATGGTCCAGAAACCCTATACCTGTGTGGACGTTAATCACTTGCGATTG
TGTGGCCTGTTCTGCTACTGCTTCTGCCTCTTTTTCTGGGAAGATCGAGTGCTCTATCGCT
AGGGGACCACCCTTTAAAGAGATCGCAATCTGAATCTTGGTTTCATTTGTAATACGCTTTA
CTAGGGCTTTCTGCTCTGTCATCTTTGCCTTCGTTTATCTTGCCTGCTCATTTTTTAGTAT
ATTCTTCGAAGAAATCACATTACTTTATATAATGTATAATTCATTATGTGATAATGCCAAT
CGCTAAGAAAAAAAAAGAGTCATCCGCTAGGGGAAAAAAAAAAATGAAAATCATTACCGAG
GCATAAAAAAATATAGAGTGTACTAGAGGAGGCCAAGAGTAATAGAAAAAGAAAATTGCGG
GAAAGGACTGTGTTATGACTTCCCTGACTAATGCCGTGTTCAAACGATACCTGGCAGTGAC
TCCTAGCGCTCACCAAGCTCTTAAAACGGGAATTTATGGTGCACTCTCAGTACAATCTGCT

CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACG
GGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATG
TGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCC
TATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCGGCCGCTCTAGAAC
TAGTGGATCAATTCCACGGACTATAGACTATACTAGTATACTCCGTCTACTGTACGATACA
CTTCCGCTCAGGTCCTTGTCCTTTAACGAGGCCTTACCACTCTTTTGTTACTCTATTGATC
CAGCTCAGCAAAGGCAGTGTGATCTAAGATTCTATCTTCGCGATGTAGTAAAACTAGCTAG
ACCGAGAAAGAGACTAGAAATGCAAAAGGCACTTCTACAATGGCTGCCATCATTATTATCC
GATGTGACGCTGCAGCTTCTCAATGATATTCGAATACGCTTTGAGGAGATACAGCCTAATA
TCCGACAAACTGTTTTACAGATTTACGATCGTACTTGTTACCCATCATTGAATTTTGAACA
TCCGAACCTGGGAGTTTTCCCTGAAACAGATAGTATATTTGAACCTGTATAATAATATATA
GTCTAGCGCTTTACGGAAGACAATGTATGTATTTCGGTTCCTGGAGAAACTATTGCATCTA
TTGCATAGGTAATCTTGCACGTCGCATCCCCGGTTCATTTTCTGCGTTTCCATCTTGCACT
TCAATAGCATATCTTTGTTAACGAAGCATCTGTGCTTCATTTTGTAGAACAAAAATGCAAC
GCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGAACAGAAATGCA
ACGCGAAAGCGCTATTTTACCAACGAAGAATCTGTGCTTCATTTTTGTAAAACAAAAATGC
AACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGAACAGAAAT
GCAACGCGAGAGCGCTATTTTACCAACAAAGAATCTATACTTCTTTTTTGTTCTACAAAAA
TGCATCCCGAGAGCGCTATTTTTCTAACAAAGCATCTTAGATTACTTTTTTTCTCCTTTGT
GCGCTCTATAATGCAGTCTCTTGATAACTTTTTGCACTGTAGGTCCGTTAAGGTTAGAAGA
AGGCTACTTTGGTGTCTATTTTCTCTTCCATAAAAAAAGCCTGACTCCACTTCCCGCGTTT
ACTGATTACTAGCGAAGCTGCGGGTGCATTTTTTCAAGATAAAGGCATCCCCGATTATATT
CTATACCGATGTGGATTGCGCATACTTTGTGAACAGAAAGTGATAGCGTTGATGATTCTTC
ATTGGTCAGAAAATTATGAACGGTTTCTTCTATTTTGTCTCTATATACTACGTATAGGAAA
TGTTTACATTTTCGTATTGTTTTCGATTCACTCTATGAATAGTTCTTACTACAATTTTTTT
GTCTAAAGAGTAATACTAGAGATAAACATAAAAAATGTAGAGGTCGAGTTTAGATGCAAGT
TCAAGGAGCGAAAGGTGGATGGGTAGGTTATATAGGGATATAGCACAGAGATATATAGCAA
AGAGATACTTTTGAGCAATGTTTGTGGAAGCGGTATTCGCAATATTTTAGTAGCTCGTTAC
AGTCCGGTGCGTTTTTGGTTTTTTGAAAGTGCGTCTTCAGAGCGCTTTTGGTTTTCAAAAG
CGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAAGCGT
TTCCGAAAACGAGCGCTTCCGAAAATGCAACGCGAGCTGCGCACATACAGCTCACTGTTCA
CGTCGCACCTATATCTGCGTGTTGCCTGTATATATATATACATGAGAAGAACGGCATAGTG
CGTGTTTATGCTTAAATGCGTACTTATATGCGTCTATTTATGTAGGATGAAAGGTAGTCTA
GTACCTCCTGTGATATTATCCCATTCCATGCGGGGTATCGTATGCTTCCTTCAGCACTACC
CTTTAGCTGTTCTATATGCTGCCACTCCTCAATTGGATTAGTCTCATCCTTCAATGCTATC
ATTTCCTTTGATATTGGATCATATGCATAGTACCGAGAAACTAGTGCGAAGTAGTGATCAG
GTATTGCTGTTATCTGATGAGTATACGTTGTCCTGGCCACGGCAGAAGCACGCTTATCGCT
CCAATTTCCCACAACATTAGTCAACTCCGTTAGGCCCTTCATTGAAAGAAATGAGGTCATC
AAATGTCTTCCAATGTGAGATTTTGGGCCATTTTTTATAGCAAAGATTGAATAAGGCGCAT
TTTTCTTCAAAGCTGCGGCCGCACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACC
CCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCGT
GATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
CCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTG
AAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCA
ACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTT
TAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGT
CGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATC
TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACAC
TGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCAC
AACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATAC
CAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATT

AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGAT
AAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGA
CAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACT
CATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGAT
CCTTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCA
GACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCT
GCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACC
AACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTA
GTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTC
TGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGA
CTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACA
CAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAG
AAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG
AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTC
GGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCC
TATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGC
TCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAG
TGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG
CGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAG
CTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGT
TAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTG
GAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCT
CGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGTACCGGGCCGGCCGTCGGGCCGTCGA
GCTTGATGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCC
CAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA
CACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTC
TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACT
CGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAA
CAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCAT
ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATAC
ATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG
TGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTAT
CACGAGGCCCTTTCGTCTTCAAGAATTGGGGATCTACGTATGGTCATTTCTTCTTCAGATT
CCCTCATGGAGAAAGTGCGGCAGATGTATATGACAGAGTCGCCAGTTTCCAAGAGACTTTA
TTCAGGCACTTCCATGATAGGCAAGAGAGAAGACCCAGAGATGTTGTTGTCCTAGTTACAC
ATGGTATTTATTCCAGAGTATTCCTGATGAAATGGTTTAGATGGACATACGAAGAGTTTGA
ATCGTTTACCAATGTTCCTAACGGGAGCGTAATGGTGATGGAACTGGACGAATCCATCAAT
AGATACGTCCTGAGGACCGTGCTACCCAAATGGACTGATTGTGAGGGAGACCTAACTACAT
AGTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGCCATTTTTTTGAGTTATAA
TAATCCTACGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCTTAATACATTCAGACACTT
CTGCGGTATCACCCTACTTATTCCCTTCGAGATTATATCTAGGAACCCATCAGGTTGGTGG
AAGATTACCCGTTCTAAGACTTTTCAGCTTCCTCTATTGATGTTACACCTGGACACCCCTT
TTCTGGCATCCAGTTTTTAATCTTCAGTGGCATGTGAGATTCTCCGAAATTAATTAAAGCA
ATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGACAGGTGGTTTGTTACGCATGC
TAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAACAGGGAATATAAAGGGCAG
CATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTTCTTACGTAAATATT

TTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTTTTCTTGCTTAA
ATCTATAACTACAAAAAACACATACAGGAATTCACCATGGCGTTTACCGATTAAGCACAGT
ACCTTTACGTTATATATAGGATTGGTGTTTAGCTTTTTTTCCTGAGCCCCTGGTTGACTTG
TGCATGAACACGAGCCATTTTTAGTTTGTTTAAGGGAAGTTTTTTGCCACCCAAAACGTTT
AAAGAAGGAAAAGTTGTTTCTTAAACCG

A.1.4. pYX222-CrPv(IRES)-$A_{2A}R$



TTGAGTTATAATAATCCTACGTTAGTGTGAGCGGGATTTAAACTGTGAGGACCTTAATACA
TTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTCGAGATTATATCTAGGAACCCAT
CAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTCAGCTTCCTCTATTGATGTTACACCT
GGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTGGCATGTGAGATTCTCCGAAAT
TAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCGGTTGAAACTGACAGGTGGTTT
GTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGCTCGGCTGCTGTAACAGGGAAT
ATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAACATTTACTATTTTCCCTTCTT
ACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTTCAATTTTTTGTTTGTATTCTT
TTCTTGCTTAAATCTATAACTACAAAAAACACATACAGGAATTCACCATGGAAAGCAAAAA
TGTGATCTTGCTTGTAAATACAATTTTGAGAGGTTAATAAATTACAAGTAGTGCTATTTTT

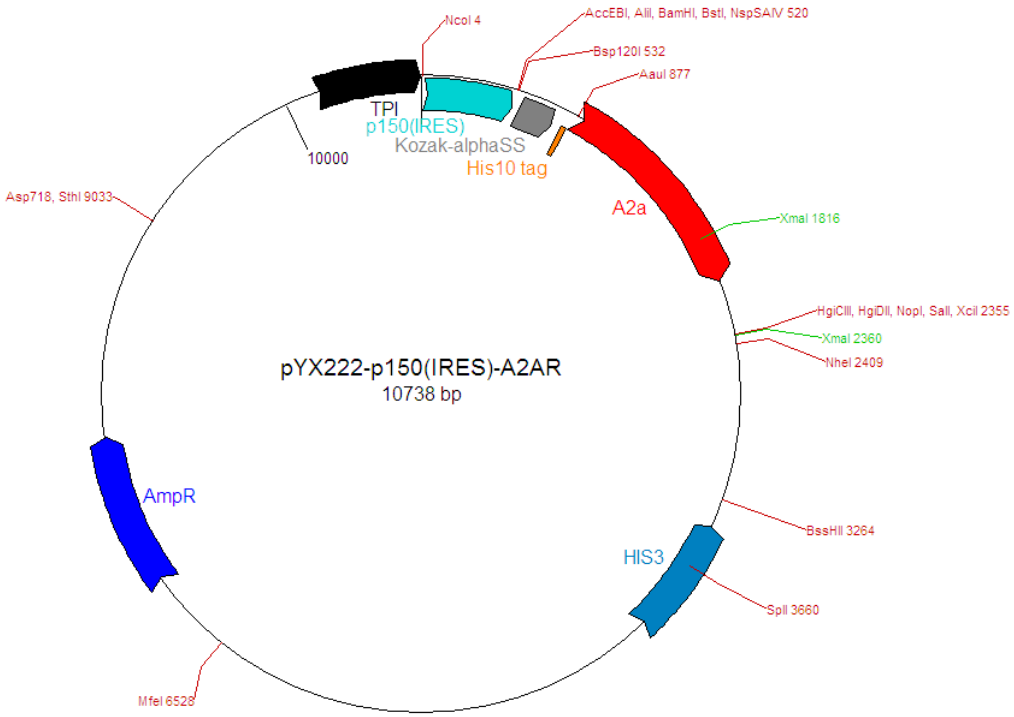
GTATTTAGGTTAGCTATTTAGCTTTACGTTCCAGGATGCCTAGTGGCAGCCCCACAATATC
CAGGAAGCCCTCTCTGCGGTTTTTCAGATTAGGTAGTCGAAAAACCTAAGAAATTTACCTG
GATCCATCCTAGGGCCCACAAGCTTAACAAAATGAGATTTCCTTCAATTTTTACTGCAGTT
TTATTCGCAGCATCCTCCGCATTAGCTGCTCCAGTCAACACTACAACAGAAGATGAAACGG
CACAAATTCCGGCTGAAGCTGTCATCGGTTACTCAGATTTAGAAGGGGATTTCGATGTTGC
TGTTTTGCCATTTTCCAACAGCACAAATAACGGGTTATTGTTTATAAATACTACTATTGCC
AGCATTGCTGCTAAAGAAGAAGGGGTATCTCTCGAGAAAAGAGAGGCTGAAGCTCATCATC
ATCATCATCATCATCATCATCATGAATTCATGCCCATCATGGGCTCCTCGGTGTACATCAC
GGTGGAGCTGGCCATTGCTGTGCTGGCCATCCTGGGCAATGTGCTGGTGTGCTGGGCCGTG
TGGCTCAACAGCAACCTGCAGAACGTCACCAACTACTTTGTGGTGTCACTGGCGGCGGCCG
ACATCGCAGTGGGTGTGCTCGCCATCCCCTTTGCCATCACCATCAGCACCGGGTTCTGCGC
TGCCTGCCACGGCTGCCTCTTCATTGCCTGCTTCGTCCTGGTCCTCACGCAGAGCTCCATC
TTCAGTCTCCTGGCCATCGCCATTGACCGCTACATTGCCATCCGCATCCCGCTCCGGTACA
ATGGCTTGGTGACCGGCACGAGGGCTAAGGGCATCATTGCCATCTGCTGGGTGCTGTCGTT
TGCCATCGGCCTGACTCCCATGCTAGGTTGGAACAACTGCGGTCAGCCAAAGGAGGGCAAG
CAGCACTCCCAGGGCTGCGGGGAGGGCCAAGTGGCCTGTCTCTTTGAGGATGTGGTCCCCA
TGAACTACATGGTGTACTTCAACTTCTTTGCCTGTGTGCTGGTGCCCCTGCTGCTCATGCT
GGGTGTCTATTTGCGGATCTTCCTGGCGGCGCGACGACAGCTGAAGCAGATGGAGAGCCAG
CCTCTGCCGGGGGAGCGGGCACGGTCCACACTGCAGAAGGAGGTCCATGCTGCCAAGTCAC
TGGCCATCATTGTGGGGCTCTTTGCCCTCTGCTGGCTGCCCCTACACATCATCAACTGCTT
CACTTTCTTCTGCCCCGACTGCAGCCACGCCCCTCTCTGGCTCATGTACCTGGCCATCGTC
CTCTCCCACACCAATTCGGTTGTGAATCCCTTCATCTACGCCTACCGTATCCGCGAGTTCC
GCCAGACCTTCCGCAAGATCATTCGCAGCCACGTCCTGAGGCAGCAAGAACCTTTCAAGGC
AGCTGGCACCAGTGCCCGGGTCTTGGCAGCTCATGGCAGTGACGGAGAGCAGGTCAGCCTC
CGTCTCAACGGCCACCCGCCAGGAGTGTGGGCCAACGGCAGTGCTCCCCACCCTGAGCGGA
GGCCCAATGGCTACGCCCTGGGGCTGGTGAGTGGAGGGAGTGCCCAAGAGTCCCAGGGGAA
CACGGGCCTCCCAGACGTGGAGCTCCTTAGCCATGAGCTCAAGGGAGTGTGCCCAGAGCCC
CCTGGCCTAGATGACCCCCTGGCCCAGGATGGAGCAGGAGTGTCCGCGGCCGCTGAAAATC
TGTATTTCCAGAGTGCCGGTAAGGCCGGAGAGGGCGAGATTCCCGCTCCGCTGGCCGGCAC
CGTCTCCAAGATCCTCGTGAAGGAGGGTGACACGGTCAAGGCTGGTCAGACCGTGCTCGTT
CTCGAGGCCATGAAGATGGAGACCGAGATCAACGCTCCCACCGACGGCAAGGTCGAGAAGG
TCCTGGTCAAGGAGCGTGACGCGGTGCAGGGCGGTCAGGGTCTCATCAAGATCGGGTGATC
TAGAGTCGACCCGGGTATCCGTATGATGTGCCTGACTACGCATGATATCTCGAGCTCAGCT
AGCTAACTGAATAAGGAACAATGAACGTTTTTCCTTTCTCTTGTTCCTAGTATTAATGACT
GACCGATACATCCCTTTTTTTTTTTGTCTTTGTCTAGCTCCAATTCGCCCTATAGTGAGTC
GTATTACAATTCACTGGCCGTCGTTTTACAACGTCGTGACTGGGAAAACCCTGGCGTTACC
CAACTTAATCGCCTTGCAGCACATCCCCCTTTCGCCAGCTGGCGTAATAGCGAAGAGGCCC
GCACCGATCGCCCTTCCCAACAGTTGCGCAGCCTGAATGGCGAATGGACGCGCCCTGTAGC
GGCGCATTAAGCGCGGCGGGTGTGGTGGTTACGCGCAGCGTGACCGCTACACTTGCCAGCG
CCCTAGCGCCCGCTCCTTTCGCTTTCTTCCCTTCCTTTCTCGCCACGTTCGCCGGCTTTCC
CCGTCAAGCTCTAAATCGGGGGCTCCCTTTAGGGTTCCGATTTAGTGCTTTACGGCACCTC
GACCCCAAAAAACTTGATTAGGGTGATGGTTCACGTAGTGGGCCATCGCCCTGATAGACGG
TTTTTCGCCCTTTGACGTTGGAGTCCACGTTCTTTAATAGTGGACTCTTGTTCCAAACTGG
AACAACACTCAACCCTATCTCGGTCTATTCTTTTGATTTATAAGGGATTTTGCCGATTTCG
GCCTATTGGTTAAAAAATGAGCTGATTTAACAAAAATTTAACGCGAATTTTAACAAAATAT
TAACGCTTACAATTTCCTGATGCGGTATTTTCTCCTTACGCATCTGTGCGGTATTTCACAC
CGCATAGATCCGTCGAGTTCAAGAGAAAAAAAAAGAAAAAGCAAAAAGAAAAAAGGAAAGC
GCGCCTCGTTCAGAATGACACGTATAGAATGATGCATTACCTTGTCATCTTCAGTATCATA
CTGTTCGTATACATACTTACTGACATTCATAGGTATACATATATACACATGTATATATATC
GTATGCTGCAGCTTTAAATAATCGGTGTCACTACATAAGAACACCTTTGGTGGAGGGAACA
TCGTTGGTTCCATTGGGCGAGGTGGCTTCTCTTATGGCAACCGCAAGAGCCTTGAACGCAC

TCTCACTACGGTGATGATCATTCTTGCCTCGCAGACAATCAACGTGGAGGGTAATTCTGCT
TGCCTCTGCAAAACTTTCAAGAAAATGCGGGATCATCTCGCAAGAGAGATCTCCTACTTTC
TCCCTCTGCAAACCAAGTTCGACAACTGCGTACGGCCTGTTCGAAAGATCTACCACCGCTC
TGGAAAGTGCCTCATCCAAAGGCGCAAATCCTGATCCAAACCTTTTTACTCCACGCACGGC
CCCTAGGGCCTCTTTAAATGCTTGACCGAGAGCAATCCCGCAGTCTTCAGTGGTGTGATGG
TCGTCTATGTGTAAGTCACCAATGCACTCAACGATTAGCGACCAGCCGGAATGCTTGGCCA
GAGCATGTATCATATGGTCCAGAAACCCTATACCTGTGTGGACGTTAATCACTTGCGATTG
TGTGGCCTGTTCTGCTACTGCTTCTGCCTCTTTTTCTGGGAAGATCGAGTGCTCTATCGCT
AGGGGACCACCCTTTAAAGAGATCGCAATCTGAATCTTGGTTTCATTTGTAATACGCTTTA
CTAGGGCTTTCTGCTCTGTCATCTTTGCCTTCGTTTATCTTGCCTGCTCATTTTTTAGTAT
ATTCTTCGAAGAAATCACATTACTTTATATAATGTATAATTCATTATGTGATAATGCCAAT
CGCTAAGAAAAAAAAAAGAGTCATCCGCTAGGGGAAAAAAAAAAATGAAAATCATTACCGAG
GCATAAAAAAATATAGAGTGTACTAGAGGAGGCCAAGAGTAATAGAAAAAGAAAATTGCGG
GAAAGGACTGTGTTATGACTTCCCTGACTAATGCCGTGTTCAAACGATACCTGGCAGTGAC
TCCTAGCGCTCACCAAGCTCTTAAAACGGGAATTTATGGTGCACTCTCAGTACAATCTGCT
CTGATGCCGCATAGTTAAGCCAGCCCCGACACCCGCCAACACCCGCTGACGCGCCCTGACG
GGCTTGTCTGCTCCCGGCATCCGCTTACAGACAAGCTGTGACCGTCTCCGGGAGCTGCATG
TGTCAGAGGTTTTCACCGTCATCACCGAAACGCGCGAGACGAAAGGGCCTCGTGATACGCC
TATTTTTATAGGTTAATGTCATGATAATAATGGTTTCTTAGACGTCGGCCGCTCTAGAAC
TAGTGGATCAATTCCACGGACTATAGACTATACTAGTATACTCCGTCTACTGTACGATACA
CTTCCGCTCAGGTCCTTGTCCTTTAACGAGGCCTTACCACTCTTTTGTTACTCTATTGATC
CAGCTCAGCAAAGGCAGTGTGATCTAAGATTCTATCTTCGCGATGTAGTAAAACTAGCTAG
ACCGAGAAAGAGACTAGAAATGCAAAAGGCACTTCTACAATGGCTGCCATCATTATTATCC
GATGTGACGCTGCAGCTTCTCAATGATATTCGAATACGCTTTGAGGAGATACAGCCTAATA
TCCGACAAACTGTTTTACAGATTTACGATCGTACTTGTTACCCATCATTGAATTTTGAACA
TCCGAACCTGGGAGTTTTCCCTGAAACAGATAGTATATTTGAACCTGTATAATAATATATA
GTCTAGCGCTTTACGGAAGACAATGTATGTATTTCGGTTCCTGGAGAAACTATTGCATCTA
TTGCATAGGTAATCTTGCACGTCGCATCCCCGGTTCATTTTCTGCGTTTCCATCTTGCACT
TCAATAGCATATCTTTGTTAACGAAGCATCTGTGCTTCATTTTGTAGAACAAAAATGCAAC
GCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGAACAGAAATGCA
ACGCGAAAGCGCTATTTTACCAACGAAGAATCTGTGCTTCATTTTTGTAAAACAAAAATGC
AACGCGAGAGCGCTAATTTTTCAAACAAAGAATCTGAGCTGCATTTTTACAGAACAGAAAT
GCAACGCGAGAGCGCTATTTTACCAACAAAGAATCTATACTTCTTTTTTGTTCTACAAAAA
TGCATCCCGAGAGCGCTATTTTTCTAACAAAGCATCTTAGATTACTTTTTTTCTCCTTTGT
GCGCTCTATAATGCAGTCTCTTGATAACTTTTTGCACTGTAGGTCCGTTAAGGTTAGAAGA
AGGCTACTTTGGTGTCTATTTTCTCTTCCATAAAAAAAGCCTGACTCCACTTCCCGCGTTT
ACTGATTACTAGCGAAGCTGCGGGTGCATTTTTTCAAGATAAAGGCATCCCCGATTATATT
CTATACCGATGTGGATTGCGCATACTTTGTGAACAGAAAGTGATAGCGTTGATGATTCTTC
ATTGGTCAGAAAATTATGAACGGTTTCTTCTATTTTGTCTCTATATACTACGTATAGGAAA
TGTTTACATTTTCGTATTGTTTTCGATTCACTCTATGAATAGTTCTTACTACAATTTTTTT
GTCTAAAGAGTAATACTAGAGATAAACATAAAAAATGTAGAGGTCGAGTTTAGATGCAAGT
TCAAGGAGCGAAAGGTGGATGGGTAGGTTATATAGGGATATAGCACAGAGATATATAGCAA
AGAGATACTTTTGAGCAATGTTTGTGGAAGCGGTATTCGCAATATTTTAGTAGCTCGTTAC
AGTCCGGTGCGTTTTTGGTTTTTTGAAAGTGCGTCTTCAGAGCGCTTTTGGTTTTCAAAAG
CGCTCTGAAGTTCCTATACTTTCTAGAGAATAGGAACTTCGGAATAGGAACTTCAAAGCGT
TTCCGAAAACGAGCGCTTCCGAAAATGCAACGCGAGCTGCGCACATACAGCTCACTGTTCA
CGTCGCACCTATATCTGCGTGTTGCCTGTATATATATATACATGAGAAGAACGGCATAGTG
CGTGTTTATGCTTAAATGCGTACTTATATGCGTCTATTTATGTAGGATGAAAGGTAGTCTA
GTACCTCCTGTGATATTATCCCATTCCATGCGGGGTATCGTATGCTTCCTTCAGCACTACC
CTTTAGCTGTTCTATATGCTGCCACTCCTCAATTGGATTAGTCTCATCCTTCAATGCTATC
ATTTCCTTTGATATTGGATCATATGCATAGTACCGAGAAACTAGTGCGAAGTAGTGATCAG

GTATTGCTGTTATCTGATGAGTATACGTTGTCCTGGCCACGGCAGAAGCACGCTTATCGCT
CCAATTTCCCACAACATTAGTCAACTCCGTTAGGCCCTTCATTGAAAGAAATGAGGTCATC
AAATGTCTTCCAATGTGAGATTTTGGGCCATTTTTTATAGCAAAGATTGAATAAGGCGCAT
TTTTCTTCAAAGCTGCGGCCGCACGTCAGGTGGCACTTTTCGGGGAAATGTGCGCGGAACC
CCTATTTGTTTATTTTTCTAAATACATTCAAATATGTATCCGCTCATGAGACAATAACCGT
GATAAATGCTTCAATAATATTGAAAAAGGAAGAGTATGAGTATTCAACATTTCCGTGTCGC
CCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTTTTTGCTCACCCAGAAACGCTGGTG
AAAGTAAAAGATGCTGAAGATCAGTTGGGTGCACGAGTGGGTTACATCGAACTGGATCTCA
ACAGCGGTAAGATCCTTGAGAGTTTTCGCCCCGAAGAACGTTTTCCAATGATGAGCACTTT
TAAAGTTCTGCTATGTGGCGCGGTATTATCCCGTATTGACGCCGGGCAAGAGCAACTCGGT
CGCCGCATACACTATTCTCAGAATGACTTGGTTGAGTACTCACCAGTCACAGAAAAGCATC
TTACGGATGGCATGACAGTAAGAGAATTATGCAGTGCTGCCATAACCATGAGTGATAACAC
TGCGGCCAACTTACTTCTGACAACGATCGGAGGACCGAAGGAGCTAACCGCTTTTTTGCAC
AACATGGGGGATCATGTAACTCGCCTTGATCGTTGGGAACCGGAGCTGAATGAAGCCATAC
CAAACGACGAGCGTGACACCACGATGCCTGTAGCAATGGCAACAACGTTGCGCAAACTATT
AACTGGCGAACTACTTACTCTAGCTTCCCGGCAACAATTAATAGACTGGATGGAGGCGGAT
AAAGTTGCAGGACCACTTCTGCGCTCGGCCCTTCCGGCTGGCTGGTTTATTGCTGATAAAT
CTGGAGCCGGTGAGCGTGGGTCTCGCGGTATCATTGCAGCACTGGGGCCAGATGGTAAGCC
CTCCCGTATCGTAGTTATCTACACGACGGGGAGTCAGGCAACTATGGATGAACGAAATAGA
CAGATCGCTGAGATAGGTGCCTCACTGATTAAGCATTGGTAACTGTCAGACCAAGTTTACT
CATATATACTTTAGATTGATTTAAAACTTCATTTTTAATTTAAAAGGATCTAGGTGAAGAT
CCTTTTTGATAATCTCATGACCAAAATCCCTTAACGTGAGTTTTCGTTCCACTGAGCGTCA
GACCCCGTAGAAAAGATCAAAGGATCTTCTTGAGATCCTTTTTTTCTGCGCGTAATCTGCT
GCTTGCAAACAAAAAAACCACCGCTACCAGCGGTGGTTTGTTTGCCGGATCAAGAGCTACC
AACTCTTTTTCCGAAGGTAACTGGCTTCAGCAGAGCGCAGATACCAAATACTGTTCTTCTA
GTGTAGCCGTAGTTAGGCCACCACTTCAAGAACTCTGTAGCACCGCCTACATACCTCGCTC
TGCTAATCCTGTTACCAGTGGCTGCTGCCAGTGGCGATAAGTCGTGTCTTACCGGGTTGGA
CTCAAGACGATAGTTACCGGATAAGGCGCAGCGGTCGGGCTGAACGGGGGGTTCGTGCACA
CAGCCCAGCTTGGAGCGAACGACCTACACCGAACTGAGATACCTACAGCGTGAGCTATGAG
AAAGCGCCACGCTTCCCGAAGGGAGAAAGGCGGACAGGTATCCGGTAAGCGGCAGGGTCGG
AACAGGAGAGCGCACGAGGGAGCTTCCAGGGGGAAACGCCTGGTATCTTTATAGTCCTGTC
GGGTTTCGCCACCTCTGACTTGAGCGTCGATTTTTGTGATGCTCGTCAGGGGGGCGGAGCC
TATGGAAAAACGCCAGCAACGCGGCCTTTTTACGGTTCCTGGCCTTTTGCTGGCCTTTTGC
TCACATGTTCTTTCCTGCGTTATCCCCTGATTCTGTGGATAACCGTATTACCGCCTTTGAG
TGAGCTGATACCGCTCGCCGCAGCCGAACGACCGAGCGCAGCGAGTCAGTGAGCGAGGAAG
CGGAAGAGCGCCCAATACGCAAACCGCCTCTCCCCGCGCGTTGGCCGATTCATTAATGCAG
CTGGCACGACAGGTTTCCCGACTGGAAAGCGGGCAGTGAGCGCAACGCAATTAATGTGAGT
TAGCTCACTCATTAGGCACCCCAGGCTTTACACTTTATGCTTCCGGCTCGTATGTTGTGTG
GAATTGTGAGCGGATAACAATTTCACACAGGAAACAGCTATGACCATGATTACGCCAAGCT
CGAAATTAACCCTCACTAAAGGGAACAAAAGCTGGTACCGGGCCGGCCGTCGGGCCGTCGA
GCTTGATGGCATCGTGGTGTCACGCTCGTCGTTTGGTATGGCTTCATTCAGCTCCGGTTCC
CAACGATCAAGGCGAGTTACATGATCCCCCATGTTGTGCAAAAAAGCGGTTAGCTCCTTCG
GTCCTCCGATCGTTGTCAGAAGTAAGTTGGCCGCAGTGTTATCACTCATGGTTATGGCAGC
ACTGCATAATTCTCTTACTGTCATGCCATCCGTAAGATGCTTTTCTGTGACTGGTGAGTAC
TCAACCAAGTCATTCTGAGAATAGTGTATGCGGCGACCGAGTTGCTCTTGCCCGGCGTCAA
CACGGGATAATACCGCGCCACATAGCAGAACTTTAAAAGTGCTCATCATTGGAAAACGTTC
TTCGGGGCGAAAACTCTCAAGGATCTTACCGCTGTTGAGATCCAGTTCGATGTAACCCACT
CGTGCACCCAACTGATCTTCAGCATCTTTTACTTTCACCAGCGTTTCTGGGTGAGCAAAAA
CAGGAAGGCAAAATGCCGCAAAAAAGGGAATAAGGGCGACACGGAAATGTTGAATACTCAT
ACTCTTCCTTTTTCAATATTATTGAAGCATTTATCAGGGTTATTGTCTCATGAGCGGATAC
ATATTTGAATGTATTTAGAAAAATAAACAAATAGGGGTTCCGCGCACATTTCCCCGAAAAG

TGCCACCTGACGTCTAAGAAACCATTATTATCATGACATTAACCTATAAAAATAGGCGTAT
CACGAGGCCCTTTCGTCTTCAAGAATTGGGGATCTACGTATGGTCATTTCTTCTTCAGATT
CCCTCATGGAGAAAGTGCGGCAGATGTATATGACAGAGTCGCCAGTTTCCAAGAGACTTTA
TTCAGGCACTTCCATGATAGGCAAGAGAGAAGACCCAGAGATGTTGTTGTCCTAGTTACAC
ATGGTATTTATTCCAGAGTATTCCTGATGAAATGGTTTAGATGGACATACGAAGAGTTTGA
ATCGTTTACCAATGTTCCTAACGGGAGCGTAATGGTGATGGAACTGGACGAATCCATCAAT
AGATACGTCCTGAGGACCGTGCTACCCAAATGGACTGATTGTGAGGGAGACCTAACTACAT
AGTGTTTAAAGATTACGGATATTTAACTTACTTAGAATAATGCCATTTTT

A.1.5. pYX222-p150(IRES)-$A_{2A}R$



TCACCATGGGAAATCATTTTTTGAAGATTACATTAATAAGGCTTTTTTCAATATCTCTGGA
ACAACAGTTTTTTTTTACTTACTAATAGCTTTAAGGACCCTCTTGGACATCATGATGGCAG
ACTTCCATCGTAATGGGATGATCATATGATGGGCGCTATCCTCATCGCGACTCGATAACGA
CGTGAGAAACGATTTTTTTTTTCTTTTTCACCGTATTTTTGTGCGTCCTTTTTCAATTATA
GCTTTTTTTTTATTTTTTTTTTTTCTCGTACTGTTTCACTGACAAAAGTTTTTTTTTCAAG
AAAAATTTTCGATGCCGCGTTCTCTGTGTGCAACGGATGGATGGTAGATGGAATTTCAATA
TGTTGCTTGAAATTTTACCAATCTTGATATTGTGATAATTTACTTAATTATGATTCTTCCT
CTTCCCTTCAATTTCTTAAAGCTTCTTACTTTACTCCTTCTTGCTCATAAATAAGCAAGGT
AAGAGGACAACTGTAATTACCTATTACAATAGGATCCATCCTAGGGCCCACAAGCTTAACA

AAATGAGATTTCCTTCAATTTTTACTGCAGTTTTATTCGCAGCATCCTCCGCATTAGCTGC
TCCAGTCAACACTACAACAGAAGATGAAACGGCACAAATTCCGGCTGAAGCTGTCATCGGT
TACTCAGATTTAGAAGGGGATTTCGATGTTGCTGTTTTGCCATTTTCCAACAGCACAAATA
ACGGGTTATTGTTTATAAATACTACTATTGCCAGCATTGCTGCTAAAGAAGAAGGGGTATC
TCTCGAGAAAAGAGAGGCTGAAGCTCATCATCATCATCATCATCATCATCATCATGAATTC
ATGCCCATCATGGGCTCCTCGGTGTACATCACGGTGGAGCTGGCCATTGCTGTGCTGGCCA
TCCTGGGCAATGTGCTGGTGTGCTGGGCCGTGTGGCTCAACAGCAACCTGCAGAACGTCAC
CAACTACTTTGTGGTGTCACTGGCGGCGGCCGACATCGCAGTGGGTGTGCTCGCCATCCCC
TTTGCCATCACCATCAGCACCGGGTTCTGCGCTGCCTGCCACGGCTGCCTCTTCATTGCCT
GCTTCGTCCTGGTCCTCACGCAGAGCTCCATCTTCAGTCTCCTGGCCATCGCCATTGACCG
CTACATTGCCATCCGCATCCCGCTCCGGTACAATGGCTTGGTGACCGGCACGAGGGCTAAG
GGCATCATTGCCATCTGCTGGGTGCTGTCGTTTGCCATCGGCCTGACTCCCATGCTAGGTT
GGAACAACTGCGGTCAGCCAAAGGAGGGCAAGCAGCACTCCCAGGGCTGCGGGGAGGGCCA
AGTGGCCTGTCTCTTTGAGGATGTGGTCCCCATGAACTACATGGTGTACTTCAACTTCTTT
GCCTGTGTGCTGGTGCCCCTGCTGCTCATGCTGGGTGTCTATTTGCGGATCTTCCTGGCGG
CGCGACGACAGCTGAAGCAGATGGAGAGCCAGCCTCTGCCGGGGGAGCGGGCACGGTCCAC
ACTGCAGAAGGAGGTCCATGCTGCCAAGTCACTGGCCATCATTGTGGGGCTCTTTGCCCTC
TGCTGGCTGCCCCTACACATCATCAACTGCTTCACTTTCTTCTGCCCCGACTGCAGCCACG
CCCCTCTCTGGCTCATGTACCTGGCCATCGTCCTCTCCCACACCAATTCGGTTGTGAATCC
CTTCATCTACGCCTACCGTATCCGCGAGTTCCGCCAGACCTTCCGCAAGATCATTCGCAGC
CACGTCCTGAGGCAGCAAGAACCTTTCAAGGCAGCTGGCACCAGTGCCCGGGTCTTGGCAG
CTCATGGCAGTGACGGAGAGCAGGTCAGCCTCCGTCTCAACGGCCACCCGCCAGGAGTGTG
GGCCAACGGCAGTGCTCCCCACCCTGAGCGGAGGCCCAATGGCTACGCCCTGGGGCTGGTG
AGTGGAGGGAGTGCCCAAGAGTCCCAGGGGAACACGGGCCTCCCAGACGTGGAGCTCC TTA
GCCATGAGCTCAAGGGAGTGTGCCCAGAGCCCCCTGGCCTAGATGACCCCCTGGCCCAGGA
TGGAGCAGGAGTGTCCGCGGCCGCTGAAAATCTGTATTTCCAGAGTGCCGGTAAGGCCGGA
GAGGGCGAGATTCCCGCTCCGCTGGCCGGCACCGTCTCCAAGATCCTCGTGAAGGAGGGTG
ACACGGTCAAGGCTGGTCAGACCGTGCTCGTTCTCGAGGCCATGAAGATGGAGACCGAGAT
CAACGCTCCCACCGACGGCAAGGTCGAGAAGGTCCTGGTCAAGGAGCGTGACGCGGTGCAG
GGCGGTCAGGGTCTCATCAAGATCGGGTGATCTAGAGTCGACCCGGGTATCCGTATGATGT
GCCTGACTACGCATGATATCTCGAGCTCAGCTAGCTAACTGAATAAGGAACAATGAACGTT
TTTCCTTTCTCTTGTTCCTAGTATTAATGACTGACCGATACATCCCTTTTTTTTTTTGTCT
TTGTCTAGCTCCAATTCGCCCTATAGTGAGTCGTATTACAATTCACTGGCCGTCGTTTTAC
AACGTCGTGACTGGGAAAACCCTGGCGTTACCCAACTTAATCGCCTTGCAGCACATCCCCC
TTTCGCCAGCTGGCGTAATAGCGAAGAGGCCCGCACCGATCGCCCTTCCCAACAGTTGCGC
AGCCTGAATGGCGAATGGACGCGCCCTGTAGCGGCGCATTAAGCGCGGCGGGTGTGGTGGT
TACGCGCAGCGTGACCGCTACACTTGCCAGCGCCCTAGCGCCCGCTCCTTTCGCTTTCTTC
CCTTCCTTTCTCGCCACGTTCGCCGGCTTTCCCCGTCAAGCTCTAAATCGGGGGCTCCCTT
TAGGGTTCCGATTTAGTGCTTTACGGCACCTCGACCCCAAAAAACTTGATTAGGGTGATGG
TTCACGTAGTGGGCCATCGCCCTGATAGACGGTTTTTCGCCCTTTGACGTTGGAGTCCACG
TTCTTTAATAGTGGACTCTTGTTCCAAACTGGAACAACACTCAACCCTATCTCGGTCTATT
CTTTTGATTTATAAGGGATTTTGCCGATTTCGGCCTATTGGTTAAAAAATGAGCTGATTTA
ACAAAAATTTAACGCGAATTTTAACAAAATATTAACGCTTACAATTTCCTGATGCGGTATT
TTCTCCTTACGCATCTGTGCGGTATTTCACACCGCATAGATCCGTCGAGTTCAAGAGAAAA
AAAAAGAAAAAGCAAAAAGAAAAAAGGAAAGCGCGCCTCGTTCAGAATGACACGTATAGAA
TGATGCATTACCTTGTCATCTTCAGTATCATACTGTTCGTATACATACTTACTGACATTCA
TAGGTATACATATATACACATGTATATATATCGTATGCTGCAGCTTTAAATAATCGGTGTC
ACTACATAAGAACACCTTTGGTGGAGGGAACATCGTTGGTTCCATTGGGCGAGGTGGCTTC
TCTTATGGCAACCGCAAGAGCCTTGAACGCACTCTCACTACGGTGATGATCATTCTTGCCT
CGCAGACAATCAACGTGGAGGGTAATTCTGCTTGCCTCTGCAAAACTTTCAAGAAAATGCG
GGATCATCTCGCAAGAGAGATCTCCTACTTTCTCCCTCTGCAAACCAAGTTCGACAACTGC

GTACGGCCTGTTCGAAAGATCTACCACCGCTCTGGAAAGTGCCTCATCCAAAGGCGCAAAT
CCTGATCCAAACCTTTTTACTCCACGCACGGCCCCTAGGGCCTCTTTAAATGCTTGACCGA
GAGCAATCCCGCAGTCTTCAGTGGTGTGATGGTCGTCTATGTGTAAGTCACCAATGCACTC
AACGATTAGCGACCAGCCGGAATGCTTGGCCAGAGCATGTATCATATGGTCCAGAAACCCT
ATACCTGTGTGGACGTTAATCACTTGCGATTGTGTGGCCTGTTCTGCTACTGCTTCTGCCT
CTTTTTCTGGGAAGATCGAGTGCTCTATCGCTAGGGGACCACCCTTTAAAGAGATCGCAAT
CTGAATCTTGGTTTCATTTGTAATACGCTTTACTAGGGCTTTCTGCTCTGTCATCTTTGCC
TTCGTTTATCTTGCCTGCTCATTTTTTAGTATATTCTTCGAAGAAATCACATTACTTTATA
TAATGTATAATTCATTATGTGATAATGCCAATCGCTAAGAAAAAAAAAGAGTCATCCGCTA
GGGGAAAAAAAAAAATGAAAATCATTACCGAGGCATAAAAAAATATAGAGTGTACTAGAGG
AGGCCAAGAGTAATAGAAAAAGAAAATTGCGGGAAAGGACTGTGTTATGACTTCCCTGACT
AATGCCGTGTTCAAACGATACCTGGCAGTGACTCCTAGCGCTCACCAAGCTCTTAAAACGG
GAATTTATGGTGCACTCTCAGTACAATCTGCTCTGATGCCGCATAGTTAAGCCAGCCCCGA
CACCCGCCAACACCCGCTGACGCGCCCTGACGGGCTTGTCTGCTCCCGGCATCCGCTTACA
GACAAGCTGTGACCGTCTCCGGGAGCTGCATGTGTCAGAGGTTTTCACCGTCATCACCGAA
ACGCGCGAGACGAAAGGGCCTCGTGATACGCCTATTTTTATAGGTTAATGTCATGATAATA
ATGGTTTCTTAGACGTCGGCCGCTCTAGAACTAGTGGATCAATTCCACGGACTATAGACT
ATACTAGTATACTCCGTCTACTGTACGATACACTTCCGCTCAGGTCCTTGTCCTTTAACGA
GGCCTTACCACTCTTTTGTTACTCTATTGATCCAGCTCAGCAAAGGCAGTGTGATCTAAGA
TTCTATCTTCGCGATGTAGTAAAACTAGCTAGACCGAGAAAGAGACTAGAAATGCAAAAGG
CACTTCTACAATGGCTGCCATCATTATTATCCGATGTGACGCTGCAGCTTCTCAATGATAT
TCGAATACGCTTTGAGGAGATACAGCCTAATATCCGACAAACTGTTTTACAGATTTACGAT
CGTACTTGTTACCCATCATTGAATTTTGAACATCCGAACCTGGGAGTTTTCCCTGAAACAG
ATAGTATATTTGAACCTGTATAATAATATATAGTCTAGCGCTTTACGGAAGACAATGTATG
TATTTCGGTTCCTGGAGAAACTATTGCATCTATTGCATAGGTAATCTTGCACGTCGCATCC
CCGGTTCATTTTCTGCGTTTCCATCTTGCACTTCAATAGCATATCTTTGTTAACGAAGCAT
CTGTGCTTCATTTTGTAGAACAAAAATGCAACGCGAGAGCGCTAATTTTTCAAACAAAGAA
TCTGAGCTGCATTTTTACAGAACAGAAATGCAACGCGAAAGCGCTATTTTACCAACGAAGA
ATCTGTGCTTCATTTTTGTAAAACAAAAATGCAACGCGAGAGCGCTAATTTTTCAAACAAA
GAATCTGAGCTGCATTTTTACAGAACAGAAATGCAACGCGAGAGCGCTATTTTACCAACAA
AGAATCTATACTTCTTTTTTGTTCTACAAAAATGCATCCCGAGAGCGCTATTTTTCTAACA
AAGCATCTTAGATTACTTTTTTTCTCCTTTGTGCGCTCTATAATGCAGTCTCTTGATAACT
TTTTGCACTGTAGGTCCGTTAAGGTTAGAAGAAGGCTACTTTGGTGTCTATTTTCTCTTCC
ATAAAAAAAGCCTGACTCCACTTCCCGCGTTTACTGATTACTAGCGAAGCTGCGGGTGCAT
TTTTTCAAGATAAAGGCATCCCCGATTATATTCTATACCGATGTGGATTGCGCATACTTTG
TGAACAGAAAGTGATAGCGTTGATGATTCTTCATTGGTCAGAAAATTATGAACGGTTTCTT
CTATTTTGTCTCTATATACTACGTATAGGAAATGTTTACATTTTCGTATTGTTTTCGATTC
ACTCTATGAATAGTTCTTACTACAATTTTTTTGTCTAAAGAGTAATACTAGAGATAAACAT
AAAAAATGTAGAGGTCGAGTTTAGATGCAAGTTCAAGGAGCGAAAGGTGGATGGGTAGGTT
ATATAGGGATATAGCACAGAGATATATAGCAAAGAGATACTTTTGAGCAATGTTTGTGGAA
GCGGTATTCGCAATATTTTAGTAGCTCGTTACAGTCCGGTGCGTTTTTGGTTTTTTGAAAG
TGCGTCTTCAGAGCGCTTTTGGTTTTCAAAAGCGCTCTGAAGTTCCTATACTTTCTAGAGA
ATAGGAACTTCGGAATAGGAACTTCAAAGCGTTTCCGAAAACGAGCGCTTCCGAAAATGCA
ACGCGAGCTGCGCACATACAGCTCACTGTTCACGTCGCACCTATATCTGCGTGTTGCCTGT
ATATATATATACATGAGAAGAACGGCATAGTGCGTGTTTATGCTTAAATGCGTACTTATAT
GCGTCTATTTATGTAGGATGAAAGGTAGTCTAGTACCTCCTGTGATATTATCCCATTCCAT
GCGGGGTATCGTATGCTTCCTTCAGCACTACCCTTTAGCTGTTCTATATGCTGCCACTCCT
CAATTGGATTAGTCTCATCCTTCAATGCTATCATTTCCTTTGATATTGGATCATATGCATA
GTACCGAGAAACTAGTGCGAAGTAGTGATCAGGTATTGCTGTTATCTGATGAGTATACGTT
GTCCTGGCCACGGCAGAAGCACGCTTATCGCTCCAATTTCCCACAACATTAGTCAACTCCG
TTAGGCCCTTCATTGAAAGAAATGAGGTCATCAAATGTCTTCCAATGTGAGATTTTGGGCC

ATTTTTTATAGCAAAGATTGAATAAGGCGCATTTTTCTTCAAAGCTGCGGCCGCACGTCAG
GTGGCACTTTTCGGGGAAATGTGCGCGGAACCCCTATTTGTTTATTTTTCTAAATACATTC
AAATATGTATCCGCTCATGAGACAATAACCGTGATAAATGCTTCAATAATATTGAAAAAGG
AAGAGTATGAGTATTCAACATTTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCC
TTCCTGTTTTTGCTCACCCAGAAACGCTGGTGAAAGTAAAAGATGCTGAAGATCAGTTGGG
TGCACGAGTGGGTTACATCGAACTGGATCTCAACAGCGGTAAGATCCTTGAGAGTTTTCGC
CCCGAAGAACGTTTTCCAATGATGAGCACTTTTAAAGTTCTGCTATGTGGCGCGGTATTAT
CCCGTATTGACGCCGGGCAAGAGCAACTCGGTCGCCGCATACACTATTCTCAGAATGACTT
GGTTGAGTACTCACCAGTCACAGAAAAGCATCTTACGGATGGCATGACAGTAAGAGAATTA
TGCAGTGCTGCCATAACCATGAGTGATAACACTGCGGCCAACTTACTTCTGACAACGATCG
GAGGACCGAAGGAGCTAACCGCTTTTTTGCACAACATGGGGGATCATGTAACTCGCCTTGA
TCGTTGGGAACCGGAGCTGAATGAAGCCATACCAAACGACGAGCGTGACACCACGATGCCT
GTAGCAATGGCAACAACGTTGCGCAAACTATTAACTGGCGAACTACTTACTCTAGCTTCCC
GGCAACAATTAATAGACTGGATGGAGGCGGATAAAGTTGCAGGACCACTTCTGCGCTCGGC
CCTTCCGGCTGGCTGGTTTATTGCTGATAAATCTGGAGCCGGTGAGCGTGGGTCTCGCGGT
ATCATTGCAGCACTGGGGCCAGATGGTAAGCCCTCCCGTATCGTAGTTATCTACACGACGG
GGAGTCAGGCAACTATGGATGAACGAAATAGACAGATCGCTGAGATAGGTGCCTCACTGAT
TAAGCATTGGTAACTGTCAGACCAAGTTTACTCATATATACTTTAGATTGATTTAAAACTT
CATTTTTAATTTAAAAGGATCTAGGTGAAGATCCTTTTTGATAATCTCATGACCAAAATCC
CTTAACGTGAGTTTTCGTTCCACTGAGCGTCAGACCCCGTAGAAAAGATCAAAGGATCTTC
TTGAGATCCTTTTTTTTCTGCGCGTAATCTGCTGCTTGCAAACAAAAAAACCACCGCTACCA
GCGGTGGTTTGTTTGCCGGATCAAGAGCTACCAACTCTTTTTCCGAAGGTAACTGGCTTCA
GCAGAGCGCAGATACCAAATACTGTTCTTCTAGTGTAGCCGTAGTTAGGCCACCACTTCAA
GAACTCTGTAGCACCGCCTACATACCTCGCTCTGCTAATCCTGTTACCAGTGGCTGCTGCC
AGTGGCGATAAGTCGTGTCTTACCGGGTTGGACTCAAGACGATAGTTACCGGATAAGGCGC
AGCGGTCGGGCTGAACGGGGGGTTCGTGCACACAGCCCAGCTTGGAGCGAACGACCTACAC
CGAACTGAGATACCTACAGCGTGAGCTATGAGAAAGCGCCACGCTTCCCGAAGGGAGAAAG
GCGGACAGGTATCCGGTAAGCGGCAGGGTCGGAACAGGAGAGCGCACGAGGGAGCTTCCAG
GGGGAAACGCCTGGTATCTTTATAGTCCTGTCGGGTTTCGCCACCTCTGACTTGAGCGTCG
ATTTTTGTGATGCTCGTCAGGGGGGCGGAGCCTATGGAAAAACGCCAGCAACGCGGCCTTT
TTACGGTTCCTGGCCTTTTGCTGGCCTTTTGCTCACATGTTCTTTCCTGCGTTATCCCCTG
ATTCTGTGGATAACCGTATTACCGCCTTTGAGTGAGCTGATACCGCTCGCCGCAGCCGAAC
GACCGAGCGCAGCGAGTCAGTGAGCGAGGAAGCGGAAGAGCGCCCAATACGCAAACCGCCT
CTCCCCGCGCGTTGGCCGATTCATTAATGCAGCTGGCACGACAGGTTTCCCGACTGGAAAG
CGGGCAGTGAGCGCAACGCAATTAATGTGAGTTAGCTCACTCATTAGGCACCCCAGGCTTT
ACACTTTATGCTTCCGGCTCGTATGTTGTGTGGAATTGTGAGCGGATAACAATTTCACACA
GGAAACAGCTATGACCATGATTACGCCAAGCTCGAAATTAACCCTCACTAAAGGGAACAAA
AGCTGGTACCGGGCCGGCCGTCGGGCCGTCGAGCTTGATGGCATCGTGGTGTCACGCTCGT
CGTTTGGTATGGCTTCATTCAGCTCCGGTTCCCAACGATCAAGGCGAGTTACATGATCCCC
CATGTTGTGCAAAAAAGCGGTTAGCTCCTTCGGTCCTCCGATCGTTGTCAGAAGTAAGTTG
GCCGCAGTGTTATCACTCATGGTTATGGCAGCACTGCATAATTCTCTTACTGTCATGCCAT
CCGTAAGATGCTTTTCTGTGACTGGTGAGTACTCAACCAAGTCATTCTGAGAATAGTGTAT
GCGGCGACCGAGTTGCTCTTGCCCGGCGTCAACACGGGATAATACCGCGCCACATAGCAGA
ACTTTAAAAGTGCTCATCATTGGAAAACGTTCTTCGGGGCGAAAACTCTCAAGGATCTTAC
CGCTGTTGAGATCCAGTTCGATGTAACCCACTCGTGCACCCAACTGATCTTCAGCATCTTT
TACTTTCACCAGCGTTTCTGGGTGAGCAAAAACAGGAAGGCAAAATGCCGCAAAAAAGGGA
ATAAGGGCGACACGGAAATGTTGAATACTCATACTCTTCCTTTTTCAATATTATTGAAGCA
TTTATCAGGGTTATTGTCTCATGAGCGGATACATATTTGAATGTATTTAGAAAAATAAACA
AATAGGGGTTCCGCGCACATTTCCCCGAAAAGTGCCACCTGACGTCTAAGAAACCATTATT
ATCATGACATTAACCTATAAAAATAGGCGTATCACGAGGCCCTTTCGTCTTCAAGAATTGG
GGATCTACGTATGGTCATTTCTTCTTCAGATTCCCTCATGGAGAAAGTGCGGCAGATGTAT

ATGACAGAGTCGCCAGTTTCCAAGAGACTTTATTCAGGCACTTCCATGATAGGCAAGAGAG
AAGACCCAGAGATGTTGTTGTCCTAGTTACACATGGTATTTATTCCAGAGTATTCCTGATG
AAATGGTTTAGATGGACATACGAAGAGTTTGAATCGTTTACCAATGTTCCTAACGGGAGCG
TAATGGTGATGGAACTGGACGAATCCATCAATAGATACGTCCTGAGGACCGTGCTACCCAA
ATGGACTGATTGTGAGGGAGACCTAACTACATAGTGTTTAAAGATTACGGATATTTAACTT
ACTTAGAATAATGCCATTTTTTTGAGTTATAATAATCCTACGTTAGTGTGAGCGGGATTTA
AACTGTGAGGACCTTAATACATTCAGACACTTCTGCGGTATCACCCTACTTATTCCCTTCG
AGATTATATCTAGGAACCCATCAGGTTGGTGGAAGATTACCCGTTCTAAGACTTTTCAGCT
TCCTCTATTGATGTTACACCTGGACACCCCTTTTCTGGCATCCAGTTTTTAATCTTCAGTG
GCATGTGAGATTCTCCGAAATTAATTAAAGCAATCACACAATTCTCTCGGATACCACCTCG
GTTGAAACTGACAGGTGGTTTGTTACGCATGCTAATGCAAAGGAGCCTATATACCTTTGGC
TCGGCTGCTGTAACAGGGAATATAAAGGGCAGCATAATTTAGGAGTTTAGTGAACTTGCAA
CATTTACTATTTTCCCTTCTTACGTAAATATTTTTCTTTTTAATTCTAAATCAATCTTTTT
CAATTTTTTGTTTGTATTCTTTTCTTGCTTAAATCTATAACTACAAAAAACACATACAGGA
AT

A.2. IRES sequences

A.2.1. *YAP1* IRES

GCGTTTACCGATTAAGCACAGTACCTTTACGTTATATATAGGATTGGTGTTTAGCTTTTTT
TCCTGAGCCCCTGGTTGACTTGTGCATGAACACGAGCCATTTTTAGTTTGTTTAAGGGAAG
TTTTTTGCCACCCAAAACGTTTAAAGAAGGAAAAGTTGTTTCTTAAACCG

A.2.2. p150 IRES

GAAATCATTTTTTGAAGATTACATTAATAAGGCTTTTTTCAATATCTCTGGAACAACAGTT
TTTTTTTACTTACTAATAGCTTTAAGGACCCTCTTGGACATCATGATGGCAGACTTCCATC
GTAATGGGATGATCATATGATGGGCGCTATCCTCATCGCGACTCGATAACGACGTGAGAAA
CGATTTTTTTTTTCTTTTTCACCGTATTTTTGTGCGTCCTTTTTCAATTATAGCTTTTTTT
TTATTTTTTTTTTTTCTCGTACTGTTTCACTGACAAAAGTTTTTTTTTCAAGAAAAATTTT
CGATGCCGCGTTCTCTGTGTGCAACGGATGGATGGTAGATGGAATTTCAATATGTTGCTTG
AAATTTTACCAATCTTGATATTGTGATAATTTACTTAATTATGATTCTTCCTCTTCCCTTC
AATTTCTTAAAGCTTCTTACTTTACTCCTTCTTGCTC

A.2.3 CrPV IRES

AAAGCAAAAATGTGATCTTGCTTGTAAATACAATTTTGAGAGGTTAATAAATTACAAGTAG
TGCTATTTTTGTATTTAGGTTAGCTATTTAGCTTTACGTTCCAGGATGCCTAGTGGCAGCC
CCACAATATCCAGGAAGCCCTCTCTGCGGTTTTTCAGATTAGGTAGTCGAAAAACCTAAGA
AATTTACCT

A.2.3.1 Synthesisied CrPV(IRES) insert (GeneArt, Life Technologies)

SpeI-CrPV(IRES)-NcoI

ACTAGTAAAGCAAAAATGTGATCTTGCTTGTAAATACAATTTTGAGAGGTTA
ATAAATTACAAGTAGTGCTATTTTTGTATTTAGGTTAGCTATTTAGCTTTAC
GTTCCAGGATGCCTAGTGGCAGCCCCACAATATCCAGGAAGCCCTCTCTGCG
GTTTTTCAGATTAGGTAGTCGAAAAACCTAAGAAATTTACCTCCATGG

A.3 Publications

Darby, R.A.J., Cartwright, S.P., Dilworth, M.V., Bill, R.M., 2012. Which yeast species shall I choose? *Saccharomyces cerevisiae* versus *Pichia pastoris* (review). Methods Mol. Biol. 866, 11–23.

Bawa, Z., Bland, C.E., Bonander, N., Bora, N., Cartwright, S.P., Clare, M., Conner, M.T., Darby, R.A.J., Dilworth, M.V., Holmes, W.J., Jamshad, M., Routledge, S.J., Gross, S.R., Bill, R.M., 2011. Understanding the yeast host cell response to recombinant membrane protein production. Biochem. Soc. Trans. 39, 719–723.