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On the use of *Pichia pastoris* for isotopic labeling of human GPCRs for NMR studies

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Abstract

NMR studies of human integral membrane proteins provide unique opportunities to probe structure and dynamics at specific locations and on multiple timescales, often with significant implications for disease mechanism and drug development. Since membrane proteins such as G protein-coupled receptors (GPCRs) are highly dynamic and regulated by ligands or other perturbations, NMR methods are potentially well suited to answer basic functional questions (such as addressing the biophysical basis of ligand efficacy) as well as guiding applications (such as novel ligand design). However, such studies on eukaryotic membrane proteins have often been limited by the inability to incorporate optimal isotopic labels for NMR methods developed for large protein/lipid complexes, including methyl TROSY. We review the different expression systems for production of isotopically labeled membrane proteins and highlight the use of the yeast *Pichia pastoris* to achieve perdeuteration and ¹³C methyl probe incorporation within isoleucine sidechains. We further illustrate the use of this method for labeling of several biomedically significant GPCRs.

Keywords

Pichia pastoris; stable isotope labeling; deuteration; solution NMR; GPCR; integral membrane proteins

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Introduction

Membrane proteins constitute a third of the human proteome and play key roles in diverse aspects of physiology and disease (Almén et al. 2009). Because they require a lipid environment or membrane mimetic to maintain native-like structure and function, integral membrane proteins are particularly challenging targets for structural and biophysical studies. Structures solved by crystallography and cryo-EM have provided molecular details of how these proteins bind to natural and artificial ligands and respond to changes in their environment. Despite these advances, most membrane proteins have remained difficult to study by NMR spectroscopy due to their high molecular weight and difficulties of producing samples labeled with specific ^2H and ^{13}C patterns that facilitate such studies. While some membrane proteins can be natively expressed or refolded from prokaryotic systems where such isotopic labeling is well-established (Casiraghi et al. 2016; Hiller et al. 2008; Park et al. 2012), most eukaryotic membrane proteins require a eukaryotic expression host for proper translation, folding, and stability.

One such expression host is the methylotrophic yeast *Pichia pastoris* ('*Pichia*'), which has been widely used to express a wide variety of proteins, including human membrane proteins (Byrne 2015). *Pichia* contains the machinery required for important post-translational modifications such as disulfide formation and glycosylation, as well as appropriate chaperones to facilitate protein folding (Cereghino and Cregg 2000). Further, *Pichia*-based overexpression is efficient enough to produce a range of human integral membrane proteins at levels sufficient for structural studies by X-ray crystallography and cryo-EM. Some examples are H_1 histamine receptor (Shimamura et al. 2011), $\text{A}_{2\text{A}}$ adenosine receptor (Hino et al. 2012), ABC G5G8 sterol transporter (Lee et al. 2016), GIRK2 potassium channel (Whorton and MacKinnon 2011), Orai store-operated calcium channel (Hou et al. 2012), P-glycoprotein (Jin et al. 2012), TPC potassium channel (Guo et al. 2016), and TAP transporter (Oldham et al. 2016).

Pichia has also proven valuable for production of isotopically labeled samples for NMR spectroscopy. The first successful demonstration of uniform $^{13}\text{C}/^{15}\text{N}$ labeling in *Pichia* was carried out for the tick anticoagulant peptide (Laroche et al. 1994), and subsequently for a number of aqueous-soluble, low-MW targets. Membrane proteins are often inherently larger on their own and even further when considering the micelle or membrane mimetic required for purification in a functional state, with accompanying slow rotational tumbling and broad NMR signals. Coupled with the poor chemical shift dispersion and substantial peak overlap associated with NMR spectra of many multiple transmembrane (TM) helix proteins (Kim et al. 2009), these considerations require sophisticated isotopic labeling patterns involving deuteration and site-specific isotope incorporation (e.g. methyl labeling) to facilitate NMR studies of these proteins.

In this brief communication, we summarize recent applications of isotopic labeling of membrane proteins in several types of eukaryotic expression hosts, including insect cells, mammalian cells, and yeast. We particularly focus on the yeast *Pichia pastoris*, and detail the general strengths and weaknesses of its use for membrane protein expression for NMR studies. Additionally, we include further background on our recently-published methyl

labeling approach in *Pichia* (Clark et al. 2015; Clark et al. 2017) and include new examples of spectra collected on labeled wild-type human GPCRs produced from this system.

Materials and Methods

Construct design

cDNAs for human CB1 (residues 74–430) and human OX₂R (residues 1–386) were cloned into the MFαOpt vector described previously (Clark et al. 2017) containing a C-terminal 10x-His tag for affinity purification. Plasmids were linearized with *PmeI* (NEB) and 5–10 µg of linearized DNA was inserted via electroporation (2.00 kV pulse) into freshly-prepared competent KM71H (Mut^s phenotype) *Pichia pastoris* cells (Invitrogen). Immediately following electroporation, 1 mL of ice-cold 1M sorbitol was added to the cuvette, and cells were transferred to a sterile culture tube and incubated at 28 °C for 60 minutes. Following incubation, 1 mL of YPD (1% yeast extract, 2% peptone, 2% dextrose) was added to each tube and cultures were shaken at 200 rpm for 4 hours at 28 °C to recover. Cells were plated on YPDS (1% yeast extract, 2% peptone, 2% dextrose, 2% agar, 1M sorbitol) agar plates containing increasing amounts of Zeocin (Invitrogen; typically 100–1000 µg/mL) and individual clones were screened for expression through western blots using an anti-His₆ antibody (Pierce). Of note, clones from the KM71H cell line gave consistently higher expression levels for all three receptors compared to GS115 and SMD1163 cell lines (data not shown). The best expressing clones were stored as 20% glycerol stocks at –80 °C.

Expression of deuterated cultures

Protein expression and culture adaptation was carried out as described for the A_{2A} receptor (Clark et al. 2017) with a few modifications. Briefly, a loopful of freshly streaked cells were inoculated into a sterile 50 mL volume of BMG (1% glycerol, 100 mM potassium phosphate pH 6.0, 1.34% YNB (yeast nitrogen base), 0.004% histidine, 4 × 10^{–5}% biotin) containing 90% D₂O/10% H₂O (Cambridge Isotope Laboratories). The 50 mL culture was shaken at 28° C until an OD₆₀₀ of ~10 was reached. At this point, 200 µL of the 90%/10% culture was inoculated into 50 mL of BMG made with 100% D₂O. The 50 mL culture was shaken at 250 rpm at 28° C until an OD₆₀₀ of ~10 was reached, and then 200 µL of that culture was used to inoculate 50 mL of BMG made with 100% D₂O and d₈-glycerol (Cambridge Isotope Laboratories). The culture was shaken until reaching an OD₆₀₀ of ~10, and then was used in its entirety to inoculate large scale cultures (typically 1–2 L) of identical media. The large-scale cultures were shaken until saturation (OD₆₀₀ of 20–30), and then spun down in sterile bottles and the media was removed. The cells were resuspended in BMM (100 mM potassium phosphate pH 6.0, 1.34% YNB, 0.004% histidine, 4 × 10^{–5}% biotin) made in 100% D₂O without methanol. The cells continued to shake at saturation density (OD₆₀₀ of 20–30) for ~12 hours to metabolize residual d₈-glycerol. One hour before induction, 200 mg/L of labeled α-ketobutyric acid (methyl-¹³C, 99%; 3,3-D₂, 98%; Cambridge Isotope Laboratories) was added to the culture. Immediately prior to induction, 500 nM taranabant (MedChemExpress) or 5 µM suvorexant (Adooq Biosciences) were added to CB1 or OX₂R cultures, respectively. Expression was induced by adding 0.5% d₄-methanol (Cambridge Isotope Laboratories) and the temperature was reduced to 20° C. Additional aliquots of 0.5% d₄-methanol were added every 12 hours, and cells were harvested after 36 hours and stored

at -80°C . Following the slow deuteration adaptation procedure as described should help reduce cellular stress from adaptation and increase the final overall deuteration level by minimizing contamination from ^1H -containing media and metabolites into later cultures.

Receptor purification

Deuterated CB1 and OX₂R cultures were purified in an identical manner to A_{2A} (Clark et al. 2017), only substituting the ligand present throughout the purification with 1 μM taranabant or 5 μM suvorexant for CB1 or OX₂R, respectively. IMAC-purified samples were injected on a Superdex200 column (GE Healthcare) equilibrated in D₂O containing 150 mM NaCl, 20 mM HEPES pH 7.5, 0.05% deuterated DDM (Anatrace), and 1 μM taranabant or 5 μM suvorexant (Fig. 1). Fractions containing receptor were collected and concentrated immediately prior to NMR experiments. Final yields ranged from 0.3–0.5 mg of purified receptor per liter of deuterated culture.

NMR experiments

Receptor samples were concentrated to a volume of $\sim 100\ \mu\text{L}$ and loaded into 3 mm Shigemi tubes. Final concentrations ranged from $\sim 30\ \mu\text{M}$ (CB1) to $\sim 100\ \mu\text{M}$ (OX₂R and A_{2A}). NMR spectra were collected at 30°C using a Bruker AVANCE III HD 800 MHz spectrometer with a cryogenically-cooled TCI probe. $^1\text{H}/^{13}\text{C}$ -HMQC spectra of the methyl region centered at 14 ppm in the ^{13}C dimension were collected with a ^{13}C spectral width of 30 ppm for CB1 and OX₂R and 22 ppm for A_{2A}. In all cases, 32 complex points were collected in the ^{13}C dimension. HMQC spectra for CB1 and OX₂R were collected as sequential experiments with 64 scans per t_1 point ($\sim 1.25\ \text{hr}$); peak intensities and locations in these spectra remained consistent for the first four experiments, so these were summed and processed with NMRpipe (Delaglio et al. 1995) and analyzed with NMRViewJ (Johnson 2004). For both proteins, peak intensities greatly decreased after four 2D spectra were acquired (ca. 5 hr), leaving only few peaks above the noise level. For A_{2A}, while the spectra remained consistent and free of extensive signal loss for a longer time ($\sim 12\ \text{hr}$), a single 2.5 hr HMQC experiment sufficed to generate enough signal-to-noise for a high quality spectrum due to higher starting concentration. All spectra were processed without linear prediction in the indirect dimension to optimize signal-to-noise ratio.

Membrane protein labeling in insect cells

Spodoptera frugiperda ('insect cells' or Sf9) remains the most common expression system used for GPCRs and other membrane proteins (Massotte 2003), with a number of routes developed to facilitate isotopic labeling for NMR studies (Franke et al. 2018). Simplest among these is supplementation of growth media with ^{13}C -labeled amino acids (e.g. methionine), enabling 80–90% labeling efficiencies, as demonstrated for NMR studies of the β_2 -adrenergic receptor (Kofuku et al. 2012; Kofuku et al. 2018; Nygaard et al. 2013). Similarly, insect cells will uptake deuterated amino acids from (protonated) growth media and incorporate them into recombinant proteins albeit with backprotonation at certain sites (Kofuku et al. 2014; Kofuku et al. 2018). Such samples have been used to probe the conformational equilibrium of the β_2 -adrenergic receptor (Kofuku et al. 2014), the μ -opioid receptor (Okude et al. 2015), and the P2X₄ purinergic receptor (Minato et al. 2016) in various membrane mimetics. As an alternative to ^{13}C labeling, several groups have

successfully carried out ^{15}N labeling in insect cells (Brüggert et al. 2003; Gossert et al. 2011; Strauss et al. 2003; Strauss et al. 2005). Isogai *et. al.* produced the first backbone NMR spectra of a GPCR with the addition of ^{15}N -valine (Isogai et al. 2016). Finally, uniformly labeled samples can be purified from insect cells grown in media formulated with “homemade” yeast extract from labeled *Pichia pastoris* cultures (Opitz et al. 2015). It is likely that further developments in media formulation will permit other isotopic labeling schemes and broaden the utility of insect cells for NMR sample preparation.

Membrane protein labeling in mammalian cells

Some eukaryotic membrane proteins require a mammalian expression host for efficient overexpression. Like insect cells, mammalian cells are unable to efficiently grow in minimal media or media containing high concentrations of D_2O (Murphy et al. 1977), complicating attempts at isotopic labeling. Further, low yields of purified protein can make NMR samples prohibitively expensive. Nevertheless, specifically-labeled amino acids can be taken up by mammalian cells and incorporated into recombinant proteins, exemplified by NMR analyses of rhodopsin from a stable HEK293 cell line (Ahuja et al. 2009; Dutta et al. 2012; Goncalves et al. 2010; Werner et al. 2007). Depending on the formulation of the amino acid lysate added to the culture media, isotopic labeling efficiency can reach up to 90% as determined by FTIR spectroscopy (Egorova-Zachernyuk et al. 2010). While cost concerns have limited the use of this approach to NMR studies of rhodopsin at this time, advances in labeling efficiency and NMR sensitivity may enable broader application in the future.

Membrane protein labeling in yeast

Yeast are the most commonly used eukaryotic systems for expression of NMR samples. Although a variety of yeast can be employed for protein expression and NMR isotope labeling (Kamiya et al. 2011; Miyazawa-Onami et al. 2013; Sugiki et al. 2012), the most commonly used host is the methylotrophic yeast *Pichia pastoris*. *Pichia* can be grown to high cell densities in shake flasks or fermentation, and robust levels of expression are achieved through target gene insertion under control of the AOX promoters and subsequent induction with methanol (Cereghino and Cregg 2000). Proteins can be expressed in a variety of *Pichia* cell strains with wild-type AOX1 and AOX2 proteins (Mut^+ phenotype), or strains with a mutant AOX1, facilitating slow growth upon induction with methanol (Mut^s phenotype) (Byrne 2015); due to strain-to-strain variations in protein expression, it is common to empirically test several options in parallel to identify those with optimal yield. Contrary to plasmid expression in bacteria, the gene-of-interest is integrated into the *Pichia* genome to create a stable cell line, often with multiple copy integration. After integration, individual clones are typically screened for copy number through increasing antibiotic concentration on selection media. This said, it is not recommended to rely on copy number (e.g. through PCR quantitation) as a proxy for protein expression level (Aw and Polizzi 2013), and subsequent screening should be performed by small-scale expression tests in either protonated or deuterated media. For more detailed reviews of available *Pichia* vectors and expression lines, see several recent reports and review articles (Byrne 2015; Cos et al. 2006; Fan et al. 2015).

Pichia is advantageous for the expression of isotopically-labeled samples, due to its capacity to grow in either protonated or deuterated minimal media (Massou et al. 1999; Morgan et al.

2000; Sugiki et al. 2012). Accordingly, uniform ^{15}N labeling is straightforwardly accomplished with the addition of 0.5% to 1% w/w of ^{15}N ammonium sulfate to yeast nitrogen base lacking amino acids and ammonium sulfate (Rodriguez and Krishna 2001); correspondingly, different ^{13}C -labeled carbon sources can be used to achieve various labeling patterns as well (Wood and Komives 1999). Examples of these approaches as applied to membrane proteins include solid state NMR studies of U- ^{13}C , ^{15}N labeled aquaporin-1 (Emami et al. 2013) and rhodopsin (Fan et al. 2011), both of which were reconstituted into proteoliposomes. More complex ^{13}C labeling patterns are achievable through the use of specifically-labeled carbon sources, such as ^{13}C sparse labeling from media containing alternatively-labeled glycerol and ^{13}C methanol to reduce peak overlap and improve spectral resolution (Liu et al. 2016), or by the combination of amino-acid auxotrophic *Pichia* strains and labeled amino acids, leading to >99% labeling efficiencies (Whittaker and Whittaker 2005).

We summarize the general pros and cons of using insect, mammalian, and yeast expression systems in Table 1.

Application of methyl labeling in *Pichia* to GPCRs

With these advantages, *Pichia* has proven a viable expression host for the production of a range of eukaryotic membrane proteins, including ion channels and GPCRs, for X-ray crystallography efforts (Guo et al. 2016; Hino et al. 2012; Shimamura et al. 2011; Yurugi-Kobayashi et al. 2009). *Pichia*-generated samples of the adenosine $\text{A}_{2\text{A}}$ receptor have also enabled solution NMR studies, using C^{19}F_3 labels chemically added *in vitro* (Ye et al. 2016) or uniformly-labeled ^{15}N samples monitored at well-dispersed glycine backbone and tryptophan indole sites (Eddy et al. 2018).

To extend the applicability of these studies, we sought to take advantage of the favorable NMR properties and locations of methyl groups within GPCRs. While such a route requires optimizing expression conditions to produce highly deuterated, methyl-labeled samples with improved NMR relaxation characteristics, it allows the use of protein samples containing solely the native wildtype sequence. In contrast, other strategies used for solution NMR studies of GPCRs have involved either post-purification chemical modifications (Ye et al. 2016) or the introduction of thermostabilizing point mutations to lengthen sample lifetimes and increase signal-to-noise by permitting NMR data collection at elevated temperatures (Isogai et al. 2016; Solt et al. 2017).

Methyl groups are highly sensitive probes for NMR, enabling NMR studies of very high-MW complexes such as the proteasome (Religa et al. 2010; Sprangers and Kay 2007). Methyl-TROSY experiments with perdeuteration capitalize on the slow relaxation properties of methyl groups to increase both resolution and signal-to-noise (Ollerenshaw et al. 2003). Methyl probes are attractive for probing different aspects of receptor activation, but applying methyl-TROSY experiments require an expression system that can facilitate specific ^{13}C -methyl labeling of samples in an otherwise deuterated background. Labeling the terminal methyl groups of the branched-chain amino acids isoleucine, leucine, and valine can be routinely performed in bacteria, through addition of ^{13}C α -ketoacids into minimal protonated or deuterated media (Gardner and Kay 1997; Goto et al. 1999). However, many

eukaryotic membrane proteins cannot be sufficiently overexpressed in bacteria, and although there has been recent success with partial deuteration in Sf9 systems (Kofuku et al. 2014; Kofuku et al. 2018; Minato et al. 2016; Okude et al. 2015), the metabolic pathways required for efficient usage of α -keto acids are not conserved in insect cells.

With this motivation, we recently developed approaches in *Pichia* to produce proteins specifically labeled with ^1H , ^{13}C $\delta 1$ methyl groups of isoleucine residues in a perdeuterated background for both challenging soluble proteins (e.g. actin) (Clark et al. 2015) and membrane proteins (Clark et al. 2017). This is achieved by addition of methyl- ^{13}C labeled α -ketobutyrate to *Pichia* cultures adapted to deuterated media, leading to ~50% labeling of Ile $\delta 1$ methyl groups in a ~90% deuterated background (Clark et al. 2015). Adaptation of *Pichia* to deuterated media takes about a week, incrementally replacing protons for deuterons in the media and in glycerol, the sole carbon source during biomass generation. Although there are cases where glucose has been successfully used with lower cost (Emami et al. 2013; Fan et al. 2015; Fan et al. 2011), glycerol is generally the preferred carbon source for *Pichia* cultures because the AOX1 promoter is severely repressed by residual glucose and can cause cell death upon methanol induction (Wood and Komives 1999).

Using this labeling strategy, we collected 2D NMR spectra on *Pichia*-expressed deuterated, methyl-labeled A_{2A} receptor and were able to assess how the ps-ns dynamics of different isoleucine side chains changed as a result of the ligand bound at the orthosteric pocket (Clark et al. 2017). Following this precedent, we collected preliminary 2D $^1\text{H}/^{13}\text{C}$ HMQC spectra on two other wild-type GPCRs, the CB1 cannabinoid receptor and the OX₂R orexin receptor bound to their respective antagonists taranabant or suvorexant (Fig. 2). CB1 and OX₂R receptors have been structurally characterized by X-ray crystallography (Shao et al. 2016; Yin et al. 2015) and are drug targets for pain and epilepsy (CB1) or sleep disorders (OX₂R). While the number of dispersed peaks in OX₂R and CB1 are fewer than for A_{2A}, we are able to distinguish specific peaks that could be monitored as probes of dynamics within the TM bundles. In each of our three samples, the number of visible methyl resonances is fewer than the number of Ile residues present in each receptor. Several possible explanations for this effect are possible. First, a substantial number of Ile residues in these proteins are pointed towards the lipid (Fig. 2), likely with few differences in local environments (and chemical shifts) among these sites, leading to highly overlapped peaks in the center of the Ile $\delta 1$ methyl region. Another contributor is likely to be chemical exchange among multiple different GPCR conformations and accompanying peak broadening. Such conformational interconversion has been documented at multiple timescales using NMR and MD approaches (Clark et al. 2017; Ye et al. 2016) and can be mitigated by changing bound ligands (including bound Na⁺), optimizing sample conditions (e.g. salt, buffer, detergent, or temperature) or reconstituting receptors into membrane mimetics such as nanodiscs. While membrane proteins reconstituted into nanodiscs would have longer rotational correlation times and slower tumbling than those in protein-detergent micelles, the protein stability and sample lifetime may be increased, allowing longer data acquisitions (and correspondingly better sensitivity in a range of NMR spectra). Additionally, new SaposinA-based nanodiscs have smaller particle sizes, leading to high quality spectra of GPCRs (Chien et al. 2017).

Isoleucines are prevalent in the TM helices of integral membrane proteins, with an average of 30 residues in Class A GPCRs (Pándy-Szekeres et al. 2018), providing abundant native NMR probes throughout receptors and other membrane proteins expressed in this manner. Notably, cell-based labeling approaches do not require any post-purification chemical modification step, as required with several ^{13}C -formaldehyde or ^{19}F labeling strategies for membrane proteins (Bokoch et al. 2010; Eddy et al. 2016; Liu et al. 2012; Manglik et al. 2015; Sounier et al. 2015; Staus et al. 2016; Ye et al. 2016). Further spectral probes are likely to be accessible in *Pichia*-expressed samples through a recently-developed protocol for ^1H , ^{13}C -methyl labeled valine and leucine using low pH to facilitate uptake of α -ketoisovalerate as a precursor to these amino acids (Suzuki et al. 2018).

Conclusions

Membrane proteins have remained challenging targets for NMR spectroscopy due to their large size and typical requirement for a eukaryotic host for heterologous expression. *Pichia pastoris* is a promising expression host for biophysical studies of membrane proteins and has been fruitful for structural and dynamic characterizations of human GPCRs. In particular, methyl labeling of isoleucine residues in a deuterated background permit sensitive methyl-TROSY experiments (Ollerenshaw et al. 2003) that can help deepen our understanding of GPCR dynamics at the atomic level, including probing the transition between inactive and active conformations. We and other groups have shown that the $\text{A}_{2\text{A}}$ receptor is a tractable model system to explore the basis of ligand modulation of GPCRs (Clark et al. 2017; Eddy et al. 2018; Ye et al. 2016), and we demonstrate here that other wild-type GPCRs can be expressed and labeled in the same manner for NMR (Fig. 1). Further developments are underway to expand our labeling capabilities in *Pichia* and help reduce costs of isotopic labeling in *Pichia* and in other eukaryotic expression systems.

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Figure 1: Sample gel filtration chromatograms of GPCR purifications.

Gel filtration traces of affinity-purified A_{2A} (A), CB1 (B), and OX_2R (C) samples injected over a Superdex200 column prior to NMR spectroscopy. In all three samples, fractions comprising the peak eluting at 15 mL were concentrated and used for NMR experiments.

Figure 2: Distribution and $^1\text{H}/^{13}\text{C}$ HMQC spectra of isoleucine $\delta 1$ methyl groups within representative GPCRs.

Top – Wire model figures of $\text{A}_{2\text{A}}$ (A) (PDB: 4EIY), CB1 (B) (PDB: 5U09), and OX_2R (C) (PDB: 4S0V) and structures with ligands shown as sticks, isoleucine sidechains as lines, and isoleucine $\delta 1$ carbon atoms as spheres. The number of isoleucine residues is shown as well. Bottom – $^1\text{H}/^{13}\text{C}$ HMQC spectra of deuterated, Ile $\delta 1$ -labeled $\text{A}_{2\text{A}}$ (A), CB1 (B), and OX_2R (C) in DDM micelles with inverse agonists ZM241385, taranabant, and suvorexant, respectively. The spectrum of $\text{A}_{2\text{A}}$ was collected on a sample independently prepared from those previously published (Clark et al. 2017), leading to slight differences. In each spectrum, an asterisk shows a peak arising from DDM detergent.

Table 1:

Summary of strengths and weaknesses of eukaryotic systems for the expression and isotopic labeling of integral membrane proteins for NMR studies.

Expression system	Pro	Con
Insect cells	• Most commonly used system for GPCRs and other MPs • Easy to grow/manipulate	• ILV methyl labeling not currently possible • High cost to deuterate • Complete deuteration not currently possible
Mammalian	• Required expression system for many MPs • Ideal host for human MPs that are sensitive to lipid environment or PTMs	• Unable to culture in deuterated media • Lack of strong precedent for difficult systems
Yeast	• Can express many different types of MPs, including GPCRs • Easy to grow/manipulate • Cultured in minimal media, with or without deuteration • Can readily label Ile δ1 methyl groups	• Complete labeling capabilities not established yet • Not suitable for all MPs