G protein-coupled receptors (GPCRs) constitute a superfamily of cell surface receptors responsible for the transduction of extracellular stimuli into intracellular signals. One sub-family of GPCRs is the adenosine family, identified as A_1R, A_2aR, A_2bR, and A_3R, which mediates the physiological effects of extracellular adenosine. Structurally, GPCRs consists of 7 transmembrane-spanning α-helices, an extracellular N-terminal domain, 3 extracellular and intracellular loops, and a cytoplasmic C-terminal domain. Despite this common architecture, adenosine receptor subtypes are subject to regulation by distinct molecular mechanisms, couple promiscuously to multiple signaling pathways, and maintain unique ligand binding properties. Although stress-response cell-signaling pathways (*e.g.* Unfolded Protein Response, UPR) and protein trafficking in yeast have been thoroughly investigated, the mechanisms underlying transport of heterologous membrane proteins from the endoplasmic reticulum (ER) to cell surface are less well understood. Notably, the regulation of receptor-mediated signaling at the plasma membrane in non-native hosts has remained elusive.

The emergence of genomic approaches coupled to recombinant DNA technologies have identified the systems that regulate proteostasis ⎯ defined as biological pathways modulating protein biogenesis, maturation, trafficking, and degradation. Recent studies have established ER quality control (ERQC) mechanisms, associated protein interactions, and trafficking motifs that regulate secretion. Despite our extensive knowledge of how these systems coordinate, recombinant protein expression and secretion has been limited. We anticipate that the most successful strategies involve integration of protein engineering strategies with host manipulation to exploit the cell's native stress-response pathways and trafficking mechanisms [1]. Here, specific recognition signals that govern quality control and secretion-dependent interactions provide a basic framework for determining trafficking mechanisms of recombinantly expressed GPCRs in yeast.

The model eukaryote, *S. cerevisiae*, has several attractive features that endorse its use for protein expression, including a well-established genome; ease of cultivation; high protein titers (*e.g.* > 1 g/L); well-developed and easily implemented genetic tools; and significant homology to the protein folding machinery and cellular process of higher eukaryotes. By implementing DNA recombination strategies using fluorescent protein variants [2,3] combined with high-resolution imaging techniques, we determined the subcellular localization of heterologous GPCRs compared to endogenous organelle markers (*i.e.* ER, plasma membrane, eisosomes) by improved spatiotemporal resolution achieved from Structured Illumination Microscopy (*i.e.* < 150 nm resolution). Alterations in organelle morphology, such as autophagy due to prolonged stress and the presence of ER-mitochondria encounter structures, are evident [4] as indicated by correlative microscopy techniques (*i.e*. a combination of d-STORM and electron microscopy).

To attain a mechanistic understanding of these phenotypic differences, we investigated processes governing the heterologous expression of 2 human adenosine receptors, hA_2aR and hA_3R, and engineered 5 chimeric receptors. Signal-mediated sorting mechanisms are conserved in yeast and higher eukaryotes, whereas ER exit and retention motifs have been identified. In this study, the implementation of select trafficking motifs to enhance recombinant protein secretion has been demonstrated. To improve functional production (*i.e.* ligand-binding yields indicative of active receptors) of GPCRs, we assessed protein structure/function by rational engineered chimeras, determined *in vivo* receptor activity using fluorescent ligands, confirmed the activity of purified GPCRs by XAC ligand chromatography, and quantified total yields of GPCR production.

Assessments of ERQC ⎯ including the UPR, ER-phagy, and ER associated degradation (ERAD) pathways were completed by time course analyses, quantitative PCR, and co-immunoprecipitation of ER folding factors. The evaluation of GPCR expression profiles

facilitated optimal conditions to minimize UPR induction, while confirming the activity of hA_2aR, hA_1R, hA_2bR, hA_3R. Collectively, protein-engineering strategies guided by biological principles and an emerging view of systems analyses, have facilitated optimal yields of active human adenosine receptors in *S. cerevisiae*.

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