

BRIEF REPORT

Functional analyses and affinity-alteration of receptors and enzymes based on membrane recruitment of yeast guanine nucleotide-binding protein γ subunit

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To investigate fundamental processes conserved in all eukaryotic cells, the budding yeast *Saccharomyces cerevisiae* has been widely used as a model organism. In particular, the yeast two-hybrid system is a powerful technique for identifying protein–protein interactions and analyzing protein function in living cells. Here, we describe several approaches for investigating and regulating the activity of target proteins using the yeast guanine nucleotide-binding protein (G-protein) signaling machinery as the readout. These approaches are rapid and easy-to-use tools that support the design of regulatory factors against receptors, enzymes, and other proteins that have been identified as potential drug target molecules.

Keywords: Yeast *Saccharomyces cerevisiae*; G-protein signaling; protein function; membrane recruitment; drug design

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All vital phenomena are based on molecular interactions, and typically involve proteins with various biological functions. During the past few decades, numerous methodologies have been developed to investigate intracellular protein functions under physiological conditions [1-2]. The budding yeast *Saccharomyces cerevisiae* is commonly used a model organism for the investigation of fundamental processes of all eukaryotic cells. Notably, the yeast two-hybrid system developed by Field and Song [3] has significantly advanced molecular interaction analyses. Furthermore, modifications to the two-hybrid technique also allow for the enzymatic activity of target proteins to be evaluated [4]. In the present paper, we describe the $G\gamma$ recruitment system (GRS), which is a modified yeast two-hybrid system developed for analyses of membrane-bound proteins [5].

Heterotrimeric guanine nucleotide-binding proteins (G-proteins), which are composed of $G\alpha$, $G\beta$, and $G\gamma$ subunits, are widely conserved among eukaryotic species [2]. In *S. cerevisiae*, pheromone stimulation through G-protein-coupled receptors induces G-protein activation, which leads to disassociation of G-proteins into two components, $G\alpha$ and the $G\beta\gamma$ complex. The $G\beta\gamma$ complex induces various cellular responses, such as global changes in transcription, growth arrest in the G1 phase, and polarized morphogenesis for mating [5]. As illustrated in Figures 1A and B, G-protein signal transduction requires membrane localization of the $G\gamma$ subunit, which requires dual lipid modification (farnesylation and palmitoylation) of the $G\gamma$ C-terminus [6]. Hence, in the case of a lipidation-defective $G\gamma$ mutant ($G\gamma_{\text{cyto}}$), alternative molecular interactions are absolutely necessary for restoring G-protein signaling.

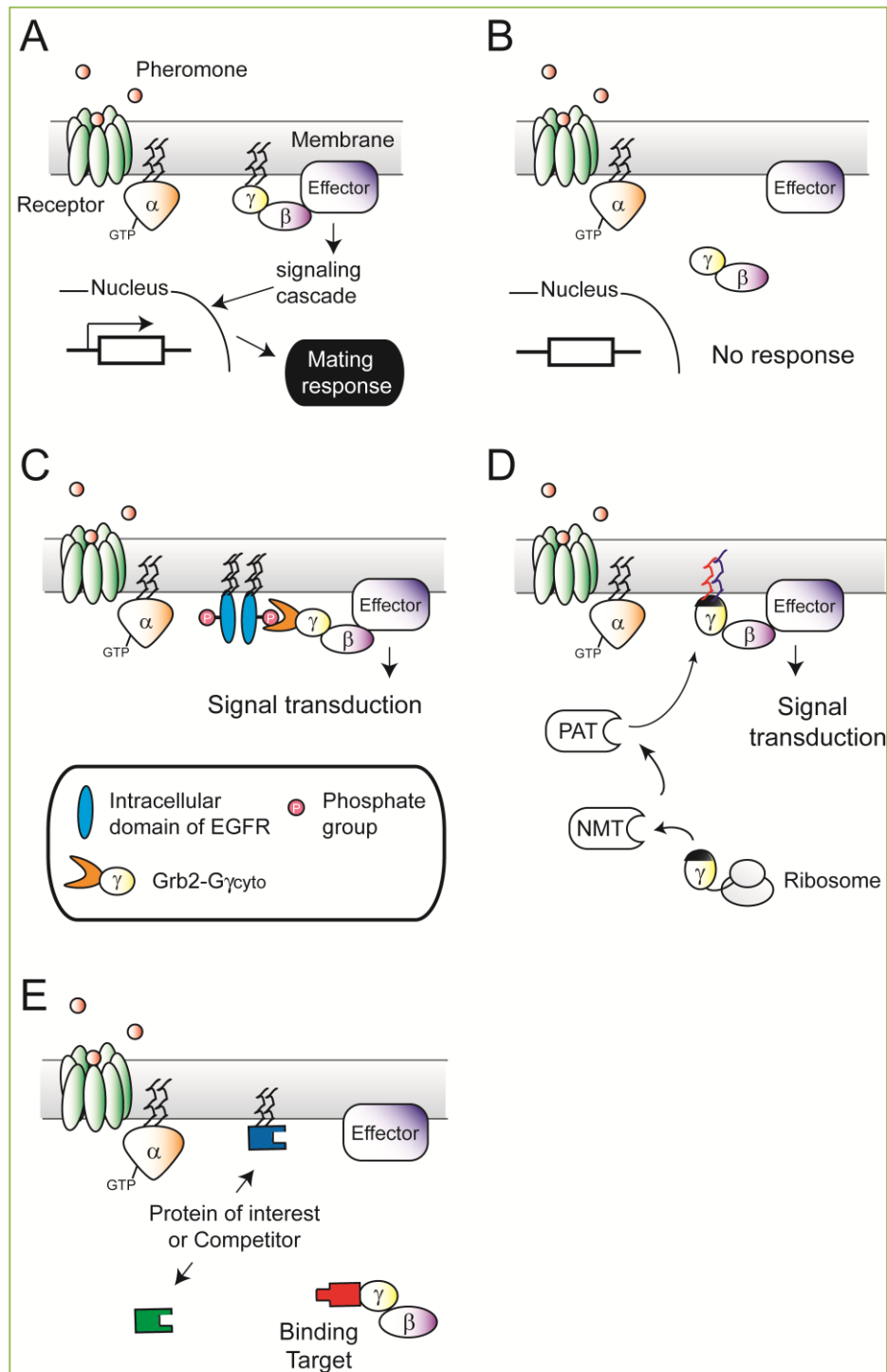


Figure 1. Schematic illustrations of yeast G-protein signaling and applications of the G γ recruitment system (GRS). (A) The wild-type G γ subunit induces pheromone-stimulated signaling in yeast. (B) In a yeast cell expressing engineered G γ lacking membrane-localization ability (G γ_{cyto}), G-protein signaling is completely blocked. (C) Two-hybrid GRS for evaluation of tyrosine kinase activity of human epidermal growth factor receptor (EGFR). (D) One-hybrid GRS for investigation of substrate sequences of both N-myristoyltransferase (NMT) and protein acyltransferases (PAT). (E) Competitor-introduced GRS for affinity-alteration of proteins.

The GRS was developed to detect protein-protein interactions in reporter and cell growth assays [5, 7]. In this system, a soluble bait protein of interest is fused to G γ_{cyto} and

the prey protein is attached to the plasma membrane, either through an intrinsic interaction or by the addition of a short recognition sequence for lipidation. Interaction between the

bait and prey proteins recruits $G\gamma_{\text{cyto}}$ to the membrane, resulting in activation of the G-protein signaling pathway and the induction of mating responses. A key feature of the GRS is its applicability for functional analyses of membrane-localized proteins, such as receptors, ion channels, and membrane-bound transporters, which often represent major drug targets^[8].

In a recent report, tyrosine kinase activity of human epidermal growth factor receptor (EGFR) using the GRS^[9]. Over-activation of the EGFR triggered by its overexpression and/or presence of activating mutations is involved in the generation and progression of various human cancers. As shown in Figure 1C, the intracellular domain of EGFR ($EGFR_{\text{cyto}}$) containing an activating mutation (L834R)^[10] was attached to the plasma membrane, while Grb2, which is an adapter protein for phosphorylated EGFR, was fused to $G\gamma_{\text{cyto}}$ (Grb2- $G\gamma_{\text{cyto}}$) in a yeast strain lacking endogenous $G\gamma$. In this experiment, G-protein signal transduction was recovered due to interaction between constitutively autophosphorylated $EGFR_{\text{cyto}}$ and Grb2- $G\gamma_{\text{cyto}}$. The generated yeast cells were used as an extremely simplified model of human cancer cells for evaluating the potential efficacy of tyrosine kinase inhibitors as anti-cancer drugs. The IC_{50} values of several inhibitors estimated using the GRS were almost equivalent to those estimated using human cell-based assays, demonstrating the applicability of this technique for drug screening or characterization^[9, 11, 12].

Recently, a yeast one-hybrid GRS was developed as a new approach for investigating protein-lipid associations^[13]. A wide range of proteins are modified with lipids as a means of regulating the membrane trafficking of proteins^[14]. As the abnormal activation of lipid-attached proteins or their biosynthetic enzymes is associated with serious diseases such as cancer and neurological disorders^[15], a better understanding of lipid modifications and protein trafficking is required for mitigating these adverse effects. The sequence motifs for myristoylation and palmitoylation at N-terminal regions of proteins were investigated using a yeast one-hybrid GRS (Fig. 1D). The attachment of a short peptide that serves a substrate of both N-myristoyltransferase (NMT) and protein acyltransferases (PAT) to the N-terminus of $G\gamma_{\text{cyto}}$ (sp- $G\gamma_{\text{cyto}}$) results in the transport of sp- $G\gamma_{\text{cyto}}$ to the plasma membrane following the attachment of myristate and palmitate, and the restoration of G-protein signaling. Using this system, substrate sequences for dual lipidation mediated by NMT and PAT were successfully identified by the screening of a random peptide library^[13].

Further modifications to the GRS are possible depending on the research question. For example, a competitor-introduced GRS was developed as a tool for

directed evolution^[7]. Because the cytosolic expression of binding competitors disrupts interactions between the membrane-attached protein of interest and $G\gamma_{\text{cyto}}$ -fused binding target, only affinity-enhanced mutants can effectively recruit the $G\gamma_{\text{cyto}}$ fusion protein to the plasma membrane (Fig. 1E). Recently, an affinity-attenuation system was established by swapping the positions of the competitor and the protein of interest^[16], with the aim of developing multi-target drugs^[17] or ‘dirty’ drugs^[18]. Attachment of the competitor to the plasma membrane results in membrane recruitment of the $G\gamma_{\text{cyto}}$ -fused binding target if the latter is not bound by the affinity-attenuated mutant in the cytosol. Using these approaches, it is possible to screen for desirable affinity-altered (affinity-enhanced or -attenuated) protein variants.

The GRS-based approaches described here are useful for the functional analyses and engineering of proteins involved in intracellular biological processes. In addition, because these techniques are based on well-established genetic manipulation in yeast cells, they are also suitable for the large-scale screening and characterization of pharmaceutical targets. We believe that the application of GRS-based approaches will become more widespread with continued advancements that make these techniques more user-friendly and accessible.

Conflict of interests

We declare that all authors are inventors on a pending patent related to aspects of the GRS for evaluating tyrosine kinase activity, and the first author is an inventor on a patent related to aspects of competitor-introduced GRS.

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References

1. Hamdi A, Colas P. Yeast two-hybrid methods and their applications in drug discovery. *Trends Pharmacol Sci* 2012; 33:109-118.
2. Ishii J, Fukuda N, Tanaka T, Ogino C, Kondo A. Protein-protein interactions and selection: yeast-based approaches that exploit guanine nucleotide-binding protein signaling. *FEBS J* 2010; 277:1982-1995.
3. Fields S, Song O. A novel genetic system to detect protein-protein interactions. *Nature* 1989; 340:245-246.
4. Stynen B, Tournu H, Tavernier J, Van Dijck P. Diversity in genetic in vivo methods for protein-protein interaction studies: from the yeast two-hybrid system to the mammalian

- split-luciferase system. *Microbiol Mol Biol Rev* 2012; 76:331-382.
5. Fukuda N, Ishii J, Tanaka T, Fukuda H, Kondo A. Construction of a novel detection system for protein-protein interactions using yeast G-protein signaling. *FEBS J* 2009; 276:2636-2644.
 6. Manahan CL, Patnana M, Blumer KJ, Linder ME. Dual lipid modification motifs in G γ and G β subunits are required for full activity of the pheromone response pathway in *Saccharomyces cerevisiae*. *Mol Biol Cell* 2000; 18:957-968.
 7. Fukuda N, Ishii J, Tanaka T, Kondo A. The competitor-introduced G γ recruitment system, a new approach for screening affinity-enhanced proteins. *FEBS J* 2010; 277:1704-1712.
 8. Emmerstorfer A, Wriessnegger T, Hirz M, Pichler H. Overexpression of membrane proteins from higher eukaryotes in yeasts. *Appl Microbiol Biotechnol* 2014; 98:7671-7698.
 9. Fukuda N, Honda S. Rapid Evaluation of Tyrosine Kinase Activity of Membrane-Integrated Human Epidermal Growth Factor Receptor Using the Yeast G γ Recruitment System. *ACS Synth Biol* 2014; doi:10.1021/sb500083t.
 10. Zhang X, Gureasko J, Shen K, Cole PA, Kuriyan J. An allosteric mechanism for activation of the kinase domain of epidermal growth factor receptor. *Cell* 2006; 125:1137-1149.
 11. Bojko A, Reichert K, Adamczyk A, Ligęza J, Ligęza J, Klein A. The effect of tyrphostins AG494 and AG1478 on the autocrine growth regulation of A549 and DU145 cells. *Folia Histochem Cytobiol* 2012; 50:186-195.
 12. Richards KN, Zweidler-McKay PA, Van Roy N, Speleman F, Trevino J, Zage PE *et al*. Signaling of ERBB receptor tyrosine kinases promotes neuroblastoma growth in vitro and in vivo. *Cancer* 2010; 116:3233-3243.
 13. Fukuda N, Doi M, Honda S. Yeast one-hybrid G γ recruitment system for identification of protein lipidation motifs. *PLoS One* 2013; 8:e70100.
 14. Resh MD. Targeting protein lipidation in disease. *Trends Mol Med* 2012; 18:206-214.
 15. Charron G, Wilson J, Hang HC. Chemical tools for understanding protein lipidation in eukaryotes. *Curr Opin Chem Biol* 2009; 13:382-391.
 16. Kaishima M, Fukuda N, Ishii J, Kondo A. Desired Alteration of Protein Affinities: Competitive Selection of Protein Variants Using Yeast Signal Transduction Machinery. *PLoS One* 2014; 9:e108229.
 17. Csermely P, Agoston V, Pongor S. The efficiency of multi-target drugs: the network approach might help drug design. *Trends Pharmacol Sci* 2005; 26:178-182.
 18. Frantz S. Drug discovery: Playing dirty. *Nature* 2005; 437:942-943.