Gγ recruitment systems specifically select PPI and affinity-enhanced candidate proteins that interact with membrane protein targets

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Protein-protein interactions (PPIs) are crucial for the vast majority of biological processes. We previously constructed a Gγ recruitment system to screen PPI candidate proteins and desirable affinity-altered (affinity-enhanced and affinity-attenuated) protein variants. The methods utilized a target protein fused to a mutated G-protein γ subunit (Gγcyto) lacking the ability to localize to the inner leaflet of the plasma membrane. However, the previous systems were adapted to use only soluble cytosolic proteins as targets. Recently, membrane proteins have been found to form the principal nodes of signaling involved in diseases and have attracted a great deal of interest as primary drug targets. Here, we describe new protocols for the Gγ recruitment systems that are specifically designed to use membrane proteins as targets to overcome previous limitations. These systems represent an attractive approach to exploring novel interacting candidates and affinity-altered protein variants and their interactions with proteins on the inner side of the plasma membrane, with high specificity and selectivity.

Protein-protein interactions (PPIs) are attracting increased attention in drug discovery studies. PPIs have functions in the regulation of cellular states involved in various diseases¹². In particular, membrane-mediated PPIs play central roles in vital biological processes and are prime drug targets. For example, tumorigenesis is often the result of gene mutations that lead to alterations in membrane PPIs and aberrant signaling cascades³. Because the molecules that control (inhibit or activate) these membrane PPIs can be used as drug candidates, rapid and unbiased screening of these molecules is essential for drug development.

The major targets of membrane proteins are G-protein-coupled receptors (GPCRs), ion channels, transporters, receptor serine/threonine and tyrosine protein kinases⁴⁻⁵ (e.g. epidermal growth factor receptor (EGFR)⁶⁻⁷, human epidermal growth factor receptor 2 (HER2)⁸⁻⁹, and vascular endothelial growth factor receptor (VEGFR)¹⁰⁻¹¹). The extracellular domains of these transmembrane proteins are commonly targeted to identify agonistic and antagonistic ligands. However, recently developed drug therapies have increasingly targeted the intracellular domains (kinase domains) of these transmembrane proteins to control interactions with the components of downstream signaling cascades¹². Similarly, membrane-associated proteins, such as guanine nucleotide-binding protein (G-protein), small GTPases,
kinase proteins and other signal transducers, hold enormous potential for use in the development of novel drugs. As a representative example, protein kinases are responsible for the reversible phosphorylation of proteins via PPIs and have a strong relationship with growth, infiltration and apoptosis in cancer cells. A multitude of these membrane-associated proteins are involved in various diseases and are often associated with the inner side of the plasma membrane. Several kinase and GTPase inhibitors have been developed in the pharmaceutical industry. More recently, intracellular antibodies (intrabodies), which can inhibit signal transducers, including membrane-associated proteins, have been studied as valuable tools for controlling PPIs inside cells. Thus, molecules that can control the PPIs of transmembrane and membrane-associated proteins on the inner side of the plasma membrane have a potential to become an important group of drug targets.

Various useful screening systems for PPIs exist and have yielded significant findings. These techniques are required for screening of large numbers of proteins and are preferable in the *in vivo* cellular context. In particular, yeast two-hybrid systems are the typical tools for such screening of candidate proteins in *vivo*. Among them, split-ubiquitin system is a well-established, useful technique to screen the candidate proteins with the PPIs for membrane target proteins. As in other yeast systems, small G-protein-based methods, including the Sos recruitment system and the Ras recruitment system, are occasionally used to study the PPIs of membrane proteins. These methods remain useful alternatives to the original two-hybrid system; however, they suffer from technical complexities, such as the different temperatures required for growth and screening (25 °C and 36 °C), slow growth at suboptimal temperatures, obligatory replica-plating steps (glucose to galactose medium), and the total time required for the procedure (7 days including precultivation). In addition to bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), protein fragment complementation assays using split-GFP and split-luciferase are useful tools for detecting the association of two proteins in living cells and have the potential to resolve these limitations. Among the varied systems used, growth reporters are generally applicable to library screening because of their convenience. Our previously developed screening method using yeast heterotrimeric G-proteins, called the Gγ recruitment system, also makes it possible to screen PPIs between a target protein and candidate proteins by the mating growth assay without false-positive clones. The details of the mechanism utilized for detecting PPIs are presented below.

The Gγ recruitment system for detecting PPIs is based on the fundamental principle that yeast pheromone (mating) signaling requires the localization of a complex consisting of the β and γ-subunits of heterotrimeric G-proteins (Gγ3/Gγ) to the inner leaflet of the plasma membrane. In yeast, the G-protein-coupled receptor (GPCR) undergoes a conformational change after binding ligands and then activates heterotrimeric G-proteins. The activated G-proteins trigger the dissociation of the G3/Gγ complex from GoX concurrently with the exchange of GDP/GTP on the Go subunit. The G3 subunit (complexed with membrane-associated Gγ) then acts upon the effectors, thereby activating the downstream signaling cascade for mating. Notably, localization of the G3/Gγ complex to the inner leaflet of the plasma membrane via the lipotidation motif of the Gγ subunit is required for initiating G-protein signaling. Our Gγ recruitment system specifically makes use of a cytosolic truncated variant of Gγ (named Gγ3Δcm), that is fused to a soluble target protein of interest, X (Gγ3Δcm-X), as shown in Fig. 1A. For the library, the candidate proteins (Y1) should be attached to the artificial lipidation site to ensure localization to the membrane (Fig. 1A). When an interaction occurs between target X and candidate Y1, the Gγ3Δcm-X fusion protein brings G3 to the membrane and induces subsequent activation of the pheromone signaling pathway. The promoted signaling can be detected by a fluorescent reporter assay or a mating growth assay after growth in simple glucose media at the optimal temperature (30 °C). Briefly, the expression of GFP under the control of a pheromone-responsive *FIG1* promoter or mating with intact haploid cells of the opposite mating type permits the detection of PPIs (Fig. 1A and Fig. S1). Because the localization of Gγ3Δcm in the cytosol completely prevents this signaling activation, the Gγ recruitment system allows for extremely reliable, low-background growth screening that excludes false-positive candidates at the optimal temperature (30 °C). The procedures for screening involve simply mixing the different mating-type cells (recombinant α-cells and intact α-cells) and plating on selective media (4 days including precultivation) (Fig. S1; right). The advanced system (competitor-introduced Gγ recruitment system), which additionally expresses an interaction competitor protein (Y2) in the cytosol (Fig. 2A), can offer highly selective screening for protein variants whose affinities have been intentionally altered to exceed the set threshold. This approach is applicable to selectively screening affinity-enhanced or affinity-attenuated protein variants by exchanging the positions of the competitor protein and the library proteins (Y1 and Y2). The localization of Gγ is of key importance for the low background of the Gγ recruitment system. The previous Gγ recruitment system was limited to using only soluble cytosolic proteins as the target (X), as candidate proteins (Y1) should be expressed on the membrane (Fig. 1A). The competitor-introduced system also had a similar problem, restricting the target X to cytosolic proteins (Fig. 2A). Thus, these previous systems could not target membrane proteins. In the current study, we have reevaluated the Gγ recruitment system by changing the localization of target proteins from the cytosol to the membrane; however, the prior protocol did not work well. With the aim of expanding the applicability of the system, we considered new protocols for the Gγ recruitment systems that might be suitable for evaluating...
membrane proteins as targets. The updated method allows the Gγ recruitment system to be used in the analysis of both cytoplasmic and membrane target proteins.

Results
Selection of candidate proteins interacting with membrane protein targets using a previously established PPI-detecting Gγ recruitment system. First, we tested whether the previous Gγ recruitment system could target membrane proteins. When target protein ‘X’ fused to Gγcyto interacts with candidate protein ‘Y1’, the Gβ and Gγcyto complex (Gβγcyto) migrates to the inner leaflet of the plasma membrane and restores the signaling function. If protein ‘X’ cannot interact with protein ‘Y1’, Gβγcyto is released into the cytosol, and signaling is blocked.

(B) PPI detection system for membrane-localized target protein

Figure 1. Schematic diagram of Gγ recruitment systems to detect PPIs of cytosolic or membrane target proteins. (A) Schematic outline of the previously established Gγ recruitment system for cytosolic target proteins. When target protein ‘X’ fused to Gγcyto interacts with candidate protein ‘Y1’, the Gβ and Gγcyto complex (Gβγcyto) migrates to the inner leaflet of the plasma membrane and restores the signaling function. If protein ‘X’ cannot interact with protein ‘Y1’, Gβγcyto is released into the cytosol, and signaling is blocked. (B) Schematic outline of the Gγ recruitment system for membrane protein targets. When membrane target protein ‘X’ interacts with candidate protein ‘Y1’ fused to Gγcyto, the Gβ and Gγcyto complex (Gβγcyto) migrates to the inner leaflet of the plasma membrane and restores the signaling function. If membrane protein ‘X’ cannot interact with protein ‘Y1’, Gβγcyto is released into the cytosol, and signaling is blocked.
resulting in MC-FC and MC-FN yeast strains (Table 1). For the candidate proteins, autonomous replication plasmids for the expression of the four different Z variants (ZWT, ZK35A, ZI31A and Z955) fused to Gγ cyt(Y1) (pGK413-Gγ cyto-Y1) (Table 2) were introduced into the MC-FC and MC-FN yeast cells (Fig. 3A and Fig. S2A). Flow cytometric analysis of the transformants was conducted after incubation in medium containing the α-cell mating pheromone (α-factor) (Fig. S1; left). The engineered yeast strains expressing the Gγ cyt-YWT and Gγ cyt-ZK35A fusion proteins as candidates slightly induced the transcription of GFP reporter genes via interaction with the membrane-associated Fc fragment, although the fluorescence levels were extremely low (Fig. 3B and Fig. S2B). In mating selection with intact α-type yeast cells (Fig. S1; right), the strains expressing Gγ cyt-ZWT and Gγ cyt-ZK35A exhibited specific but negligible cell growth on selective medium (Fig. 3C and Fig. S2C). In both GFP transcription assays and mating growth selection, interactions of Gγ cyt-ZI31A (very low affinity for Fc) and Gγ cyt-Z955 (negative control) with the membrane-associated...
| Strain       | Relevant feature | Source                  |
|-------------|-----------------|-------------------------|
| BY4741      | MATa his3Δ1 leu2Δ0 met15Δ | 67                      |
| BY4742      | MATa his3Δ1 leu2Δ0 lys2Δ0 | 67                      |
| MC-F1       | BY4741 fgl1-FG1-EGFP |                         |
| MC-FC       | MC-F1 ste18Δ3:kanMX4-PREP/Fc-Ste18C | Present study |
| MC-FN       | MC-F1 ste18Δ3:kanMX4-PREP/Fc-Gpa1N-Fc | Present study |
| FC-GW       | FC-GW ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GK       | FC-GK ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-ZK35A | Present study |
| FC-GI       | FC-GI ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-ZK35A | Present study |
| FC-G9       | FC-G9 ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-ZK35A | Present study |
| FN-GW       | FN-GW ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-ZK35A | Present study |
| FN-GK       | FN-GK ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-ZK35A | Present study |
| FN-GI       | FN-GI ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-ZK35A | Present study |
| FN-G9       | FN-G9 ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-ZK35A | Present study |
| PC-W        | PC-W ste18Δ3:kanMX4-PREP/Fc-Ste18C PREP::LEU2-PREP-ZK35A | Present study |
| PC-K        | PC-K ste18Δ3:kanMX4-PREP/Fc-Ste18C PREP::LEU2-PREP-ZK35A | Present study |
| PC-I        | PC-I ste18Δ3:kanMX4-PREP/Fc-Ste18C PREP::LEU2-PREP-ZK35A | Present study |
| PC-9        | PC-9 ste18Δ3:kanMX4-PREP/Fc-Ste18C PREP::LEU2-PREP-ZK35A | Present study |
| FN-W        | FN-W ste18Δ3:kanMX4-PREP/Gpa1N-Fc PREP::LEU2-PREP-ZK35A | Present study |
| FN-K        | FN-K ste18Δ3:kanMX4-PREP/Gpa1N-Fc PREP::LEU2-PREP-ZK35A | Present study |
| FN-I        | FN-I ste18Δ3:kanMX4-PREP/Gpa1N-Fc PREP::LEU2-PREP-ZK35A | Present study |
| FN-9        | FN-9 ste18Δ3:kanMX4-PREP/Gpa1N-Fc PREP::LEU2-PREP-ZK35A | Present study |
| FC-GWW      | FC-GWW ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GWK      | FC-GWK ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GWI      | FC-GWI ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GW9      | FC-GW9 ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GKW      | FC-GKW ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GKK      | FC-GKK ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-GKI      | FC-GKI ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-G9I      | FC-G9I ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-G9W      | FC-G9W ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FC-G9K      | FC-G9K ste18Δ3:kanMX4-PREP/Fc-Ste18C his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GWW      | FN-GWW ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GWK      | FN-GWK ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GW1      | FN-GW1 ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GW9      | FN-GW9 ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GKW      | FN-GKW ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-GK1      | FN-GK1 ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-G9I      | FN-G9I ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |
| FN-G9K      | FN-G9K ste18Δ3:kanMX4-PREP/Gpa1N-Fc his3Δ3::URA3-PREP-Gpa1N-Fc | Present study |

Continued
Table 1. Yeast strains used in this study.

| Strain | Relevant feature | Source |
|--------|-----------------|--------|
| FN-G9W | MC-F1 ste18Δ::kanMX4-PGK1-Gpa1N-Fc his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955-PHOP2P | Present study |
| FN-G9K | MC-F1 ste18Δ::kanMX4-PGK1-Gpa1N-Fc his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955A-PHOP2P | Present study |
| FN-G9I | MC-F1 ste18Δ::kanMX4-PGK1-Gpa1N-Fc his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955A-PHOP2P | Present study |
| FN-G99 | MC-F1 ste18Δ::kanMX4-PGK1-Gpa1N-Fc his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955P-PHOP2P | Present study |
| MC-ErC | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC | Present study |
| MC-EsC | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ste18ΔC | Present study |
| MC-EgN | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, cyto-Ras1C his3Δ::URA3-PGK1-Grb2-GPHOP2P | Present study |
| ErC-grbG | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| EsC-grbG | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ste18ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| EgN-grbG | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| ErC-Ggrb | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| EsC-Ggrb | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ste18ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| EgN-Ggrb | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, his3Δ::URA3-PY2328-Grb2-GPHOP2P | Present study |
| ErC-grbG-E89K | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955P-PHOP2P | Present study |
| ErC-grbG-R86G | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955P-PHOP2P | Present study |
| ErC-grbG-LEU | MC-F1 ste18Δ::kanMX4-PGK1-EGFR1L834R, ras1ΔC his3Δ::URA3-PY2328-Grb2-GPHOP2P::LEU2-PGK1-Z955P-PHOP2P | Present study |

Fc fragment were not detected. These results showed that the previous protocol was not sufficient to screen the interactions between membrane-associated target ‘X’ and candidate ‘Y1’-fused Gγcyto proteins.

PPI-detecting Gγ recruitment system for the selection of candidate proteins interacting with membrane protein targets. Next, we tested the new protocol, in which we changed the method used to introduce the Gγcyto-Y1 candidate genes. The DNA cassettes for cytosolic expression of the Gγcyto-fused candidate Z variants (ZWT, ZK35A, and Z955) as a library were stably integrated into the MC-FC and MC-FN yeast chromosomes, generating the Fc fragment and the Gγcyto-fused Z domain (Fig. 3D–F and Fig. S2D–F). These results were clearly different from those following expression of Gγcyto-fused candidate ‘Y1’ using autonomous replicating plasmids (Fig. 3A–C and Fig. S2A–C).

Competitive selection of affinity-enhanced protein variants interacting with membrane protein targets using a previous protocol. Previously, we established the competitor-introduced Gγ recruitment system for selective screening of protein variants that exceed a specified affinity threshold41 (Fig. 2A). In the conventional Gγ recruitment system, additional expression of a cytosolic parental (known) protein (Y2) that binds to Gγcyto-fused target protein ‘X’ competes with artificially membrane-associated protein variants as a candidate library (Y1), thereby permitting the selective screening of affinity-enhanced protein variants (Fig. 2A).

To test whether the previous competitor-introduced Gγ recruitment system allows for the use of membrane proteins as target ‘X’ (Fig. 2B and S3A), we consistently used the membrane-associated Fc fragment and the Gγcyto-fused Z variants as target ‘X’ and candidate ‘Y1’ proteins, respectively (Figs. 4A and S4A). ZWT (low affinity for Fc; 8.0 × 10^4 M^-1) was utilized as the model of the competitive parental ‘Y2’ protein (Figs. 4B and S4B). Therefore, the ZWT and ZK35A, candidate proteins (Y1), with higher affinities, should have outcompeted the interaction between membrane-associated Fc (X) and cytosolic ZWT (Y2), recovering the signaling in the system (Fig. S3A). In the previous system, the DNA cassette for ZWT expression as a competitor ‘Y2’ protein in the cytosol was stably integrated into the yeast chromosome of MC-FC, in which the C-terminally membrane-associated Fc fragment (X) (with the ste18p lipida tion motif) was expressed, generating an FC-1 strain (Table 1). Autonomous replication plasmids for expression of the Gγcyto-fused Z variants as candidate ‘Y1’ (pGK413-Gγ-EZW1, pGK413-Gγ-EZK35A, pGK413-Gγ-EZK35A, and pGK413-Gγ-EZK35A).
pGK413-Gγ-EZI31A and pGK413-Gγ-EZ955) (Table 2) were then introduced into the FC-I strain. However, both flow cytometric analysis and mating selection were barely able to detect the interactions between the membrane-associated Fc fragment (target ‘X’) and the Gγcyto-fused Z variants (candidate ‘Yγ’) relative to the interactions between the membrane-associated Fc fragment and cytosolic Z31A in all transformants (Fig. 4B). Additionally, when using an FN-1 strain chromosomally expressing an N-terminally membrane-associated Fc fragment (X) (with a Gpa1p lipidation motif) and competitive Z31A protein (Yγ) (Table 1), the transformants in which the candidate autonomous plasmids were introduced to express the Gγcyto-fused Z variants (Yγ) provided similar results to the C-terminally membrane-associated Fc fragment (Fig. S4B). These results showed that the previous system was unable to screen the interactions between membrane-associated target ‘X’ and candidate ‘Yγ’-fused Gγcyto proteins relative to the interactions between membrane target ‘X’ and the cytosolic ‘Yγ’ competitor.

Competitor-introduced Gγ recruitment system that specifically selects affinity-enhanced protein variants interacting with membrane protein targets. Similar to what was described in the previous section, we attempted to change the protocol by introducing the expression cassettes for Gγcyto-Yγ candidate genes into the competitor-introduced Gγ recruitment system (Figs. 4C and S4C). As competitive parental ‘Yγ’ proteins, the genes for expressing the four different Z variants (ZWT, Z35A, Z31A and Z955) in the cytosol were integrated into the MC-FC yeast chromosome (also expressing the C-terminally membrane-associated Fc fragment with the Ste18p lipidation motif as target ‘X’), generating FC-W, FC-K, FC-I and FC-9. The DNA cassettes for expressing the Gγcyto-fused candidate Z variants as model library Yγ proteins were then stably integrated into the chromosome of the four yeast strains, generating 16 engineered yeast strains (FC-GWW through FC-G99; Table 1) (Fig. 4C).

Both flow cytometric analysis and mating selection revealed the interactor combinations between membrane-associated Fc and the Gγcyto-fused Z variants serving as candidate Yγ proteins, with higher affinities than when the cytosolic Z variants served as competitor Yγ proteins (Fig. S5A). ZWT was utilized as the model of the competitive parental Yγ protein. Therefore, Gγcyto-fused ZWT (Yγ) should have outcompeted the interactions between membrane-associated Fc (X) and the ZWT candidate proteins (Yγ), which have lower affinities, recovering the signaling in the system.

In the previous system, autonomous replication plasmids for expression of the Z variants in the cytosol as candidate Yγ proteins (pGK-LsZWTc, pGK-LsZ35Ac, pGK-LsZ31Ac and pGK-LsZ955c) (Table 2) were then introduced into the FC-GW strain, which chromosomally expresses Fc-Ste18C as X’ and Gγcyto-ZWT as competitor Yγ (Table 1). Both flow cytometric analysis and mating selection revealed the interactor combinations between membrane-associated Fc and the cytosolic Z variants serving as candidate Yγ proteins, whose affinities were lower than that of Gγcyto-fused ZWT as the competitor Yγ protein (Fig. 5B,C). Additionally, when using the FN-GW strain chromosomally expressing Gpa1pN-Fc as X’ and Gγcyto-fused ZWT as competitor Yγ (Table 1), the transformants in which the candidate autonomous plasmids were introduced to express the Z variants in the cytosol (Yγ) provided similar results (Fig. S5B,C). In contrast to the affinity-enhanced system, these results showed that the previous competitor-introduced Gγ recruitment system was able to screen affinity-attenuated protein variants using membrane proteins as the target proteins.

Competitive selection of affinity-attenuated protein variants interacting with membrane protein targets using a previous protocol. Previously, we also established a system that permits the selective screening of affinity-attenuated protein variants. In the conventional Gγ recruitment system, by setting the cytosolic protein (Yγ) as the candidate library and the artificially membrane-associated protein (Yγ) as the parental (known) competitor that binds to Gγcyto-fused target protein ‘X’, the system permits the selective screening of affinity-attenuated protein variants (Fig. 2A).

To test whether the previous competitor-introduced Gγ recruitment system allows for the use of membrane proteins as target ‘X’ (Fig. 2B and S3B), we consistently used the membrane-associated Fc fragment and the cytosolic Z variants as target ‘X’ and candidate Yγ proteins, respectively (Fig. 5A and S5A). ZWT was utilized as the model of the competitive parental Yγ protein. Therefore, Gγcyto-fused ZWT (Yγ) should have outcompeted the interactions between membrane-associated Fc (X) and the ZWT candidate proteins (Yγ), which have lower affinities, recovering the signaling in the system.

In the previous system, autonomous replication plasmids for expression of the Z variants in the cytosol as candidate Yγ proteins (pGK-LsZWTc, pGK-LsZ35Ac, pGK-LsZ31Ac and pGK-LsZ955c) (Table 2) were then introduced into the FC-GW strain, which chromosomally expresses Fc-Ste18C as X’ and Gγcyto-ZWT as competitor Yγ (Table 1). Both flow cytometric analysis and mating selection revealed the interactor combinations between membrane-associated Fc and the cytosolic Z variants serving as candidate Yγ proteins, whose affinities were lower than that of Gγcyto-fused ZWT as the competitor Yγ protein (Fig. 5B,C). Additionally, when using the FN-GW strain chromosomally expressing Gpa1pN-Fc as X’ and Gγcyto-fused ZWT as competitor Yγ (Table 1), the transformants in which the candidate autonomous plasmids were introduced to express the Z variants in the cytosol (Yγ) provided similar results (Fig. S5B,C). In contrast to the affinity-enhanced system, these results showed that the previous competitor-introduced Gγ recruitment system was able to screen affinity-attenuated protein variants using membrane proteins as the target proteins.

Demonstration of applicability of our system using intracellular domain of EGFR and Grb2. To demonstrate the applicability of our system, we selected the intracellular domain of EGFR (EGFRcyto), which contains a tyrosine kinase domain and tyrosine phosphorylation sites, and the adaptor
| Plasmids       | Genotype                                           | Reference |
|---------------|---------------------------------------------------|-----------|
| pGK425        | Expression vector containing PGK1 promoter, 2μ origin and LEU2 marker | 68        |
| pGK425-Gpa1N  | N-terminus of Gpa1 (9 a.a.) expression, in pGK425 | This study|
| pGK425-Stel18C| C-terminus of Ste18 (9 a.a.) expression, in pGK425 | This study|
| pGK425-Ras1C  | C-terminus of Ras1 (10 a.a.) expression, in pGK425 | This study|
| pGK425-Gpa1N-Fc| Fc protein expression, in pGK425-Gpa1N | This study|
| pGK425-Fc-Stel18C| Fc protein expression, in pGK425-Stel18C | This study|
| pGK426-GPTK   | URA3-STE18 promoter-kanMX4-STE18 terminator in pGK426 | 42        |
| pUMGPTK-Gpa1N-Fc| URA3-STE18p-PGK1 promoter -Gpa1N (9 a.a.)-Fc- PGK1 terminator -kanMX4-STE18 terminator in pGK426-GPTK | This study|
| pUMGPTK-Fc-Stel18C| URA3-STE18p-PGK1 promoter -Fc-Stel18C (9 a.a.)- PGK1 terminator -kanMX4-STE18 terminator in pGK426-GPTK | This study|
| pGK413        | Expression vector containing PGK1 promoter, CEN/ARS single-copy origin and HIS3 marker | 68        |
| pGK413-G7-EZWT| G715A-ZWT fusion expression, in pGK413 | This study|
| pGK413-G7-EZK35A| G715A-ZK35A fusion expression, in pGK413 | This study|
| pGK413-G7-EZI31A| G715A-ZI31A fusion expression, in pGK413 | This study|
| pGK413-G7-EZ955| G715A-Z955 fusion expression, in pGK413 | This study|
| pUSTE18p-G7-cyto| URA3-STE18 promoter-G715A-PGK1 terminator in pGK426 | This study|
| pUSTE18p-G7-cyto-HIS3t| URA3-STE18 promoter-G715A-PGK1 terminator-HIS3 terminator in pGK426 | This study|
| pUSTE18p-G7-cyto-ZWT-H| URA3-STE18 promoter-G715A-ZWT-PGK1 terminator in pUSTE18p-G7-cyto-HIS3t | This study|
| pUSTE18p-G7-cyto-ZK35A-H| URA3-STE18 promoter-G715A-ZK35A-PGK1 terminator in pUSTE18p-G7-cyto-HIS3t | This study|
| pUSTE18p-G7-cyto-ZI31A-H| URA3-STE18 promoter-G715A-ZI31A-PGK1 terminator in pUSTE18p-G7-cyto-HIS3t | This study|
| pUSTE18p-G7-cyto-Z955-H| URA3-STE18 promoter-G715A-Z955-PGK1 terminator in pUSTE18p-G7-cyto-HIS3t | This study|
| pGK414        | Expression vector containing PGK1 promoter, CEN/ARS single-copy origin and LEU2 marker | 68        |
| pGK-LaZWTc    | ZWT expression, in pGK414 | 51        |
| pGK-LaZK35Ac  | ZK35A expression, in pGK415 | 41        |
| pGK-LaZI31Ac  | ZI31A expression, in pGK415 | 41        |
| pGK-LaZ955c   | Z955 expression, in pGK415 | 41        |
| pGK-LaZWTc-HOP2p| LEU2-PGK promoter -ZWT-PGK terminator -HOP2 promoter in pGK415 | This study|
| pGK-LaZK35Ac-HOP2p| LEU2-PGK promoter -ZK35A-PGK terminator -HOP2 promoter in pGK415 | This study|
| pGK-LaZI31Ac-HOP2p| LEU2-PGK promoter -ZI31A-PGK terminator -HOP2 promoter in pGK415 | This study|
| pGK-LaZ955c-HOP2p| LEU2-PGK promoter -Z955-PGK terminator -HOP2 promoter in pGK415 | This study|
| pGK425-Gpa1N-EGFR(LR) | EGFR834R,L834R expression, in pGK425-Gpa1N | This study|
| pGK425-EGFR(LR)-Stel18C| EGFR834R,L834R expression, in pGK425-Stel18C | This study|
| pGK425-EGFR(LR)-Ste18C| EGFR834R,L834R expression, in pGK425-Ste18C | This study|
| pUMGPTK-Gpa1N-EGFR(LR)| URA3-STE18p-PGK1 promoter -Gpa1N (9 a.a.)-EGFR834R,L834R-PGK1 terminator -kanMX4-STE18 terminator in pGK426-GPTK | This study|
| pUMGPTK-EGFR(LR)-Stel18C| URA3-STE18p-PGK1 promoter-EGFR834R,L834R-Ste18C (9 a.a.)-PGK1 terminator -kanMX4-STE18 terminator in pGK426-GPTK | This study|
| pUMGPTK-EGFR(LR)-Ras1C| URA3-STE18p-PGK1 promoter -EGFR834R,L834R-Ras1C (10 a.a.)-PGK1 terminator -kanMX4-STE18 terminator in pGK426-GPTK | This study|
| pGK413-Grb2-G7| Grb2-G715A fusion expression, in pGK413 | This study|
| pGK416        | Expression vector containing PGK1 promoter, CEN/ARS single-copy origin and URA3 marker | 68        |
| Ste18p-416    | URA3-STE18 promoter-PGK1 terminator in pGK416 | This study|
| pUSTE18p-c-G7-cyto| URA3-STE18 promoter-G715A (w/ stop codon)-PGK1 terminator in pGK416 | This study|
| pUSTE18p-c-G7-cyto-HIS3t| URA3-STE18 promoter-G715A (w/ stop codon)-PGK1 terminator-HIS3 terminator in pGK416 | This study|
| pUSTE18p-Grb2-G7-cyto-HIS3t| URA3-STE18 promoter-Grb2-G715A-PGK1 terminator in pUSTE18p-c-G7-cyto-HIS3t | This study|
| pUSTE18p-Grb2-Grb2-HIS3t| URA3-STE18 promoter-Grb2-G715A-Grb2-PGK1 terminator in pUSTE18p-Grb2-cyto-HIS3t | This study|
| pUSTE18p-Grb2(R86G)-G7-cyto-HIS3t| URA3-STE18 promoter-Grb2(R86G)-G715A-PGK1 terminator in pUSTE18p-Grb2-cyto-HIS3t | This study|
| pUSTE18p-Grb2(E89K)-G7-cyto-HIS3t| URA3-STE18 promoter-Grb2(E89K)-G715A-PGK1 terminator in pUSTE18p-Grb2-cyto-HIS3t | This study|
Table 2. List of plasmids used in this study.

| Plasmids                  | Genotype                                                                 | Reference  |
|---------------------------|---------------------------------------------------------------------------|------------|
| pGK415-HOP2p              | URA3-PGK1 promoter-PGK1 terminator-HOP2 promoter in pGK415               | This study |
| pGK-LsGrb2-HOP            | LEU2-PGK1 promoter-Grb2-PGK1 terminator-HOP2 promoter in pGK415         | This study |
| pGK-LsGrb2[R86G]-HOP     | LEU2-PGK1 promoter-Grb2[R86G]-PGK1 terminator-HOP2 promoter in pGK415   | This study |
| pGK-LsGrb2[E89K]-HOP     | LEU2-PGK1 promoter-Grb2[E89K]-PGK1 terminator-HOP2 promoter in pGK415   | This study |

Protein Grb2 protein for the PPI pair. In normal cells, binding of the epidermal growth factor (EGF) to the extracellular domain of EGFR leads to dimerization of the receptor and autophosphorylation of the receptor intracellular domain. Grb2 binds to the phosphotyrosines of EGFR and links to the activation of subsequent intracellular signaling cascades. In yeast, the intracellular domain of EGFR and its mutant derivatives have been often used to test the interaction with Grb2 protein. To assay the interaction between EGFR and Grb2 in yeast, we used the intracellular domain of EGFR with L834R mutation (EGFRL834Rcyto) that is constitutively dimerized and activated even in the absence of EGF. As the membrane protein by fusing several types of lipidation motifs at both the N-terminus (Gpa1p motif; Gpa1N) and the C-terminus (Ras1p motif; Ras1C and Ste18p motif; Ste18C). The Grb2 adaptor was fused to G\gamma\,cyto at the N-terminus and the C-terminus (G\gamma\,cyto-Grb2 and Grb2-G\gamma\,cyto) were stably integrated into the MC-ErC, MC-EsC and MC-EgN yeast chromosomes, generating ErC-Ggrb(grbG), EsC-Ggrb(grbG) and EgN-Ggrb(grbG) (Table 1) (Fig. S6A–D). As a consequence of GFP transcription assays and mating selection, the engineered strains co-expressing the EGFRL834Rcyto with C-terminal lipidation motifs (Ras1C and Ste18C) and the C-terminally G\gamma\,cyto-fused Grb2 (Grb2-G\gamma\,cyto) specifically showed apparent fluorescence and cell growth on the selective medium (Fig. S6A–F). The accessibility between the phosphotyrosines of membrane-associated EGFRL834Rcyto and the SH2 domains of Grb2 or the distance of G\gamma\,cyto complex from the membrane might have influenced the interactions of these proteins or to the subsequent membrane-anchored effector molecule. Compared with the MC-ErC strain introducing the Grb2-G\gamma\,cyto-expressing autonomous replicating plasmid (pGK413-Grb2-G\gamma\,cyto) (Table 2), the ErC-grbG strain that chromosomally expressed Grb2-G\gamma\,cyto was determinably more suitable for recovering the signaling (Fig. 6A–E).

To further test whether the competitor-introduced G\gamma recruitment system that has designed to select the affinity-enhanced protein variants interacting with membrane target proteins is applicable to the intracellular domain of EGFR, we consistently used the membrane-associated EGFRL834Rcyto and the G\gamma\,cyto-fused Grb2 as membrane target ‘X’ and candidate ‘Y’ proteins, respectively (Fig. 6F). Several Grb2 variants (Grb2, Grb2[E89K] and Grb2[R86G]) with different affinities for the phosphotyrosines of EGFR were utilized for the competitive parental ‘Y’ proteins (K_{\gamma}\,cyto Grb2 > Grb2[E89K] > Grb2[R86G]).

Similar to what was described in the previous section, we tested the new protocol by chromosomally integrating the expression cassettes for Y\gamma\,cyto candidate genes (Fig. 6F). As competitive parental ‘Y’ proteins, the genes for expressing the three different Grb2 variants (Grb2, Grb2[E89K] and Grb2[R86G]) in the cytosol were integrated into the ErC-grbG yeast chromosome (also co-expressing the membrane-associated EGFRL834Rcyto with the Ras1p lipidation motif as target ‘X’ and the Grb2-G\gamma\,cyto fusion protein as candidate ‘Y\gamma\,cyto’), generating ErC-grbG-grb, ErC-grbG-E89K and ErC-grbG-R86G (Table 1). ErC-grbG-LEU yeast strain never expressing any competitor proteins was also generated as positive control (Table 1).

Both flow cytometric analysis and mating selection displayed the consistent results with the Z variants as expected (Fig. 6G,H). When using the strains respectively expressing Grb2[E89K] and Grb2[R86G] as the competitive parental ‘Y’ proteins (ErC-grbG-E89K and ErC-grbG-R86G), the G\gamma\,cyto-fused Grb2 expressed as candidate ‘Y\gamma\,cyto’ (Grb2-G\gamma\,cyto) predictably recovered the signaling in accordance with the order of difference in the affinity strengths between the competitive proteins and the candidate proteins. Similarly, the strain co-expressing the same Grb2 protein as the candidate ‘Y\gamma\,cyto’ and the parental ‘Y’ proteins (ErC-grbG-grb) barely showed GFP fluorescence and cell growth on the selective medium. Thus, we demonstrated that our systems were applicable to the membrane protein, which linked to the cellular states involved in various diseases.

Discussion. In this study, we found that the previously established G\gamma recruitment systems were basically unable to utilize membrane proteins as target protein ‘X’. The new systems described here successfully enable the use of membrane proteins as target ‘X’, both in the conventional (for screening
of PPI candidate 'Y₁' proteins) and competitor-introduced (for screening of affinity-enhanced candidate 'Y₁' protein variants) Gγ recruitment systems. In the new systems, only the protocol for expression of Gγcyto-fused candidate 'Y₁' proteins was changed: instead of autonomous replicating plasmids,
Figure 4. Competitive selection of Z variants with higher affinities for membrane-associated target Fc using previous and new methods for affinity-enhanced systems. (A) Previous affinity-enhanced system for membrane proteins as targets. (B) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated Fc and competitor $Z_{31A}$ as cytosolic ‘$Y_2$’ via stable integration into the yeast chromosome as well as cytosolic Z variants ‘$Y_1$’ fused to $G_{\gamma_{cyto}}$ via autonomous replication plasmids. Control yeast strains lacked the expression of ‘$Y_1$’ fused to $G_{\gamma_{cyto}}$ (transformed with pGK413 mock vector). (C) New affinity-enhanced system for membrane proteins as target. (D,E) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated Fc, competitor cytosolic Z variants ‘$Y_2$’ and cytosolic Z variants ‘$Y_1$’ fused to $G_{\gamma_{cyto}}$ via stable integration into the yeast chromosome. The control yeast shows the strain without the expression of ‘$Y_1$’ fused to $G_{\gamma_{cyto}}$. 
chromosomal integration was employed. These new systems are therefore very simple but highly useful. The results of the intracellular domain of EGFR and Grb2 interaction showed that our Gγ recruitment systems could be exploited as a convenient heterologous system to discern the strong binders to the phosphotyrosines in the intracellular domain of EGFR, and therefore would provide the basis for studying other receptor tyrosine kinases as well. In this manner, the screening of binding partners and affinity-enhanced variants targeted to the inner domains of these membrane proteins has great potential for applications in the treatment of human diseases.

Previously, we demonstrated that Gγ recruitment systems enabled extremely reliable screening that could completely exclude false-positive candidates. Generally, membrane yeast two-hybrid systems and protein fragment complementation assays sometimes exhibit background readouts due to the use of directly fused artificial transcription factors and automatic self-associations of the split proteins. These background readouts are a critical problem, even when they are negligible, especially in the case of growth screening using a large-scale library. The exclusive selection in Gγ recruitment systems is made possible by using the signal transduction machinery, which requires the localization of Gβ/Gγ in GFP transcription assays and mating selection (Figs. 3–5). This extremely disciplined selection machinery makes Gγ recruitment systems worth using.

In the Gγ recruitment system that has designed for membrane proteins as the target, ZDHA with extremely low affinity could not be detected in both cases of the flow cytometric analysis and the mating selection (Fig. 3). Due to the very low affinity between ZDHA and the Fc region (8.0 × 10⁻¹⁰ M⁻¹), the migration of Gγcyto to the membrane was likely insufficient for the recovering of the signal transduction. This affinity (8.0 × 10⁻¹⁰ M⁻¹) seems to be less than a lower limit of our present system, although it is unlikely that a protein mutant exhibiting such extremely low affinity would be required.

From the perspective of screening for a target membrane protein ‘X’, the new methods that chromosomally integrate the DNA cassettes expressing Gγcyto-fused candidate ‘Y₁’ proteins might have a handicap in constructing a library. Specifically, the transformation efficiencies of homologous integrations into the yeast chromosome are commonly 10⁻⁷–10⁻⁸ fold lower than those of autonomous replicating plasmids (approximately 10⁻⁵–10⁻⁶ cfu/µg)⁶⁰–⁶². Therefore, constructing a large-scale library might require a little ingenuity to increase the transformation efficiencies, such as via the use of large amounts of DNA, the electroproporation method⁶¹,⁶³, the spheroplasting method⁶⁴⁶⁵, and use of I-SceI meganuclease⁶⁵. Even allowing for this additional effort, however, the conventional Gγ recruitment system is a powerful tool because of its extremely reliable selection of binding partners. In addition, the competitor-introduced Gγ recruitment system, which allows for the specific screening of affinity-enhanced protein variants (specifically excluding protein variants showing equal or lower affinities⁴¹), is valuable as a unique and irreplaceable growth selection technique.

A similar approach for screening for affinity-attenuated protein variants among membrane proteins serving as target ‘X' made it possible to apply the previous method using autonomous replicating plasmids to express the candidate ‘Y₂' in the cytosol (Fig. 5). We believe that the unstable expression of ‘Y₁'-fused Gγcyto using autonomous replicating plasmids rendered the Gγ recruitment system useless. Because it has been reported that plasmid retentions become unstable during signal-promoted states⁶⁶, ‘Y₁'-fused Gγcyto might be more affected by this unstable plasmid retention than cytosolic ‘Y₂'. In any event, the chromosomal expression of ‘Y₁'-fused Gγcyto is favorable in our Gγ recruitment systems.

In summary, new Gγ recruitment systems make it possible for membrane proteins to be target protein ‘X'. These systems permit reliable and specific screens for binding partners and affinity-enhanced protein variants. We envision that our selection method will provide a powerful, broadly applicable tool for studying biological processes, creating new opportunities to develop new drugs targeting a wide range of membrane-associated proteins and inner domains of transmembrane proteins.

Methods

Strains and media. The genotypes of Saccharomyces cerevisiae BY4741⁶⁷, MC-F1⁶⁵, and BY4742⁶⁷ and the other recombinant strains used in this study are provided in Table 1. The yeast strains were grown in YPD medium containing 1% (w/v) yeast extract, 2% peptone and 2% glucose or in SD medium containing 0.67% yeast nitrogen base without amino acids (BD Diagnostic Systems, Sparks, MD, USA) and 2% glucose. The SD medium was supplemented with amino acids and nucleotides (20 mg/L histidine, 60 mg/L leucine, 20 mg/L methionine, or 20 mg/L uracil), as required by the auxotrophic strains. Agar (2%; w/v) was added to the medium to produce YPD and SD solid media.

Construction of plasmids. All plasmids and primers used in this study are listed in Table 2 and Table S1. Plasmids inserting lipidation motifs were constructed as follows. The fragments of the PGK1 promoter (PPGK) fused to the lipidation motif of Gpa1p (9 a.a. of N-terminus) and the multi-cloning site were amplified from pGK425⁶⁸ using primer 1, primer 2 and primer 3 and inserted into the Xhol-BglII sites of the autonomous replication plasmid pGK425⁶⁸, yielding plasmid pGK425-Gpa1N. The fragments of the PGK1 promoter fused to the lipidation motif of Ste18p (9 a.a. of C-terminus) and the multi-cloning site were amplified from pGK425⁶⁸ using primer 1, primer 4 and primer 5 and inserted into the Xhol-BglII sites of the autonomous replication plasmid pGK425⁶⁸, yielding plasmid pGK425-Ste18C. The fragments of the PGK1 promoter fused to the lipidation motif of Ras1p (10 a.a. of C-terminus) and the multi-cloning site were amplified from pGK425⁶⁸ using primer 1, primer 6 and...
The plasmids used for the expression of the Fc fragment on the membrane were constructed as follows. The fragments encoding the Fc protein were amplified from pUMGP-G\(\gamma\)MFcH42 using primers 8 and 9 or primers 10 and 11 and inserted into the SalI-BamHI sites of the autonomous replication plasmid pGK425-Gpa1N or pGK425-Ste18C, yielding pGK425-Gpa1N-Fc and pGK425-Fc-Ste18C, respectively. The cassettes for expression of the membrane-associated Fc protein for integration at the ste18 locus on the yeast chromosome were then amplified from pGK425-Gpa1N-Fc or pGK425-Fc-Ste18C using primer 12 and primer 13 and inserted into the XhoI sites of pGK426-GPTK42 using an In-Fusion HD Cloning Kit (Clontech Laboratories – Takara Bio, Shiga, Japan), yielding pUMGPTK-Gpa1N-Fc and pUMGPTK-Fc-Ste18C, respectively.

The plasmids used for the expression of the G\(\gamma\)cyto-Z domain variants in the cytosol were constructed as follows. The fragment encoding G\(\gamma\)\(\text{cyto}\) lacking the lipidation sites (G\(\gamma\)\(\text{cyto}\)) was amplified from pUMGP-G\(\gamma\)MFcH42 using primer 14 and primer 15. The fragments encoding the Z variants (ZWT, ZK35A, ZI31A and Z955) were amplified from pGK-LsZWTc, pGK-LsZK35Ac, pGK-LsZI31Ac and pGK-LsZ955c41 using primer 16 and primer 17. The fusion fragments encoding the G\(\gamma\)\(\text{cyto}\)-Z domain were then amplified by overlap PCR using primer 14 and primer 17 and inserted into the SalI-EcoRI sites of the autonomous replication plasmid pGK41368, yielding plasmids pGK413-G\(\gamma\)-EZWT, pGK413-G\(\gamma\)-EZK35A, pGK413-G\(\gamma\)-EZI31A and pGK413-G\(\gamma\)-EZ955, respectively.

The plasmids used for the expression of the G\(\gamma\)\(\text{cyto}\)-Z domain variants in the cytosol were constructed as follows. The fragment encoding G\(\gamma\)\(\text{cyto}\) lacking the lipidation sites (G\(\gamma\)\(\text{cyto}\)) was amplified from pUMGP-G\(\gamma\)MFcH42 using primer 14 and primer 15. The fragments encoding the Z variants (ZWT, ZK35A, ZI31A and Z955) were amplified from pGK-LsZWTc, pGK-LsZK35Ac, pGK-LsZI31Ac and pGK-LsZ955c41 using primer 16 and primer 17. The fusion fragments encoding the G\(\gamma\)\(\text{cyto}\)-Z domain were then amplified by overlap PCR using primer 14 and primer 17 and inserted into the SalI-EcoRI sites of the autonomous replication plasmid pGK41368, yielding plasmids pGK413-G\(\gamma\)-EZWT, pGK413-G\(\gamma\)-EZK35A, pGK413-G\(\gamma\)-EZI31A and pGK413-G\(\gamma\)-EZ955, respectively. Subsequently, the cassettes for expression of the G\(\gamma\)\(\text{cyto}\)-Z variants for integration at the his3 locus on the yeast chromosome were constructed as follows. The fragment containing the STE18 promoter (P\(_{\text{STE18}}\)) and the gene encoding G\(\gamma\)\(\text{cyto}\) were amplified from pUMGP-G\(\gamma\)MFcH42 using primer 18 and primer 19 and inserted into the XhoI-NheI sites of pGK42668, yielding plasmid pUSTE18p-G\(\gamma\)cyto. The fragment encoding HIS3 terminator (T\(_{\text{HIS3}}\)) was amplified from the BY4741 genome using primer 20 and primer 21 and inserted into the NotI-Sacl sites of pUSTE18p-G\(\gamma\)cyto, yielding plasmid pUSTE18p-G\(\gamma\)cyto-HIS3t. Finally, the fragments encoding the Z variants were amplified from pGK-LsZWTc, pGK-LsZK35Ac, pGK-LsZI31Ac and pGK-LsZ955c41 using primer 22 and primer 23 and inserted into the SalI-BamHI sites of pUSTE18p-G\(\gamma\)cyto-HIS3t, yielding plasmids pUSTE18p-G\(\gamma\)cyto-ZWT-HIS3t, pUSTE18p-G\(\gamma\)cyto-ZK35A-HIS3t, pUSTE18p-G\(\gamma\)cyto-ZI31A-HIS3t and pUSTE18p-G\(\gamma\)cyto-Z955-HIS3t, respectively.

Figure 5. Competitive selection of Z variants with lower affinities for membrane-associated target Fc using the previous affinity-attenuated system. (A) Previous affinity-attenuated system for membrane proteins as targets. (B,C) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated Fc and competitor Z\(_{\text{WT}}\) as cytosolic ‘Y\(_1\)’ fused to G\(\gamma\)\(\text{cyto}\) via stable integration into the yeast chromosome and cytosolic Z variants ‘Y\(_2\)’ via autonomous replication plasmids. The control yeast shows the strain without the expression of ‘Y\(_1\)’ fused to G\(\gamma\)\(\text{cyto}\) and cytosolic Z variants ‘Y\(_2\)’.
Figure 6. Competitive selection of Grb2 for membrane-associated intracellular domain of EGFR.

(A) Previous Gγ recruitment system for intracellular domain of EGFR as the membrane target. (B) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated intracellular domain of EGFR L834R mutant (EGFR_L834R_cyto) via stable integration into the yeast chromosome as well as cytosolic Grb2 fused to Gγ cyto ‘Y1’ (Grb2-Gγ cyto) via autonomous replication plasmids. The control yeast shows the strain without the expression of Grb2-Gγ cyto (transformed with pGK413 mock vector).

(C) New Gγ recruitment system for intracellular domain of EGFR as the membrane target. (D,E) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated EGFRL834R,cyto and cytosolic Grb2-Gγ cyto via stable integration into the yeast chromosome. The control yeast shows the strain without the expression of Grb2-Gγ cyto (MC-ErC in Table 1).

(F) New affinity-enhanced system for intracellular domain of EGFR as the membrane target. (G,H) Flow cytometric analyses and mating growth assay. The fluorescence and growth intensities of the engineered strains expressing C-terminally membrane-associated EGFR_L834R_cyto and cytosolic Grb2-Gγ cyto via stable integration into the yeast chromosome. The control yeast shows the strain without the expression of any competitive ‘Y2’ proteins (ErC-grbG-LEU in Table 1). The negative control yeast shows the strain without the expression of ‘Y1’ fused to Gγ cyto.
The cassettes for expression of the cytosolic Z variants as competitors for integration upstream of the HO2 gene locus ($P_{HOP2}$-HOP2 promoter region) on the yeast chromosome were constructed as follows. The fragments encoding $P_{HOP2}$ were amplified using primer 24 and primer 25 and inserted into the NotI-SacI sites of pGK-LsZWTc, pGK-LsZK35Ac, pGK-LsZI31Ac and pGK-LsZ955c, yielding plasmids pGK-LsZW1c-HOP, pGK-LsZK35Ac-HOP, pGK-LsZI31Ac-HOP and pGK-LsZ955c-HOP, respectively.

The plasmids used for the expression of the intracellular domain of EGFR L834R mutant (EGFR$_{L834Rcyto}$) on the membrane were constructed as follows. The fragments encoding the intracellular domain of EGFR$_{L834Rcyto}$ mutant were amplified from the BY4741 strain using primers 26 and 27 or primers 28 and 29 and inserted into the Sall-MluI sites of pGK426-GPTK and pGK426 Ste18C and pGK426 Ras1C, yielding pGK426-Gpa1N-EGFR(LR), pGK426-EGFR(LR)-Ste18C and pGK426-EGFR(LR)-Ras1C, respectively. The cassettes for expression of the membrane-associated EGFR$_{L834Rcyto}$ for integration at the ste18 locus on the yeast chromosome were then amplified from pGK426-Gpa1N-EGFR(LR), pGK426-EGFR(LR)-Ste18C and pGK426-EGFR(LR)-Ras1C using primer 12 and primer 13 and inserted into the Xhol sites of pGK426-GPTK-Gpa1N using an In-Fusion HD Cloning Kit, yielding plasmid pUMGPTK-Gpa1N-EGFR(LR), pUMGPTK-EGFR(LR)-Ste18C and pUMGPTK-EGFR(LR)-Ras1C, respectively.

The plasmids used for the expression of the Grb2-G$_{cyto}$ in the cytosol were constructed as follows. The fragment encoding the Grb2-G$_{cyto}$ was amplified from pB1U-GL$^{68}$ using primer 30 and primer 31 and inserted into the Sall-EcoRI sites of the autonomous replication plasmid pGK413$^{68}$ using an In-Fusion HD Cloning Kit, yielding plasmid pGK413-Grb2-G$_{cyto}$. Subsequently, the cassettes for expression of the Grb2-G$_{cyto}$ for integration at the his3 locus on the yeast chromosome were constructed as follows. The fragment containing the STE18 promoter ($P_{STE18}$) was amplified from pUMGP-G$^{85,14}$-MchI using primer 32 and primer 33 and inserted into the Xhol-Nhel sites of pGK416$^{68}$, yielding plasmid pSTE18p-G$^{85,14}$-MchI. The fragment containing the gene encoding G$_{cyto}$ was amplified from pUMGP-G$^{85,14}$-MchI using primer 34 and primer 35 and inserted into the Xhol-EcoRI sites of Ste18p-416, yielding a plasmid pUSTE18p-$G_{cyto}$-HIS3t. The fragment encoding His3 terminator ($T_{HIS3}$) was amplified from the BY4741 strain using primer 20 and primer 21 and inserted into the NotI-SacI sites of pUSTE18p-$G_{cyto}$-HIS3t, yielding plasmid pUSTE18p-$G_{cyto}$-HIS3t.

The plasmids for the expression of the G$_{cyto}$-Grb2 for integration at the his3 locus on the yeast chromosome were constructed as follows. The fragment encoding Grb2 was amplified from pGK413-Grb2-G$_{cyto}$ using primer 36 and primer 37 and inserted into the Nhel-XmaI sites of pUSTE18p-$G_{cyto}$-HIS3t, yielding plasmid pUSTE18p-$G_{cyto}$-HIS3t.

The plasmids used for the expression of the Grb2 variants as competitors for integration at the upstream of the HO2 gene locus ($P_{HOP2}$-HOP2 promoter region) on the yeast chromosome were constructed as follows. The fragments encoding $P_{HOP2}$ were amplified using primer 20 and primer 21 and inserted into the NotI-Sacl sites of pGK415$^{68}$, yielding plasmid pGK415-HOP2p. The fragment encoding Grb2 was amplified from pGK413-Grb2-G$_{cyto}$ using primers 38 and 39 and inserted into the Sall-XmaI sites of pGK415-HOP2p using an In-Fusion HD Cloning Kit, yielding plasmid pGK415-Grb2-HOP2p. The fragment encoding Grb2$_{BSEG}$ Mutant was amplified from pGK413-Grb2-G$_{cyto}$ using primers 40 and 42, primers 41 and 43 and the fragments encoding the Grb2$_{BSEG}$ Mutant was amplified from these two fragments by overlap PCR using primer 40 and primer 41 and inserted into the Sall-XmaI sites of pGK415-HOP2p using an In-Fusion HD Cloning Kit, yielding plasmid pGK415-Grb2$_{BSEG}$-HOP2p. The fragment encoding Grb2$_{E89K}$ was amplified from pGK413-Grb2-G$_{cyto}$ using primers 40 and 44, primers 41 and 45 and the fragments encoding the Grb2$_{E89K}$ mutant was amplified from these two fragments by overlap PCR using primer 40 and primer 41, and inserted into the Sall-XmaI sites of pGK415-HOP2p using an In-Fusion HD Cloning Kit, yielding plasmid pGK415-Grb2$_{E89K}$-HOP2p.

**Construction of yeast strains.** All strains used in this study are listed in Table 1. Integration of the DNA cassettes for expressing the membrane-associated Fc protein was achieved as follows. The DNA fragments containing $P_{STEV}$-Fgk1-Fc-Ste18C-Tfgk1-kamMX4-Tste18 and $P_{STE18}$-Pfgk1-Gpa1N-Fc-Tfgk1-kamMX4-Tste18 were amplified from pUMGPTK-Fc-Ste18C and pUMGPTK-Gpa1N-Fc using primer 46 and primer 47. The amplified DNA fragments were then used to transform MC-F1$^{69}$ using the lithium acetate method. The transformants were selected on a YPD-G418 plate to yield MC-FC and MC-FN (Table 1).

Integration of the DNA cassettes for expressing the Z variants (Z$_{wt}$, Z$_{K35A}$, Z$_{I31A}$ and Z$_{955C}$) in the cytosol was achieved as follows. The DNA fragments containing URA3-Pfgk1-G$_{cyto}$-ZWT(-ZK35A,-ZI31A and -Z955C)-Tfgk1-HIS3t were amplified from pUSTE18p-G$_{cyto}$-ZWT(-ZK35A,-ZI31A and -Z955C)-HIS3t using primer 48 (containing the homologous regions of the HIS3 promoter) and primer 49. The amplified DNA fragments were used to transform MC-FC and MC-FN using the lithium acetate method.$^{69}$ The transformants were then selected on an SD-Ura plate (containing leucine, histidine and methionine) to yield MC-GW, MC-GK, MC-GI and MC-GN and FN-GW, FN-GK, FN-GI and FN-GN (Table 1).

Integration of the DNA cassettes for expressing the Z variants (Z$_{wt}$, Z$_{K35A}$, Z$_{I31A}$ and Z$_{955C}$) as competitors in the cytosol was achieved as follows. The DNA fragments containing LEU2-Pfgk1-ZWT(-ZK35A,-ZI31A and -Z955C)-Tfgk1-HOP2 were amplified from pGK-LsZW1c(-LsZK35Ac,-LsZI31Ac and -LsZ955c)-HOP2 using primer 50 (containing the homologous regions of $P_{HOP2}$ upstream) and primer 51. The amplified DNA fragments were then used to transform MC-F1$^{69}$ using the lithium acetate method.$^{69}$ The transformants were selected on a YPD-G418 plate to yield MC-FC and MC-FN (Table 1).
DNA fragments were used to transform FC-GW, FC-GK, FC-GI, and FC-G9 and FN-GW, FN-GK, FN-GI and FN-G9. The transformants were then selected on an SD-Leu/-Ura plate (containing histidine and methionine) to yield FC-GGW, FC-GWK, FC-GWI, and FC-GGW; FC-GKW, FC-GKK, FC-GKI, and FC-GK9; FC-GIW, FC-GIK, FC-GII, and FC-GI9; and FC-GGW, FC-GWK, FC-GWI, and FC-GGW as well as FN-GGW, FN-GWK, FN-GWI, and FN-GGW; FN-GKW, FN-GKK, FN-GKI, and FN-GK9; FN-GIW, FN-GIK, FN-GII, and FN-GI9; and FN-GGW and FN-GWK and FN-GWI and FN-GGW (Table 1).

Integration of the DNA cassettes for expressing the membrane-associated intracellular domain of EGFR L834R mutant (EGFR\(_{L834R_{cyto}}\)) was achieved as follows. The DNA fragments containing \(F_\text{STE18}\gamma\text{-EGFR}_{L834R_{cyto}}\gamma\)-Ras1C-TPGK1-kanMX4-\(T_\text{STE18}\gamma\text{-EGFR}_{L834R_{cyto}}\gamma\)-Ste18C were amplified from pGK425-EGFR(LR)-Ste18C using primer 46 and primer 47. The amplified DNA fragments were then used to transform MC-F16 using the lithium acetate method49. The transformants were selected on a YPD+G418 plate to yield MC-ErC, MC-EsC and MC-EgN (Table 1).

Integration of the DNA cassettes for the Grb2-\(G_{cyto}\) in the cytosol was achieved as follows. The DNA fragments containing URA3-\(P_{\text{PGRK}}\text{-Grb2-}G_{cyto}\text{-}T_{\text{PGRK}}\text{-}T_{\text{HIS3}}\) was amplified from pUSTE18p-Grb2-\(G_{cyto}\)-HIS3t using primer 48 (containing the homologous regions of the HIS3 promoter) and primer 49. The amplified DNA fragments were then used to transform MC-ErC, MC-EsC and MC-EgN using the lithium acetate method49. The transformants were selected on an SD-Ura plate to yield ErC-grbG, EsC-grbG and EgC-grbG (Table 1). Integration of the DNA cassettes for the \(G_{cyto}\)-Grb2 in the cytosol was achieved as follows. The DNA fragments containing URA3-\(P_{\text{PGRK}}\text{-Grb2-}G_{cyto}\text{-}T_{\text{PGRK}}\text{-}T_{\text{HIS3}}\) was amplified from pUSTE18p-Grb2-\(G_{cyto}\)-HIS3t using primer 48 (containing the homologous regions of the HIS3 promoter) and primer 49. The amplified DNA fragments were used to transform MC-ErC, MC-EsC and MC-EgN using the lithium acetate method49. The transformants were then selected on an SD-Ura plate to yield ErC-grbG, EsC-grbG and EgC-grbG (Table 1).

Integration of the DNA cassettes for expressing Grb2 variants (Grb2, Grb2\(_{269K}\) and Grb2\(_{268G}\)) and positive control (no competitor expression) as the competitor in the cytosol was achieved as follows. The DNA fragments containing LEU2-\(P_{\text{PGRK}}\text{-}Grb2\text{-}G_{cyto}\text{-}P_{\text{HOP2}}\) and LEU2-\(P_{\text{PGRK}}\text{-}G_{cyto}\text{-}P_{\text{HOP2}}\) were amplified from pGK-LsGrb2(-LsGrb2(R89K) and -LsGrb2(R86G))-HOP and pGK415-HOP2p using primer 50 (containing the homologous regions of \(P_{\text{HOP2}}\) upstream) and primer 51. The amplified DNA fragments were used to transform ErC-grbG. The transformants were then selected on a SD-Leu/-Ura plate to yield ErC-grbG-grb, ErC-grbG-E89K, ErC-grbG-R86G and ErC-grbG-LEU (Table 1).

All transformants were obtained by introducing the autonomous replicating plasmids (Table 2) into these yeast strains using the lithium acetate method49.

**GFP reporter expression analysis.** GFP reporter expression analysis basically followed previous methods41, with certain modifications. In the case of the previous method, the engineered yeast a-cells were grown in 5 mL of SD-His medium (for the PPI detection system), SD-His/-Leu medium (for the affinity-enhanced system) or SD-Leu/-Ura medium (for the affinity-attenuated system) at 30°C overnight. The cultured cells were then inoculated in 2 mL of fresh SD-His, SD-His/-Leu or SD-Leu/-Ura medium containing 5 \(\mu\)M \(\alpha\)-factor (Zymo Research, Orange, CA, USA) to obtain an initial OD\(_{600}\) of 0.1 (OD\(_{600}\) = 0.1). In the case of the new method, the engineered yeast a-cells were grown in 5 mL of YPD medium (for the PPI detection system and affinity-enhanced system) at 30°C overnight. The cultured cells were then inoculated in 2 mL of fresh YPD medium containing 5 \(\mu\)M \(\alpha\)-factor (Zymo Research, Orange, CA, USA) to obtain an initial OD\(_{600}\) of 0.1 (OD\(_{600}\) = 0.1). The expression of the FIG1-EGFP fusion reporter gene was then stimulated by growth at 30°C for 6 hours.

The fluorescence intensities of the cultured cells were measured using a BD FACSCanto II flow cytometer equipped with a 488-nm blue laser (BD Biosciences, San Jose, CA, USA)70. The GFP fluorescence signal was specifically collected through a 530/30-nm band-pass filter. The mean fluorescence intensity was defined as the GFP-A mean of 10,000 cells. The data were analyzed using BD FACSDiva software (version 5.0, BD Biosciences).

**Mating growth spotting assay.** The mating growth spotting assay basically followed a previous method41, with certain modifications. For the previous method, each engineered yeast a-cell was grown in 5 mL of SD-His media (for PPI detection system), SD-His/-Leu medium (for the affinity-enhanced system) or SD-Leu/-Ura medium (for the affinity-attenuated system) at 30°C overnight. The initial OD\(_{600}\) of each haploid cell was set at 0.1 (OD\(_{600}\) = 0.1). For the new method, each engineered yeast a-cell was grown in 5 mL of YPD medium (for the PPI detection system and the affinity-enhanced system) at 30°C overnight and then cultivated in 5 mL of YPD medium with the mating partner, or the BY4742 \(\alpha\)-cell77, at 30°C for 3 hours. The initial OD\(_{600}\) of each haploid cell was set at 0.1 (OD\(_{600}\) = 0.1). After cultivation, the yeast cells were harvested, washed, and resuspended in distilled water. To quantify the mating ability of each strain, a dilution series of each yeast cell suspension was prepared (OD\(_{600}\) = 1.0, 0.1, 0.01, 0.001 and 0.0001), and 40 \(\mu\)L of each dilution was then spotted on a selective SD-Ura/Leu plate (lacking methionine, lysine and histidine; for the PPI detection system generated by the previous method), SD-Ura plate (lacking methionine, lysine, histidine and leucine; for the
affinity-enhanced system generated by the previous method), SD-His plate (lacking methionine, lysine, leucine and uracil; for the affinity-attenuated system generated by the previous method), SD-His/Leu plate (lacking methionine, lysine and uracil; for the PPI detection system generated by the new method) or SD-His plate (lacking methionine, lysine, uracil and leucine; for the affinity-enhanced system generated by the new method).

References

1. Brannigan, J. A. & Wilkinson, A. J. Protein engineering 20 years on. Nat. Rev. Mol. Cell Biol. 3, 964–970 (2002).

2. Valkov, E., Sharpe, T., Marsh, M., Greive, S. & Hyvönen, M. In Top Curr Chem. 11, 145–179 (2011).

3. Adjei, a. a. & Hidalgo, M. Intracellular signal transduction pathway proteins as targets for cancer therapy. J. Clin. Oncol. 23, 5386–5403 (2005).

4. Hopkins, A. L. & Groom, C. R. The druggable genome. Nat. Rev. Drug Discov. 1, 727–730 (2002).

5. Sawyers, C. Targeted cancer therapy. Nature 432, 294–297 (2004).

6. Cardillo, F. & Tortora, G. EGFR antagonists in cancer treatment. N. Engl. J. Med. 358, 1160–1174 (2008).

7. Normanno, N. et al. Epidermal growth factor receptor (EGF) signaling in cancer. Gene 366, 2–16 (2006).

8. Gravalos, C. & Jimeno, A. HER2 in gastric cancer: a new prognostic factor and a novel therapeutic target. Ann. Oncol. 19, 1523–1529 (2008).

9. Nahta, R., Yu, D., Hung, M.-C., Hortobagyi, G. N. & Esteva, F. J. Mechanisms of Disease: understanding resistance to HER2-targeted therapy in human breast cancer. Nat. Clin. Pract. Oncol. 3, 269–280 (2006).

10. Valstola, R. et al. VEGFR-3 and Its Ligand VEGF-C Are Associated With Angiogenesis in Breast Cancer. Am. J. Pathol. 154, 1381–1390 (1999).

11. Siotto, B., Nagy, J. A. & Dvorak, H. F. Anti-VEGF/VEGFR Therapy for Cancer: Reassessing the Target. Cancer Res. 72, 1909–1914 (2012).

12. Tan, S., Tan, H. T. & Chung, M. C. M. Membrane proteins and membrane proteomics. Proteomics 8, 3924–3932 (2008).

13. Overington, J. P. et al. How many drug targets are there? Nat. Rev. Drug Discov. 5, 993–6 (2006).

14. Zhang, J., Yang, P. L. & Gray, N. S. Targeting cancer with small molecule Kv1.5 inhibitors. Nat. Rev. Cancer 9, 28–39 (2009).

15. Höller, C., Freimuth, M. & Nanoff, C. G proteins as drug targets. Cell. Mol. Life Sci. 55, 257–270 (1999).

16. Kimple, A. J., Bosch, D. E., Giguère, P. M. & Siderovski, D. P. Regulators of G-protein signaling and their G0 substrates: promises and challenges in their use as drug discovery targets. Pharmacol. Rev. 63, 728–749 (2011).

17. Butler, D. C., Mclear, J. A. & Messer, A. Engineered antibody therapies to counteract mutant huntingtin and related toxic intracellular proteins. Prog. Neurobiol. 97, 190–204 (2012).

18. Ma, L. et al. Generation of intracellular single-chain antibodies directed against polyepitope GalNAC-transferase using a yeast two-hybrid system. Biochem. Biophys. Res. Commun. 418, 628–633 (2012).

19. Tanaka, T., Lobato, M. N. & Rabbits, T. H. Single domain intracellular antibodies: A minimal fragment for direct in vivo selection of antigen-specific intrabodies. J. Mol. Biol. 331, 1109–1120 (2003).

20. 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. 277, 1982–1993 (2010).

21. Fields, S. & Song, O. A novel genetic system to detect protein-protein interactions. Nature 340, 245–246 (1989).

22. Suh, S. S. & Koide, S. Phage display for engineering and analyzing protein interaction interfaces. Curr. Opin. Struct. Biol. 17, 481–487 (2007).

23. Stynen, B., Tournu, H., Tavernier, J. & Van Dijck, P. Diversity in Genetic In Vivo Protein-Protein Interaction Studies: from the Yeast Two-Hybrid System to the Mammalian Split-Luciferase System. Microbiol. Mol. Biol. Rev. 76, 331–382 (2012).

24. Vidal, M., Braun, P., Chen, E., Boeke, J. D. & Harlow, E. Genetic characterization of a mammalian protein-protein interaction domain by using a yeast reverse two-hybrid system. Proc. Natl. Acad. Sci. USA. 93, 10321–10326 (1996).

25. Vidal, M. & Endoh, H. Prospects for drug screening using the reverse two-hybrid system. Trends Biotechnol. 17, 374–381 (1999).

26. Kondo, E., Suzuki, H., Horii, A. & Fukushige, S. A yeast two-hybrid assay provides a simple way to evaluate the vast majority of hMLH1 germ-line mutations. Cancer Res. 63, 3302–3308 (2003).

27. Young, K. et al. Identification of a calcium channel modulator using a high throughput yeast two-hybrid screen. Nat. Biotechnol. 16, 924–925 (1998).

28. Stagljar, I. et al. A genetic system based on split-ubiquitin for the analysis of interactions between membrane proteins in vivo. Proc. Natl. Acad. Sci. USA 95, 5187–92 (1998).

29. Nakamura, Y., Ishii, J. & Kondo, A. Rapid, Facile Detection of Heterodimer Partners for Target Human G-Protein-Coupled Receptors Using a Modified Split-Ubiquitin Membrane Yeast Two-Hybrid System. PLoS One 8, 2–9 (2013).

30. Broder, Y. C., Katz, S. & Aronheim, A. The ras recruitment system, a novel approach to the study of protein-protein interactions. Curr. Biol. 8, 1121–1124 (1998).

31. Kruse, C., Hanke, S., Vasiliev, S. & Hennemann, H. Protein-protein interaction screening with the Ras-recruitment system. Signal Transduct. 6, 198–208 (2006).

32. Aronheim, A., Zandi, E., Hennemann, H., Elledge, S. J. & Karin, M. Isolation of an AP-1 repressor by a novel method for detecting protein-protein interactions. Mol. Cell. Biol. 17, 3094–3102 (1997).

33. Huang, W., Wang, S. L., Lozano, G. & De Crombrugghe, B. cDNA library screening using the SOS recruitment system. Biotechniques 30, 94–100 (2001).

34. Aronheim, A. Improved efficiency Osu recruitment system: Expression of the mammalian GAP reduces isolation of Ras GTPase false positives. Nucleic Acids Res. 25, 3373–3374 (1997).

35. Bacart, J., Corbel, C., Jockers, R., Bach, S. & Couturier, C. The BRET technology and its application to screening assays. Biotechnol. J. 3, 311–324 (2008).

36. Lalonde, S. et al. Molecular and cellular approaches for the detection of protein-protein interactions: Latest techniques and current limitations. Plant J. 53, 610–635 (2008).

37. Villalobos, V., Naik, S. & Piwnica-Worms, D. Current state of imaging protein-protein interactions in vivo with genetically encoded reporters. Annu. Rev. Biomed. Eng. 9, 321–349 (2007).

38. Shyu, Y. J. & Hu, C. D. Fluorescence complementation: an emerging tool for biological research. Trends Biotechnol. 26, 622–630 (2008).

39. Fields, S. High-throughput two-hybrid analysis: The promise and the peril. FEBS J. 272, 5391–5399 (2005).

40. Pichler, J. New methodologies for measuring protein interactions in vivo and in vitro. Curr. Opin. Struct. Biol. 15, 4–14 (2005).

41. 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 9, e108229 (2014).

42. 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. 276, 2636–2644 (2009).
43. Fukuda, N., Ishii, J. & Kondo, A. Gγ recruitment system incorporating a novel signal amplification circuit to screen transient protein-protein interactions. FEBS J. 278, 3086–3094 (2011).
44. Manahan, C. L., Patmana, M., Blumer, K. J. & Linder, M. E. Dual lipid modification motifs in G(α) and G(γ) subunits are required for full activity of the pheromone response pathway in Saccharomyces cerevisiae. Mol. Biol. Cell. 11, 957–968 (2000).
45. Fukuda, N., Ishii, J., Tanaka, T. & Kondo, A. The competitor-introduced Gγ recruitment system, a new approach for screening affinity-enhanced proteins. FEBS J. 277, 1704–1712 (2010).
46. Nilsson, B. et al. A synthetic IgG-binding domain based on staphylococcal protein a. Protein Eng. Des. Sel. 1, 107–113 (1987).
47. Cedergren, L., Andersson, R., Lanson, B. Uhlen, M. & Nilsson, R. Mutational analysis of the interaction between staphylococcal protein a and human IgG1. Protein Eng. 6, 441–448 (1993).
48. Nordberg, E. et al. Cellular studies of binding, internalization and retention of a radiolabeled EGFR-binding affibody molecule. Nucl. Med. Biol. 34, 609–618 (2007).
49. Fukuda, N. & Honda, S. Rapid Evaluation of Tyrosine Kinase Activity of Membrane-Integrated Human Epidermal Growth Factor Receptor Using the Yeast Gγ Recruitment System. ACS Synth. Biol. 4, 121–129 (2015).
50. Schlessinger, J. Ligand-induced, receptor-mediated dimerization and activation of EGFR receptor. Cell 110, 669–672 (2002).
51. Lemmon, M. A. & Schlessinger, J. Cell Signaling by Receptor Tyrosine Kinases. Cell 141, 1117–1134 (2010).
52. Lowenstein, E. J. et al. The SH2 and SH3 domain-containing protein GRB2 links receptor tyrosine kinases to ras signaling. Cell 70, 431–442 (1992).
53. Batzer, A. G., Rotin, D., Ureña, J. M., Skolnik, E. Y. & Schlessinger, J. Hierarchy of binding sites for Grb2 and Shc on the epidermal growth factor receptor. Mol. Cell. Biol. 14, 5192–5201 (1994).
54. Köhler, F. & Müller, K. M. Adaptation of the Ras-recruitment system to the analysis of interactions between membrane-associated proteins. Nucleic Acids Res. 31, e28 (2003).
55. Gunde, T. & Barberis, A. Yeast growth selection system for detecting activity and inhibition of dimerization-dependent receptor tyrosine kinase. Biotechniques 39, 341–349 (2005).
56. Kohler, F. A co-localization assay for the analysis of protein–protein interactions. Gene 388, 14–18 (2007).
57. Zhang, X., Gureasko, J., Shen, K., Cole, P. A. & Kurtian, J. An Allosteric Mechanism for Activation of the Kinase Domain of Epidermal Growth Factor Receptor. Cell 125, 1137–1149 (2006).
58. Cussac, D., Frech, M. & Chardin, P. Binding of the Grb2 SH2 domain to phosphotyrosine motifs does not change the affinity of its SH3 domains for Sos proline-rich motifs. EMBO J. 13, 4011–4021 (1994).
59. Koegl, M. & Uetz, P. Improving yeast two-hybrid screening systems. Briefings Funct. Genomics Proteomics 6, 302–312 (2007).
60. Manivasakam, P., Weber, S. C., McElver, J. & Schiestl, R. H. Micro-homology mediated PCR targeting in Saccharomyces cerevisiae. Nucleic Acids Res. 23, 2799–2800 (1995).
61. Gietz, R. D. & Woods, R. A. Genetic transformation of yeast. Biotechniques 3, 1458–1463 (1987).
62. Pan, X. et al. A robust toolkit for functional profiling of the yeast genome. Mol. Cell 16, 487–496 (2004).
63. Benatui, L., Perez, J. M., Belk, J. & Hsieh, C. M. An improved yeast transformation method for the generation of very large human antibody libraries. Protein Eng. Des. Sel. 23, 155–159 (2010).
64. Zillig, P. M. & Percival, K. J. Transformation of yeast spheroplasts without cell fusion. Anal. Biochem. 163, 391–397 (1987).
65. Kuijpers, N. G. A. et al. One-step assembly and targeted integration of multigene constructs assisted by the I-SceI meganuclease in Saccharomyces cerevisiae. FEBS Lett. 13, 769–781 (2013).
66. Ishii, J. et al. Yeast-based fluorescence reporter assay of G protein-coupled receptor signalling for flow cytometric screening: FAK-1 disruption recovers loss of episomal plasmid caused by signalling in yeast. J. Biochem. 143, 667–674 (2008).
67. Brachmann, C. B. et al. Designer deletion strains derived from Saccharomyces cerevisiae S288C: A useful set of strains and plasmids for PCR-mediated gene disruption and other applications. Yeast 14, 115–132 (1998).
68. Ishii, J. et al. A simple and immediate method for simultaneously evaluating expression level and plasmid maintenance in yeast. J. Biochem. 145, 701–708 (2009).
69. Gietz, D., St Jean, A., Woods, R. A. & Schiestl, R. H. Improved method for high efficiency transformation of intact yeast cells. Nucl Acid Res 20, 1425 (1992).
70. Ishii, J. et al. Cell wall trapping of autocrine peptides for human G-protein-coupled receptors on the yeast cell surface. PLoS One 7, e37136 (2012).

Acknowledgements
This work was supported in part by a Research Fellowship for Young Scientists and a Grant-in-Aid for Young Scientists (B) from the Japan Society for the Promotion of Science (JSPS).

Author Contributions
M.K., N.F., J.I. and A.K. designed the experiments; M.K. performed the experiments and collected data; M.K., N.F. and J.I. analyzed and interpreted the data; and M.K. and J.I. wrote the manuscript with contributions from N.F. and A.K.

Additional Information
Supplementary information accompanies this paper at http://www.nature.com/srep

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Kaishima, M. et al. Gγ recruitment systems specifically select PPI and affinity-enhanced candidate proteins that interact with membrane protein targets. Sci. Rep. 5, 16723; doi: 10.1038/srep16723 (2015).

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