An improved fluorescent tag and its nanobodies for membrane protein expression, stability assay, and purification

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Green fluorescent proteins (GFPs) are widely used to monitor membrane protein expression, purification, and stability. An ideal reporter should be stable itself and provide high sensitivity and yield. Here, we demonstrate that a coral (\textit{Galaxea fascicularis}) thermostable GFP (TGP) is by such reasons an improved tag compared to the conventional jellyfish GFPs. TGP faithfully reports membrane protein stability at temperatures near 90 °C (20-min heating). By contrast, the limit for the two popular GFPs is 64 °C and 74 °C. Replacing GFPs with TGP increases yield for all four test membrane proteins in four expression systems. To establish TGP as an affinity tag for membrane protein purification, several high-affinity synthetic nanobodies (sybodies), including a non-competing pair, are generated, and the crystal structure of one complex is solved. Given these advantages, we anticipate that TGP becomes a widely used tool for membrane protein structural studies.

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Current strategies for membrane protein structural studies require functional isolation in large quantities but their hydrophobicity brings great challenges in almost every step, such as expression, purification, and crystallization or cryo-electron microscopy\textsuperscript{1-8}. Overcoming these challenges often relies on multilevel optimization. To increase expression or reduce degradation, homologs and mutants sometimes in dozens to hundreds\textsuperscript{8-13}, as well as hosts\textsuperscript{14-22} and culture conditions\textsuperscript{23-27}, are evaluated. To increase stability, numerous constructs by scanning mutagenesis or rational design are screened\textsuperscript{28-35}. To find supportive purification conditions, detergent type\textsuperscript{36-44}, lipid additive type and ratio\textsuperscript{44-46}, as well as salts\textsuperscript{45}, ligands\textsuperscript{46}, and pH\textsuperscript{47,48}, are assessed\textsuperscript{46,48}. Such optimization brings a large number of screen variables, which can be time-consuming and costly. Improving throughput is crucial.

Fluorescent proteins, mostly green fluorescent proteins (GFPs), fit this purpose as a fusion partner for the study of protein of interest (POI). Its bright fluorescence in the visible wavelengths allows real-time monitor of expression level, folding, chromatography efficiency and profile, and stability of membrane protein\textsuperscript{27,46,50}. Of particular interest is the fluorescence-detection size exclusion chromatography (FSEC)-based thermostability assay (FSEC-TS)\textsuperscript{46} for measuring apparent melting temperature (T_m). This method is generally applicable; i.e., does not require specific enzymatic or ligand-binding assays. An additional advantage is that the assay can be done without purification, the failure of which is sometimes the very reason for stability assays in the first place. Since its introduction to the field in 2012, FSEC-TS has been used successfully, along with mutagenesis, for the thermostabilization of several membrane proteins for structural studies\textsuperscript{46,51-54}. With an automated HPLC system, ~100 constructs/conditions can be screened in an overnight run.

One limitation of the most commonly used GFPs, however, is their modest thermostability. It fails to serve as a reporter for FSEC-TS of membrane proteins with apparent T_m exceeding the T_m of GFP itself which is ~76 °C (10-min heating)\textsuperscript{46}. While this appears to be reasonably high, it is important to note that the actual T_m may be far below the apparent T_m (or more appropriately termed as T_aggregation) because proteins can undergo denaturation long before aggregation\textsuperscript{37,53}. Therefore, sometimes conditions leading to apparent T_m of higher-than-76 °C need to be sought. FSEC-TS assays in such cases have relied on intrinsic tryptophan fluorescence\textsuperscript{46}. This inevitably requires the purification of POIs, which could be challenging for membrane proteins. A more stable fluorescent protein for FSEC-TS assays at high temperatures will be welcome.

We were curious to see whether a thermostable GFP (TGP, from coral)\textsuperscript{56-58} can fulfill this demand. Here, our systematic survey study with several membrane proteins demonstrates that TGP is indeed a robust and reliable FSEC-TS reporter as it enables assays at ~90 °C with T_m values in close agreement with established methods. Our data also show improved expression for TGP fusion of four test membrane proteins in four systems. High-affinity synthetic nanobodies (sybodies) against TGP have been generated using ribosome and phage display\textsuperscript{59}, enabling purification of a membrane protein to comparable purity (~90%) with that from immobilized metal affinity chromatography. The binding mode of one of the sybodies to TGP has been revealed in detail by X-ray crystallography. The overall superior characteristics of TGP over GFPs, together with the sybody tools, should encourage the use of TGP as a fusion partner for not only membrane proteins but may also for soluble proteins.

Results

TGP was brighter than the conventional GFPs. Two GFP variants, commonly used as fusion tags in Escherichia coli\textsuperscript{50,80} (ecGFP, NCBI AGT98535.1) and Saccharomyces cerevisiae\textsuperscript{27}, insect cells\textsuperscript{61,62}, and mammal cells\textsuperscript{63} (scGFP, NCBI AFI82039.1), were used for comparison. The engineered Azami-Green\textsuperscript{55-58}, although also a GFP, is referred to as TGP. In a plate reader with excitation/emission wavelength pair of 488/512 nm, the brightness ratio was 1: 0.84: 0.43 (TGP: ecGFP: scGFP) (Fig. 1a), agreeing with the fact that TGP has a higher extinction coefficient (ε\textsubscript{493} TGP = 64,600 M\textsuperscript{-1}cm\textsuperscript{-1}, ε\textsubscript{490} ecGFP = 49,550 M\textsuperscript{-1}cm\textsuperscript{-1}) and a higher quantum yield (Φ\textsubscript{TGP} = 0.66, Φ\textsubscript{ecGFP} = 0.60) than GFPs\textsuperscript{46}. In in-gel fluorescence, TGP was as bright as ecGFP, and twice as bright as scGFP, with a ratio of 1: 0.99: 0.46 (TGP: ecGFP: scGFP) (Fig. 1b, c, Supplementary Fig. 1a). The slight discrepancies between the two methods may be due to different filter settings between the two systems, or possible differences in their response to the chaotropic sodium dodecyl sulfate (SDS) in the electrophoresis buffer. In addition, the scGFP contains mutations\textsuperscript{27} that cause yellow-shift of the excitation wavelength (λ_max = 502 nm), which is partly responsible for its relatively low intensity in both counting and in-gel fluorescence analysis.

TGP was more stable than GFPs. As expected, TGP displayed the highest T_m at 95.1 °C, which was 18.0 °C above ecGFP and 26.7 °C above scGFP (Fig. 1d). Because membrane proteins are generally solubilized in detergents for purification, we also measured their T_m in dodecylmaltoside, a mild detergent, and lauryldimethylamine oxide, a harsher zwitterionic detergent, at 1%(w/v) concentration. The T_m for fluorescent proteins decreased by 1.8–2.6 °C in dodecylmaltoside (Fig. 1d) and by 4.9–5.3 °C in lauryldimethylamine oxide (Fig. 1d), reflecting the modest chaotropic effects of these detergents.

TGP was a robust and reliable reporter for stability assays. To test TGP as a reporter for stability assays, its T_m was measured using FSEC-TS (Fig. 1e, Supplementary Fig. 2a) and compared to that obtained by fluorescence counting. Similar to previous findings for GFPs\textsuperscript{43,46}, the T_m values by the two methods were similar (Fig. 1e), suggesting FSEC-TS was reliable for monitoring TGP unfolding. Consistent with the fluorescence counting method, TGP showed higher stability than both GFPs (Fig. 1e, Supplementary Fig. 2b, 2c). Notably, the main-peak profile remained unchanged even after heating at 99 °C (Supplementary Fig. 2d), which is an important characteristic for a reporter.

Next, we performed three sets of experiments to test its reliability as a FSEC-TS reporter. First, we compared the FSEC-TS profiles between GFP and TGP using three membrane protein fusions: a microbial (Mycobacterium smegmatis) homolog of the human Δ8–7 sterol isomerase (msSI, 29.0 kDa, six predicted transmembrane helices) expressed in E. coli, and the human Δ8–7 sterol isomerase (hsSI, 26.4 kDa, five transmembrane helices) and the human CDP-diacylglycerol-inositol 3-phosphatidyltransferase (PIS, 23.5 kDa, four transmembrane helices) expressed in S. cerevisiae. As shown in Fig. 2a, b, and Supplementary Fig. 3a, 3b, the FSEC-TS melting profile for msSI and PIS did not show notable changes upon the replacement of GFP by TGP. For hsSI, the TGP signal decreased at a lower rate than scGFP, displaying a ~5-°C increase in the apparent T_m (Fig. 2c, Supplementary Fig. 3c). The reason for this will be discussed.

Second, we compared the FSEC-TS profile of membrane proteins with and without TGP fusion using purified samples, which are necessary for the tryptophan-based assay. For this we tested hsSI and its homolog from Thermosthlmomyces thermophilus (rhs)\textsuperscript{64}. As shown in Fig. 2d and Supplementary Fig. 3d, 3e, the TGP-based pseudo-melting curve for the fusion protein was nearly superimposable with the tryptophan-based curve for the
TGP-free protein in both of the cases. Therefore, the TGP fusion did not change the apparent $T_m$ of tSI or hSI.

Third, an orthogonal validation of TGP for FSEC-TS was performed using the ‘gold-standard’ enzymatic assay of the glycerol 3-phosphate acyltransferase from Staphylococcus pneumoniae (SpPlsY, 22.9 kDa, seven transmembrane helices). The melting profiles from the two methods were overall similar but differed in fine details (Fig. 2e). Specifically, the activity dropped gradually upon heating, whilst the fluorescence increased before dropping. Compared to the enzymatic assay, the FSEC-TS curve entered the denaturation phase later but fell more quickly, resulting in a slightly lower $T_m$ (by ~5 °C). Taken together, the results demonstrated that TGP was an overall faithful FSEC-TS reporter despite slight variations.

Next, we tested the robustness of TGP for FSEC-TS assay using the ultra-stable PlsY from Aerococcus aquifex (aaPlsY, 20.9 kDa, 7 transmembrane helices), which has a half-life of 30 min at 90 °C as revealed by an activity-based assay.65 As shown in Fig. 2f and Supplementary Fig. 3f, the fluorescence of aaPlsY-TGP remained unchanged at 85 °C. Because both aaPlsY (Fig. 2f) and TGP (Fig. 1e) started to unfold at higher temperatures, the drop of fluorescence beyond 85 °C could be a result of denaturation of either or both proteins. Thus, the apparent $T_m$ of 91.1 °C, although agreeing with the activity-based $T_m$ value, may or may not be the true apparent $T_m$ of aaPlsY. Nevertheless, it showed that the FSEC profile of TGP, in this case as a fusion to a membrane protein, was unchanged at 85 °C. As an additional example, we repeated the experiment with the thermostable conSI (α-sterol Δ8-7 isomerase thermostabilized by consensus mutagenesis64). The result showed that TGP fluorescence only dropped by ~20–30% at 90 °C, reporting an apparent $T_m$ value of 94.4 °C (Supplementary Fig. 4), which was close to that of TGP itself (Fig. 1e). Again, the FSEC shape remained unchanged even at 99 °C (Supplementary Fig. 4d). Thus, fusing to membrane proteins did not change the stability or FSEC behavior of TGP. Based on this, we suggest the upper limit for TGP-based FSEC-TS assays be at 90 °C. As expected, GFPs, even the more stable ecGFP, was incapable of monitoring membrane protein unfolding.

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Fig. 1 TGP was brighter and more stable than GFPs. a Linear correlation between fluorescence (in a relative unit, RFU) and concentration (in a 0.2-mL solution) with indicated slope (RFU for 1 mg L$^{-1}$ of fluorescent protein). Mean and standard deviation of three independent experiments are plotted. b In-gel fluorescence image with inverted colors. M: home-made fluorescent markers. Data are representative of two independent experiments. c Linear correlation between integrated in-gel fluorescence (in an arbitrary unit, AU) and loading using data in b and T as a linear correlation of fluorescent proteins in the presence of dodecylmaltoside. Graph depicts mean of three technical replicates. The unit (°C) for $T_m$ is omitted for all panels. In this report, all samples were heated for 20 min for $T_m$ measurement unless stated otherwise. In panels a, c, and e, black triangle denotes TGP, red square denote ecGFP, and blue circle denotes scGFP. In d, TGP traces are black, ecGFP traces are red, and scGFP traces are blue; Solid line with square denotes samples without detergents, dashed line with triangle denotes DDM samples, and dotted line with circle denote LDAO samples. DDM dodecylmaltoside, FSEC fluorescence-detection size exclusion chromatography, GFP green fluorescent protein, LDAO lauryldimethylamine oxide, TGP thermostable GFP, $T_m$ melting temperature.
at such temperatures, falsely reporting an under-measured $T_m$ of 73.5 °C (Fig. 2f). Thus, the results showed TGP was an improved FSEC-TS reporter owing to its ultrastability.

Replacing GFP with TGP improved membrane protein expression in *E. coli*. By now we had demonstrated TGP as an improved reporter for FSEC-TS at high temperatures. Enabling high-level expression of POI is another characteristic of an ideal fusion partner. Recently, it has been reported that the yield of membrane proteins expression in mammalian cells can vary by up to 5 folds when fused with different GFP variants. As part of the characterization, the effect of TGP on membrane protein expression was also compared to GFPs in four expression systems.

Three different methods were used for assessment: fluorescence counts of cell culture, in-gel fluorescence of cell lysate or membranes, and FSEC of solubilized fluorescent fusion proteins. The fluorescence counting method is quick but risks overestimation because the calculation does not exclude free GFP. In-gel fluorescence reveals the integrity of fusion proteins but is less accurate because the densitometry analysis is only semi-quantitative with a relatively narrow linear range. Besides, it gives little information for membrane protein folding because of the presence of denaturing detergent SDS. The FSEC method, although slightly more time-consuming, can provide more relevant information about membrane protein quality. Owing to the chromatographic separation, the profile reflects monodispersity and degradation severity. Therefore, we introduce Yield$_{FSEC}$ as a relative term to compare the effect of fluorescent protein on meaningful membrane protein expression levels (See Methods). To this end, the peak density corrected by their respective fluorescence-concentration relationship was used for comparison. Because only the fraction that was soluble in the mild detergents and eluted at a meaningful internal pore volume was used, it should provide both quantitative and qualitative information about the fusing membrane proteins.

We first ran the test for the *E. coli* system using *aaPlsY* and msSI. Based on fluorescence counting, replacing ecGFP with TGP increased the expression level to 2.5 folds for *aaPlsY* (Fig. 3a) and 5 folds for msSI (Fig. 3b). In both cases, in-gel fluorescence analysis showed a major band corresponding to the molecular weight of the fusion protein (Fig. 3c, d, Supplementary Fig. 1b, 1c), and densitometry analysis showed a ~1.5-fold expression level with TGP over ecGFP (Fig. 3c, d, Table 1). In addition, the FSEC intensity of the TGP fusion was higher than the ecGFP fusion: 1.8-fold for *aaPlsY* (Figs. 3e) and 2.5-fold for msSI (Fig. 3f), corresponding to 1.7-fold and 2.3-fold in Yield$_{FSEC}$ (Table 1), respectively. Importantly, the FSEC profiles were superimposable between the two fluorescent tags (Fig. 3g, h), suggesting tag type had no impact on the gel permeation behavior for both membrane proteins.

Replacing GFP with TGP improved membrane protein expression in *S. cerevisiae*. The test for the *S. cerevisiae* system was performed with *hSI* and PIS. Although no notable differences were observed between the TGP- and scGFP-fusion proteins based on fluorescence counting (Fig. 4a, b), in-gel fluorescence (Fig. 4c, d, Supplementary Fig. 1d, 1e) and Yield$_{FSEC}$ analysis (Fig. 4f, Table 1) showed a 2-fold expression level with TGP compared to scGFP for both membrane proteins. Similar to the *E. coli* system, the FSEC retention profile was unaffected by tag type for both *hSI* and PIS (Fig. 4g, h).

Replacing GFP with TGP improved membrane protein expression in insect cells. When tested in insect cells, replacing scGFP with TGP increased the expression of *hSI* (Fig. 5a–d) by 2 folds, based on the in-gel fluorescence (Fig. 5b, Supplementary Fig. 1f) and Yield$_{FSEC}$ analysis (Fig. 5c, Table 1). This trend was also observed for PIS, which showed a 1-fold increase when replacing scGFP with TGP (Fig. 5e–h, Supplementary Fig. 1g, Table 1). Again, the FSEC profile of the membrane proteins were the same regardless of the fluorescent tags, although more fractional degradation was observed for the TGP-tagged membrane proteins (Fig. 5d, h).
Replacing GFP with TGP improved membrane protein expression in mammalian cells. TGP showed remarkable improvement for hSI expression in the mammalian system. First, for maximal transient expression level, lower amount of plasmid was required for hSI-TGP (2 mg L\(^{-1}\)) than for hSI-scGFP (3 mg L\(^{-1}\)). When assessed under optimal conditions, the yield for hSI-TGP was over 10 folds compared with hSI-scGFP using the three aforementioned quantitative methods (Fig. 6a–d, Supplementary Fig. 1h, Table 1). The low level for hSI-scGFP (1.0 mg L\(^{-1}\)) could mean a challenging project for most crystallography groups because of high costs with the large-scale mammalian expression for membrane protein production in milligram quantities. By contrast, that for hSI-TGP (11.1 mg L\(^{-1}\)) would make a promising structural project.

Notably, the FSEC signal for hSI-scGFP was invisibly flat when plotted in scale with hSI-TGP (Fig. 6c) due to the combinational effect from weak expression and low brightness associated with scGFP. The difference in peak intensity between the two was 38 folds, translating to a ~13-fold difference in Yield\(_{\text{FSEC}}\), which was in close agreement with the other two methods. Despite the yield differences, the two hSI forms displayed nearly superimposable FSEC profiles (Fig. 6d), again suggesting a similar overall fold.

Higher expression level with TGP in comparison to scGFP was also observed for PIS, although not as dramatic as for hSI. As judged by fluorescence counting, switching from scGFP to TGP increased the expression level of PIS to 3.8 folds (Fig. 6e). In-gel fluorescence (Fig. 6f, Supplementary Fig. 1i) and FSEC results (Fig. 6g) confirmed this trend, displaying a 2-fold difference (Table 1). Despite the overall superimposable profiles (Fig. 6h), the free TGP peak was much more evident than scGFP, a trend also seen in in-gel fluorescence. Quantification of the peaks showed that a quarter of TGP-fusion protein was degraded, which means the gap between the expression yields could have been larger had the degradation be controlled.

TGP sybodies enabled affinity purification of membrane proteins. As part of the GFP toolkit, single-chain camelid antibodies (nanobodies)\(^{67,68}\) and designed ankyrin repeat proteins (DARPins)\(^{69}\) are available for affinity chromatography, contributing to the purification of several high-profile membrane proteins for structure determination\(^{70-75}\). To develop equivalents for TGP, we selected synthetic nanobodies (sybodies) from a recently established library\(^{59}\) using a strategy that combines ribosome display and phage display. Since the concave sybody library\(^{59}\) was randomized on the basis of a GFP nanobody known as the enhancer (PDB 3K1K), which bound sideways and contains a short complementarity determining regions 3 (CDR3), this library was used for performing the selections. After three rounds of panning with successively lower TGP concentrations, libraries with enriched binders were subcloned from the phage display system to expression plasmids for screening at a single-colony level.

![Graphs and images](image-url)
Table 1 Comparison of TGP with GFPs for FSEC-TS assay and membrane protein expression.

| Expression host | Protein | Fusion | App. $T_m$ (°C)a | Yield (mg L$^{-1}$)b | In-gel fluorescence | FSEC | Yield (%)c | Yield (%)d |
|-----------------|---------|--------|-------------------|----------------------|---------------------|------|------------|------------|
|                 |         |        |                   |                      | Intst. (AU)          |      | Intst. (%) | Intst. (%) |
| E. coli         | ααPlsY | TGP    | 91.1              | 11.1 ± 0.2           | 100 ± 15.2          | 100 ± 8.4 | 100 ± 8.4 |
|                 | ecGFP  |        | 73.5              | 4.4 ± 0.1            | 60.6 ± 14.3         | 55.1 ± 6.7 | 60.5 ± 6.1 |
| msSl            | TGP    |        | 33.3              | 16.4 ± 0.3           | 100 ± 17.0          | 100 ± 15.3 | 100 ± 15.3 |
| S. cerevisiae   | hSI    | TGP    | 32.0              | 2.9 ± 0.1            | 56.9 ± 7.9          | 39.2 ± 3.6 | 43.1 ± 4.0 |
|                 | ecGFP  |        | 44.3              | 1.6 ± 0.1            | 213.6 ± 6.7         | 14.3 ± 0.7 | 43.3 ± 2.1 |
|                 | scGFP  |        | 40.5              | 1.0 ± 0.0            | 21.4 ± 4.6          | 14.1 ± 0.8 | 42.7 ± 2.4 |
|                 | TGP    |        | /                 | /                    | /                   | /    | 100 ± 10  | 100 ± 10  |
|                 | scGFP  |        | /                 | /                    | 6.0 ± 0.2           | 17.4 ± 2.5 | 37.8 ± 5.4 |
| Sf9             | hSI    | TGP    | /                 | /                    | /                   | /    | 100 ± 11  | 100 ± 11  |
|                 | scGFP  |        | /                 | /                    | 12.5 ± 0.3          | 24.7 ± 3.5 | 47.8 ± 3.2 |
|                 | TGP    |        | /                 | /                    | /                   | /    | 100 ± 12  | 100 ± 12  |
|                 | scGFP  |        | /                 | /                    | 7.0 ± 0.2           | 24.7 ± 3.5 | 47.8 ± 3.2 |
|                 | scGFP  |        | /                 | /                    | 1.0 ± 0.1           | 3.0 ± 0.5  | 7.8 ± 0.4  |
|                 | TGP    |        | /                 | /                    | 2.3 ± 0.5           | 100 ± 28.3 | 100 ± 18.3 |
|                 | scGFP  |        | /                 | /                    | 0.6 ± 0.1           | 22.0 ± 1.6 | 43.0 ± 3.0 |

Fig. 4 Replacing GFP with TGP improved expression of two human membrane proteins in S. cerevisiae. Assessment of expression level was based on fluorescence counts (a, b), in-gel fluorescence (c, d), and relative FSEC intensities (e, f). Normalized FSEC traces are shown in g, h. Mean and standard deviation (a, b) or a representative (c-f) of three independent experiments on different colonies are shown. In e, g, f, and h, TGP traces are shown as black solid lines and scGFP traces are shown as red dashed lines. FSEC fluorescence-detection size exclusion chromatography, GFP green fluorescent protein, TGP thermostable GFP.

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*A Apparent $T_m$ was calculated by sigmoidal dose-response fitting of data from single experiments.

*Yield, as mean ± standard deviation, was calculated based on the fluorescence counts of cells from three independent experiments on different colonies for bacterial and yeast expression, and on cells with different passage numbers for baculovirus and mammalian expression.

*Percent yield relative to membrane protein-TGP was calculated based on integrated arbitrary intensity (intst, AU) corrected with calibrated quantity-intensity correlation. Results (mean ± standard deviation) are from three independent experiments as in b.

*Percent yield relative to membrane protein-TGP was calculated using intensity corrected with calibrated quantity-fluorescence correlation. Data (mean ± standard deviation) are from three independent experiments as in b.
Prescreening of 188 colonies using a pull-down assay (see Methods) identified 86 hits that showed higher TGP fluorescence in the elution compared to the negative control. Further screening for earlier retention volumes of TGP FSEC upon incubation with periplasmic extracts identified four binders (Fig. 7a, Supplementary Fig. 5). TGP-sybody complexes also formed in preparative size exclusion chromatography (Fig. 7b, Supplementary Fig. 1j). Biolayer interferometry assays showed that the sybodies bound to TGP with affinities in the range of 3.3–10.4 nM (Fig. 7c–f). The sybodies could all be purified from E. coli with relatively high yield ranging from 10–48 mg L\(^{-1}\).

The feasibility of using the sybodies to purify TGP-fusion membrane proteins was demonstrated using msSI-TGP expressed in E. coli. To this end, purified sybody 44 (Sb44) was conjugated to a solid support by amine coupling. msSI-TGP was then purified by standard affinity chromatography protocols, and TGP-free msSI was released from the affinity beads by enzymatic digestion at a pre-engineered 3C protease site. The purity of msSI was comparable to that purified using immobilized metal affinity chromatography (Fig. 7c, Supplementary Fig. 1k, 1l). Consistent with this, unlike those reported for GFPs, none of the sybodies changed the fluorescent property of TGP.

Nanobodies recognizing different parts of GFPs have been reported in literature\(^{76}\). Such nanobodies could be used to construct biparatopic fusion to enhance affinity\(^{69,77}\). To seek such nanobodies for TGP, we performed FSEC analysis of the Sb66-TGP complex in the presence of other binders. This identified Sb92 as a noncompeting partner for Sb66 for TGP-binding (Fig. 7h).

Crystal structure of the TGP-Sb44 complex. For further characterization, we obtained crystals of TGP-Sb44 (Supplementary Fig. 6) which diffracted to 2.0 Å at the synchrotron. The structure of the complex (Fig. 8, Table 2) was solved by molecular replacement using existing TGP and nanobody structures\(^{58,59,78}\) as search models. The asymmetric unit contained two TGP-Sb44 heterodimers, which were highly similar (C\(\alpha\) root mean square deviation of 0.29 Å). We focused on one TGP-Sb44 dimer (Chain IDs C and D) for structural analysis.

The Ca root mean square deviation between the previous TGP-alone structure (PDB 4TZ\(\alpha\)\(^{58}\)) and the Sb44-bound form was 0.20 Å. Thus, the binding did not cause noticeable conformation change of TGP, reflecting the ultrastability and thus structure rigidity of TGP. Consistent with this, Sb44 binds to one side of the TGP barrel (Fig. 8a) with the surfaces showing electrostatic complementarity (Fig. 8b).
Interestingly, although Sb44 binds at approximately the opposite side of the barrel compared to the GFP nanobody enhancer (PDB 3K1K) (Supplementary Fig. 7a), its paratope surface is also concave-shaped (Supplementary Fig. 7b–d) as anticipated by the design of the concave sybody library59. A PISA79 analysis showed an interface (835.6 Å²) that was larger than those for the GFP nanobodies68 (enhancer, 672 Å²; minimizer, 652 Å²). As typically observed for nanobodies, all three CDRs were involved in the recognition of the epitope (Fig. 8c–e, Supplementary Fig. 8, Supplementary Fig. 9) consisting of TGP residues from the β-strand 2, 4, 5, 6, and 9, which are 3-dimensionally close but primarily distant. Similar to the GFP enhancer nanobody, several scaffold (non-CDR) residues of Sb44 also participated in the binding. In detail, the interaction was mediated by two salt bridges, nine hydrogen bonds, and a rich network of hydrophobic interactions made of apolar residues and the hydrocarbon part of several hydrophilic side chains (Fig. 8d, e). Peculiarly, six of the nine hydrogen bonds involved backbone amino and carbonyl groups.

**Discussion**

FSEC-TS assays for fluorescently tagged membrane proteins are best performed at temperatures where the tags are largely unaffected (~25% drop of fluorescence) so that the decrease of fluorescence can be largely attributed to the aggregation/precipitation of protein of interest (POI). We recommend the upper limit for FSEC-TS assay as ~90 °C for TGP, ~74 °C for ecGFP, and ~64 °C for scGFP in the presence of dodecylmaltoside. Therefore, TGP broadens the FSEC-TS temperatures drastically.

Previously, FSEC-TS assays at high temperatures (>76 °C) rely on tryptophan fluorescence of pure proteins, which, for membrane proteins, can be difficult to obtain46. Alternatively, they can be performed with the modestly-stable GFPs in harsher detergents, typically those with shorter chains such as decylmaltoside and octylglucoside. Accordingly, the stability of membrane protein, but probably not of GFPs, drops, allowing Tm measurement under comprising conditions for membrane proteins. While arguably straightforward, such experiments, bring disadvantages. First, the POI may not be stable at all in harsh detergents. Second, high concentrations need to be used for shorter-chain detergents because of their high critical-micelle-concentrations. This could increase the cost of FSEC-TS by one to two orders of magnitude. Third, because purifications are usually performed in mildest detergents possible, the results obtained with harsh detergents would be less informative to guide the design of purification strategies. The use of TGP avoids such problems.

The FSEC-TS trace increases before dropping for some TGP-fusion proteins (Fig. 2b, e, f), but not for all (Fig. 2a, c, d), a phenomenon also seen in literature43,46,64. Because the increase was not observed for the free TGP, and because it appeared at different temperatures for different membrane proteins, we...
propose it is not intrinsic for TGP. Instead, it probably reflects the folding states of POI. It has been reported that GFP fluorescence can respond to subtle environmental differences caused by POI unfolding (before aggregation), and the resulted changes in intensity (decrease in those cases) can be used directly to monitor unfolding by differential scanning fluorimetry. In our assays, the rise-phase, appearing just before the drop-phase (aggregation), might also reflect local denaturation of POIs before aggregation.

The $T_m$ values measured with GFP and TGP for the same POI were mostly consistent. However, a 5-°C increase in apparent $T_m$ was observed for hSI-TGP compared with hSI-scGFP. Because hSI and the fluorescent proteins are unrelated and exist as distinct domains, it is very unlikely that the stability of hSI was intrinsically enhanced by fusion to a more stable partner (TGP); rather, like a stable chaperon, TGP may have prevented the fusion protein as a whole from precipitating during heating, thus showing a higher apparent $T_m$ or in essence, $T_{\text{aggregation}}$.

We note a 18-°C decrease of the apparent $T_m$ for hSI-TGP after purification (Fig. 2d, 31.2 °C) compared with that obtained using the membrane solubilization fraction (Fig. 2c, 49.4 °C). This is probably caused by the likely delipidation events during chromatography steps. The loss of stability after purification was also observed for PIS, in which case the PIS-TGP fusion protein precipitated during our attempts in isolating pure TGP-fused and TGP-free PIS for stability comparison as was done for ttSI and hSI (Fig. 2d).

As an indirect assay, how apparent $T_m$ values would differ from actual $T_m$ values (as determined by functional assays) is always a topic of general interest. It is widely accepted that such apparent $T_m$ values should be treated with caution. Only when denaturation coincides with aggregation would the values be the same. Occasionally, membrane proteins, even for those with a high apparent $T_m$ in the fusion form, precipitate upon the removal of the fluorescent protein, suggesting protective effect of these stable tags on membrane proteins. For crystallization, it may require changing to fluorescent tag-free constructs so that the POI did not experience compositional shock during later purification steps. Despite these limitations, FSEC-TS should be considered when absolute $T_m$ values are not necessarily required, for example, in the screening of stable homologs or mutants for structural study, or screening of lipids, ligands for purification, owing to its simplicity, general applicability, and high-throughput feature.

Interestingly, the $T_m$ of spPlsY measured by FSEC-TS only differed slightly from that measured by enzymatic assays. Probably, as a relatively small protein without multi-domains, its unfolding followed a simple path, and it remained active until heat-induced aggregation.

All the tested membrane proteins displayed higher expression levels with TGP compared to GFPs in both prokaryotic and eukaryotic systems. For hSI, substituting GFP with TGP enhanced the expression level by a remarkable 10 folds when expressed in mammalian cells. The mechanism for this remains to be studied. Possibly, the fusion with a rock-stable partner helps the membrane proteins escape the surveillance system for cellular degradation.

The increase of POI expression by the replacement of TGP for GFP should not be interpreted such that the TGP tag always increases expression of POIs in comparison with their tag-free forms. Including a chaperon may be beneficial for heterologous expression, but this will inevitably increase energy expenditure of the host. In addition, depending on the POIs, tags may interfere with POI’s folding and localization, and hence may influence
negatively on production yield\textsuperscript{14}. Therefore, the effect of fluorescent protein tags on membrane protein expression should be tested on a case-by-case basis, when needed.

We note that, although revealing the same trend in expression level, the fluorescence counting, in-gel fluorescence, and Yield\textsubscript{FSEC} methods sometimes gave inconsistent results. This is partly caused by the fact that these assays used different fractions. In some cases, the in-gel fluorescence and Yield\textsubscript{FSEC} experiments used membranes removal of free fluorescent proteins, while the fluorescence counting method did not exclude them. Overall, for comparison, we credit the Yield\textsubscript{FSEC} more than the other two methods due to reasons mentioned in the Results section, and we use this now routinely in the lab for the optimization of expression conditions.

While the generality of high expression remains to be tested, the gained sensitivity due to TGP’s high brightness should be beneficial for fluorescence-based assays, especially with membrane proteins that express poorly in costly eukaryotic or in vitro systems. This is especially true in comparison to scGFP. For example, to evaluate the yield of membrane protein-GFPs, enough signal, usually 10 times over the background of medium (150–280 relative fluorescence units depending on the medium types) are needed. This corresponds to 6–11 mg L\textsuperscript{−1} of expression level in the case of scGFP. Since membrane protein expression levels are usually below this, cells are concentrated by centrifugation and resuspension before fluorescence measurement to enhance the signal-to-noise ratio. The use of TGP should enable such measurements with less cell culture or costly cell-free reagents, by a factor of 2, when at a same expression level. Similarly, the loading for TGP-based FSEC can be reduced by a factor of 3, lowering the risk of clogging expensive columns.

Although the TGP form here has been monomerized, signs of dimer were observed in in-gel fluorescence assays (Fig. 1b) even with fused membrane proteins (Figs. 3c, d, 4c, d, 5b, f, 6b, f). In line with this, TGP showed a peak at 3.02 mL, consistent with a dimer based on calibrated molecular weight. Interestingly, the putative dimer resisted high temperatures up to 80 °C (Supplementary Fig. 2a).
The previous crystal structure contains four TGP protomers (Supplementary Fig. 10a) in the asymmetric unit while our structure contains two protomers that are inserted (Supplementary Fig. 10b). Comparing the structures revealed that the surface involved in protomer interactions in the previous structure (PDB 4TZA) overlaps with that in our structure (Supplementary Fig. 10c, 10d). Because the two interacting protomers from the two structures (Pc and Pc') had different orientations (Supplementary Fig. 10c), the interface may be a result of nonspecific crystal artifacts. Nevertheless, this common interface should be considered for mutation should further TGP monomerization be needed. Interestingly, the other major interaction surfaces for protomers in the previous structure was exploited by Sb44 for binding (Supplementary Fig. 10e, 10f).

In summary, the work here presents encouraging results for TGP as a replacement of current GFP tags for membrane protein production, purification, and stability measurement.

Methods

Molecular cloning. The coding sequence of TGP (PDB entry 4TZA, originally named as ‘Azami-Green’) was synthesized by overlapping PCR using oligos that are typically 59-pb long with 19–20 bp of annealing region. The gene encoding msl (NCBI WP_058126696.1) was amplified from a cDNA clone (9BC001444) using primer pairs 5'-GGGACCATGCGAGTTTCTGAGAACAAACCTGCTGAG-3' and 5'-GAGCTTGGTACTGCTGAGGACCTGCGCGG-3'. The PCR product was digested with BamHI and KpnI and ligated into vector p3EG and pETSG. The plasmid for his (NCBI WP_006570.1) expression was constructed previously. The gene encoding PIS (NCBI NP_006310.1) was amplified from a cDNA clone (9BC001444) using primer pairs 5'-GGGACCATGCGAGTTTCTGAGAACAAACCTGCTGAG-3' and 5'-GAGCTTGGTACTGCTGAGGACCTGCGCGG-3'. The PCR product was digested with BamHI and KpnI and ligated into vector p3EG and pETSG. The fragments of his and PIS were also subcloned into vectors of p3EG and pETSG for insect cell expression, and PmG and pPfTSG for expression in mammalian cells. Schematic maps of the vectors are shown in Supplementary Fig. 11.

Protein purification—GFPs and TGP. ecGFP was expressed in E. coli as a C-terminally His-tagged protein. BL21 (DE3) cells carrying the plasmid pEG14 was induced at OD_{600} of 0.6–0.8 for 16 h at 30 °C. Cells were suspended in a lysis buffer containing 150 mM NaCl, 50 mM Tris-HCl pH 8.0 and passed through a constant cell disruptor (Cat. TS 0.75KW, Constant System, Daventry, UK) at 25 kpsi for 3 times. The lysate was heated at 65 °C for 10 min, cooled in ice-water, and clarified by centrifugation at 20,000 × g for 30 min at 4 °C. The supernatant was incubated with 4 mL of Ni-NTA resin for 2 h with gentle agitation at 4 °C. The beads were loaded into a gravity column and washed with 20 column volume (CV) of lysis buffer supplemented with 50 mM imidazole. ecGFP was eluted using 250 mM imidazole in the lysis buffer. scGFP was expressed in Pichia pastoris GSS15 (lab collection, genotype his4) as a C-terminally His-tagged protein. The strain was checked by its auxotrophic phenotype. Cells carrying the plasmid pZG14 were cultured in 10 mL YPD medium in a 50-mL tube for culturing at 250 rpm at 30 °C. After 24 h, the cells were seeded into fresh 1 L BMGY medium (2% (w/v) peptone, 1% (w/v) yeast extract, 1.34% (w/v) glycerol, 100 mM potassium phosphate pH 5.5). When OD_{600} reached 15–20, cells were collected by centrifugation and resuspended with BMMY medium (replacement glycerol in BMGY medium with 0.5% (w/v) methanol) to OD_{600} of 3–4 for protein expression. For compendium for metabolic consumption, methanol was added again to a 0.5% (w/v) after 24 h. Cells were harvested and resuspended in the same lysis buffer as for ecGFP, and lysed by passing through a constant cell disruptor three times at 25, 28, and 30 kpsi. The purification was carried out as for ecGFP except that no heating was performed.

The expression and purification of TGP was performed using the same procedure as for ecGFP except that the lysate was heated at 80 °C instead of 65 °C before affinity chromatography. The concentration of GFPs was determined using the molar extinction coefficient of 22,015 M⁻¹ cm⁻¹ with absorbance measured at 280 nm. The concentration of TGP was determined the same way with the molar extinction coefficient of 31,985 M⁻¹ cm⁻¹ at 280 nm.

Protein purification—BirA (Uniprot P06709). E. coli BL21 (DE3) cells carrying the plasmid pET-BirA (protein sequence: His6-SSGLVPRGSH-BirA) (a kind gift from Professor Ying Gao at the authors’ institute) were induced with 0.1 mL IPTG for 15 h at 18 °C. Cell pellet from 1 L culture was resuspended with 80 mL lysis buffer containing 150 mM NaCl, 5% (v/v) glycerol, 1 mM TCEP, 20 μg mL⁻¹ DNase I, 5 mM MgCl₂, 1 mM PMSF, 10 mM imidazole, 50 mM Tris-HCl pH 8.0, and lysed with a cell disruptor. After clarification, the supernatant was stirred with 2 mL Ni-NTA resin at 4 °C for 2 h. The resin was washed with 50 mL buffer containing 150 mM NaCl, 5% (v/v) glycerol, 0.2 mM TCEP, 10 mM imidazole, 50 mM Tris-HCl pH 8.0. The protein was eluted with 300 mM imidazole. ecGFP was expressed in the periplasmic extracts, the suspension was centrifuged at 4 °C for 30 min and pellets were discarded. The supernatant was incubated with 150 mM NaCl, 5% (v/v) glycerol, 0.2 mM TCEP, 10 mM Tris-HCl pH 8.0, aliquoted, flash-frozen with liquid nitrogen and stored at −80 °C.

Protein purification—sbyodies. The expression of sbyodies in flasks was carried out by scaling-up the small-scale protocols. Cells were lysed by osmotic shock as follows. Biomass from 1 L of culture was resuspended in 20 mL TES buffer (0.5 M sucrose, 0.5 M EDTA, and 0.2 M Tris-HCl pH 8.0) for dehydration at 4 °C for 0.5 h, followed by rehydration by dilution with 40 mL of ice-cold MilliQ H₂O at 4 °C for 1 h. To collect the periplasmic extracts, the suspension was centrifuged at 25,000 × g for 4 °C for 30 min and pellets were discarded. The supernatant was adjusted to have 150 mM of NaCl, 2 mM of MgCl₂ and 20 mM of imidazole. Ni-NTA resin (2 mL), pre-equilibrated with 20 mM imidazole (all buffer contained 130 mM NaCl and 20 mM HEPEs pH 7.5), was added into the supernatant for batch binding with stirring at 4 °C for 1.5 h. The resin was packed into a gravity column and washed with 20 CV of 30 mM imidazole. The sbyody was eluted using 300 mM imidazole.

Expression of fluorescantly tagged membrane proteins in E. coli. The expression of allIFS with different fluorescent protein tags was carried out as below.

BL21 (DE3)/pETSG cells carrying pEG14-paP41 or pETSG-paP41 were cultured in Luria-Bertani (LB) medium (0.5%(w/v) yeast extract, 1%(w/v) peptone, 1%(w/v) NaCl) plus 50 mg L⁻¹ kanamycin in a 37 °C shaking incubator for overnight. This starter culture was 1:100 seeded into fresh LB-kanamycin medium for growth at 37 °C. When OD_{600} reached 0.6, IPTG was added to a final concentration of 50 μM, and the temperature was dropped to 20 °C. After 16 h of induction, cells were harvested by centrifugation at 4000 × g for 10 min at 4 °C, flash-frozen in liquid nitrogen, and stored at −80 °C until use.
27 °C, cells from 10 mL of culture were harvested by centrifugation at 300 × g for 3 min at 4 °C. The supernatant was either taken directly for stability assay or saved for TGP assay. Cells were then resuspended in 50 mM Tris-HCl pH 8.0. The fractional counts or peak intensities were plotted against temperatures. Data from a single experiment were fitted with a sigmoidal dose-response function. The Tm values are reported from the fitting.

**Stability assay---TGP- or GFP-based FSEC-TS for membrane proteins**. For FSEC-TS assay, 0.1 mL of solubilized fraction was heated at various temperatures on a PCR cycler for 20 min. The samples were cooled on ice for 10 min, and centrifuged at 21,000 × g for 30 min at 4 °C to remove precipitates before FSEC analysis. Depending on the expression yield, 1–5 mL of samples were loaded onto a Sepax Zenix C SEC-300 column (Cat. 233300–4630, Sepax Technologies, Newark, DE, USA) connected to a Shimadzu HPLC machine equipped with a fluorescence detector (RF-20A, Shimadzu) with excitation/emission wavelength of 470/512 nm. FSEC running buffers were the following. auPiY and msI, 0.3%(w/v) DDM, 0.2 mM TCEP, 150 mM NaCl, and 50 mM Tris-HCl pH 8.0. IFL, 0.3%(w/v) DDM, 0.1 mM EDTA, 0.2 mM TCEP, 150 mM NaCl, and 50 mM Tris-HCl pH 8.0, 0.1% (w/v) DDM, 0.1 M NaCl, and 50 mM Tris-HCl pH 8.0, 0.1% (w/v) DDM, 0.1 M NaCl, and 50 mM Tris-HCl pH 8.0. 0.1% (w/v) DDM, 0.1 M NaCl, and 50 mM Tris-HCl pH 8.0. Tm was determined from the zero absorbance as the temperature at which the absorbance at 450/466 nm was reduced to 50% of its maximum value. The absorbance at 280 nm of the sample was monitored at 1°C intervals. The percentage activity to the unheated sample was plotted against temperature.

**Stability assay---tryptophan-based FSEC-TS for tSI and hSI**. tSI was purified for tryptophan-based FSEC-TS assay as follows. Membranes from 4 L of S. cerevisiae cells expressing tSI-TGP were solubilized in buffer containing 1%(v/v) DDM, 0.2%(w/v) CHS, 500 mM NaCl, 1 M PMSE, 1 M TCEP, 0.1% (w/v) protease inhibitor cocktail, 20 mM HEPES pH 7.5; sPlpY, 0.03% LMNG, 150 mM NaCl, 50 mM Tris-HCl pH 8.5.

Membrane isolation and solubilization (for fluorescent protein-based FSEC-TS assay). Membranes were isolated as follows. For E. coli, cells from 10 mL of culture were resuspended in 1 mL of Buffer A (150 mM NaCl, 10%(v/v) glycerol, 0.1 mM EDTA, 0.2 mM TCEP, 0.2% (w/v) protease inhibitor cocktail, 50 mM Tris-HCl pH 8.0) for centrifugation using an ultracentrifuge homogenizer (Cat. SC2000; SBI Singapore, Singapore) and a 2-mm probe at 30% power for 10 min in ice-water with on/off cycles of 3 s. For S. cerevisiae, cells from 10 mL of culture were resuspended in 1 mL of Buffer A, incubated with 0.4–0.5 g of 0.3-micron precored glass beads (Cat. 150005 G, Aoran Technology, Shanghai, China), and mechanically broken using a Precellys Evolution homogenizer (Cat. P000062-PEVO0-A, Bertin, Germany) at 8000 rpm for 30 × 5 times. Between each cycle, the cells were cooled down on ice for at least 3 min. Insect cell and mammalian cells were lysed with solubilizing detergents. Membranes were isolated from the cell lysate by centrifugation at 55000 rpm (~150,000 × g) using a Beckman TL-55 rotor for 1 h at 4 °C.

auPiY was solubilized in buffer containing 1%(w/v) dodecylmaltoside (DDM), 150 mM NaCl, 10%(v/v) glycerol, 0.1 mM EDTA, 0.2 mM TCEP, and 50 mM Tris-HCl pH 8.0. msI was solubilized in buffer containing 1%(w/v) DDM, 150 mM NaCl, 0.1% (w/v) protease inhibitor cocktail, and 50 mM Tris-HCl pH 8.0. hSI was solubilized in the same buffer. PlsY was solubilized using 0.9%(v/v) Triton X-100, 5%(v/v) glycerol, 0.5 mM DTT, 10 mM PMSE, 30 mM MgCl2, 1 M mMMSo, and 20 mM Tris-HCl pH 8.5. tSI and conSI were solubilized in 1%(w/v) DDM, 0.2%(w/v) CHS, 500 mM NaCl, 1 M PMSE, 1 M TCEP, 0.1% (w/v) protease inhibitor cocktail, 2 mM imidazole, 50 mM Tris-HCl pH 8.0. The increase of fluorescence (excitation and emission wavelength of 425/466 nm) as the readout of sPlpY activity was monitored at 30 °C in a plate reader for 1 h at 30 intervals. The percentage activity to the unheated sample was plotted against temperatures for curve fitting.

Yield calculation—fluorescence counting. Cells from 1 mL of culture were collected in a 1.5-mL Eppendorf tube by centrifugation at 12000 × g for 3 min at 4 °C. Cell pellets were resuspended in 120 μL of buffer (150 mM NaCl, 50 mM Tris-HCl pH 8.0) for fluorescence measurement using a black 96-well plate in a SpectraMax M2e plate reader (Molecular Devices, San Jose, CA, USA) with excitation/emission wavelength pair of 488/512 nm. The fluorescence counts corrected for dilution factors were used to calculate expression yield based on a calibrated linear quantity-intensity relationship of fluorescent protein standards (Fig. 1a). Three independent experiments were performed using different colonies or transfection of cells with different passage numbers.

Yield calculation—in-gel fluorescence. Membranes were solubilized in SDS-loading buffer for electrophoresis. SDS-PAGE gels were imaged directly using a ChemiDoc MP (Bio-Rad) with the GFP-channel setting. Fluorescence markers prepared in-house were included alongside of membrane protein fusion samples. Relative intensities were integrated directly for quantification of fluorescence protein expression. The relative yield was calculated using integrated intensities applied with the correction factors based on the quantity-intensity relationship for each fluorescent protein.
Three independent experiments were performed using different colonies or transfection of cells with different passage numbers.

**Yield calculation—Yield<sub>SEC</sub>** To calculate Yield<sub>SEC</sub>, the relative fluorescence intensity of the same fluorescent proteins were incubated in the plate and analyzed using an FACSCalibur (Becton Dickinson) flow cytometer. The average fluorescence intensity (MFI) of the mixture was calculated. For each condition, at least 100,000 events were collected with an acquisition time of 10 min. The relative intensity was at 1: 0.91: 0.33 (TG: ecGFP: scGFP). This correction factor was applied to the peak intensities before calculating the relative Yield<sub>SEC</sub>. For example, a ratio of fluorescence intensity at 1: 2 translates to a Yield<sub>SEC</sub> ratio of 0.66: 1 (TG: scGFP). Three independent experiments were performed using different colonies or transfection of cells with different passage numbers.

**Sybody selection—ribosome display and phage display.** To facilitate sybody selection, TG-P was engineered to contain an Avi-tag (GLNDIFEAQKIEWHE), followed sequentially by the 3C protease site (LEVLFQGP), and an octa-histidine tag at the C-terminus. This form (TG<sub>Avi</sub>) was purified the same way as described above. For biotinylation, 4.5 mg mL<sup>−1</sup> of TG<sub>Avi</sub> was incubated with 13 mg mL<sup>−1</sup> of His-tagged BirA, 0.72 mg mL<sup>−1</sup> of His-tagged 3C protease, 5 mM ATP, 10 mM magnesium acetate, 0.23 mM biotin, 100 μM NaCl, 20 mM HEPES pH 7.4. The solution was incubated with 0.5 mL pre-equilibrated Ni-NTA resin and rotated at 4 °C for 1.5 h to remove His-tagged 3C protease and BirA. The flow-through fraction containing biotinylated TG<sub>Avi</sub> (free of His-tag) was loaded onto a Superdex 200 10/300 GL column pre-equilibrated with buffer containing 150 mM NaCl, 20 mM Tris-HCl pH 8.0 to remove ATP and biotin. Fractions with A<sub>280</sub> were pooled, adjusted to 0.6 mg mL<sup>−1</sup> aliquoted, flash-frozen with liquid nitrogen, and stored at −80 °C.

In vitro translation of the 'concave' library was performed as follows. A mix (PUREfect 2.1 kit, Cat. PF213-25-EX, Genefrontier, Chiba, Japan) containing 1.8 μg of plasmid DNA, 4 μL of solution II and 1 μL of solution III, 0.5 μM 10 mM cysteine, 0.5 μM 10 mM cysteine, 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mM cysteine, and 0.5 μM 10 mL of buffer containing 150 mM NaCl, 20 mM HEPES pH 7.4. After a 2 h pre-translation incubation at 37 °C, the mixture was translated in a black 96-well plate under 'top-read' mode to avoid interference from beads. As a positive control, a GFP nanobody<sup>14</sup> fused to GST was immobilized to glutathione resin to pull down His-tagged GFP in the same setup. A sybody against the maltose-binding protein<sup>15</sup> was used as a negative control.

**Sybody selection—FSEC assay to screen TGP binders.** Periplasmic extracts described above were mixed with 1 μg of TGP. One microliter of the mixture was loaded onto a Sepharose-6 FastPhire 300 column (Cat. 233300-4630, Sepharose Technologies, Newport, DE, USA) for analytical FSEC in the running buffer containing 50 mM NaCl and 50 mM Tris-HCl pH 8.0. A sybody against the maltose-binding protein<sup>16</sup> was used as a negative control.

**Biolayer interferometry assay.** To measure kinetics for TGP-sybody binding using biolayer interferometry assay with an Octet system, biotinylated TG<sub>Avi</sub> was immobilized onto a streptavidin SA sensor (Fortebio, Cat. 18-5019) by soaking the sensor in TG<sub>Avi</sub> (2 μg mL<sup>−1</sup>) solution in a buffer containing 0.05%<sub>v/v</sub> Tween 20, 150 mM NaCl, and 20 mM HEPES pH 7.5. After a balance phase in sybody-free buffer, SA wafers were incubated in various sybody concentrations at 27 °C, and an Octet system. After stabilization, 20 μL of the bound sybody complexes were eluted from the sensor with 200 μL of elution buffer (500 mM imidazole, 150 mM NaCl, 50 mM Tris-HCl pH 8.0). TGP fluorescence was measured with 100 μL of elution in a black 96-well plate under 'top-read' mode to avoid interference from beads. As a positive control, a GFP nanobody<sup>14</sup> fused to GST was immobilized to glutathione resin to pull down His-tagged GFP in the same setup. A sybody against the maltose-binding protein<sup>15</sup> was used as a negative control.

**Purification of msiT-GTP—by conjugated sybody 44.** 84-bp resin was prepared by incubating SB44 (4 mg) with 1 mL of swelled CNBr-activated Sepharose 4B beads (Cat. 17043001, GE Healthcare) pre-equilibrated with buffer containing 0.5 mM NaCl and 50 mM Tris-HCl pH 7.4. The solution was then washed extensively with 150 mM NaCl, 20 mM HEPES pH 7.5 before use.

Cells expressing msiT-GTP were disrupted using a Constant cell disruptor. The cell lysate was centrifuged at 20,000 × g for 30 min.

Membranes were sedimented by ultracentrifugation at 150,000 × g for 30 min, and resuspended in 16 mL of solubilized buffer (1%<sub>v/v</sub>) (DDM, 150 mM NaCl, 10%<sub>v/v</sub>) (glicer, 1 mM dodecylamine, 0.2 mM TCEP, 0.1× protease inhibitor cocktail, 50 mM Tris-HCl pH 8.0). After 2 h of mild agitation at 4 °C for solubilization, unsolubilized pellets were discarded by centrifugation at 48,000 × g for 1 h. The supernatant was then washed extensively with 150 mM NaCl, 20 mM HEPES pH 7.5 before use.

Purification of msiT-GTP was performed using a TALON resin column.

**Cryocrystallization of the TGP-SB44 complex.** The TGP construct for crystallization contained residues 1–218 of TGP with a C-terminal octa-histidine tag spaced with a (SGGO<sub>2</sub>)<sub>4</sub> linker. TGP and sybody were mixed at a molar ratio of 1:1, and the complex was purified using SEC in 150 mM NaCl, 20 mM HEPES pH 7.5. Fractions of the complex were pooled, concentrated to 20 μg mL<sup>−1</sup> using 10-KD cut-off filtration membranes (Cat. UFC501096, Merck Millipore, Burlington, MA, USA). Sitting drop crystallization was performed by first pipetting 70 μL of precipitant solution into each well as reservoir, followed by depositing 150 μL of the precipitant solution on top of 150 μL of protein with a Crystal Gryphon LCP robot (Art Robbins Instruments). Crystals were incubated at 20 °C in a Rock Imager R1000. Crystals were obtained in condition containing 0.2 mM ammonium acetate, 25%<sub>v/v</sub> polyethylene glycol 3,350, 0.1 M Bis-Tris pH 6.5.

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<sup>13</sup> Communications Biology | https://doi.org/10.1038/s42003-020-01478-z | www.nature.com/commsbio
Data collection and structure determination. Crystals were transferred sequentially from sitting drop wells to 2-μL drops (on a glass slide) of mother liquor supplemented with 2.5, 5, and 7.5% (v/v) glycerol using a MicTeGen loop (Cat. MS-L185P series, Ithaca, NY, USA). Crystals were allowed to equilibrate with the glycerol gradients for 5–10 s before sequential crystallization. Cryo-cooling was performed by the rapid plunge of cryo-protected crystals into liquid nitrogen. Crystals were screened by X-ray diffraction at beamlines 18U1 and 19U1 at Shanghai Synchrotron Radiation Facility. Diffraction data were collected with a 50 × 50 μm beam on a Pilatus detector at a distance of 300 mm, with oscillation of 0.5° and a deviation. The data were from four independent experiments. Independent experiments refer to experiments starting with different bacterial and yeast colonies, or cells with different passage numbers for insect cells and mammalian cells. Statistical analyses were performed using GraphPad Prism 8. Error bars in all figures refer to standard deviation.

Statistics and reproducibility. All experiments for the measurement of expression levels were replicated three times independently. The FSEC-TS data in Fig. 2e were from four independent experiments. Independent experiments refer to experiments starting with different bacterial and yeast colonies, or cells with different passage numbers for insect cells and mammalian cells. Statistical analyses were performed using GraphPad Prism 8. Error bars in all figures refer to standard deviation.

Reporting summary. Further information on research design is available in the Nature Research Reporting Summary linked to this article.

Data availability. Atomic coordinates and structure factors for the reported TGP-Sb4 structure are deposited in the Protein Data Bank (PDB) under accession codes of 6L2Z. Relevant plasmids and sequences have been deposited in Addgene (www.addgene.org) with the following IDs: pETSG, 159418; pYTSG, 159419; pETSG, 162389; pETSG, 159420; pSB_init_SB4, 159421; pSB_init_S66, 159422; pSB_init_S68, 159423; pSB_init_S892, 159424. Source data for Figs. 1a,c,d,e, Fig. 2, Figs. 3a,b-e, Figs. 4a,b-e, Fig. 5a,c,d,e,g, h, Figs. 6a,c,d,e,g,h, and Figs. 7c–f are available through Supplementary Data 1. There are no restrictions on data availability.

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