Molecular switching system using glycosylphosphatidylinositol to select cells highly expressing recombinant proteins

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Although many pharmaceutical proteins are produced in mammalian cells, there remains a challenge to select cell lines that express recombinant proteins with high productivity. Since most biopharmaceutical proteins are secreted by cells into the medium, it is difficult to select cell lines that produce large amounts of the target protein. To address this issue, a new protein expression system using the glycosylphosphatidylinositol (GPI)-anchor was developed. PGAP2 is involved in processing GPI-anchored proteins (GPI-APs) during transport. In PGAP2 mutant cells, most GPI-APs are secreted into the medium. Here, we established a HEK293 cell line where endogenous PGAP2 was knocked out and exogenous PGAP2 was inserted with a piggyBac transposon in the genome. Using these cells, human lysosomal acid lipase (LIPA) and α-galactosidase A (GLA) were expressed as GPI-anchored forms (LIPA-GPI and GLA-GPI) and cells expressing high levels of LIPA-GPI or GLA-GPI on the cell surface were enriched. Removal of the PGAP2 gene by piggyBac transposase or FLP recombinase converted LIPA-GPI and GLA-GPI from membrane-bound to the secreted forms. Thus, cells expressing LIPA or GLA in large amounts could be enriched using this approach. The GPI-based molecular switching system is an efficient approach to isolate cells expressing recombinant proteins with high productivity.
unsaturated fatty acid at the \(sn\)-2 position on GPI is eliminated by PGAP3 (Post-GPI-attachment to proteins factor 3), followed by the transfer of a saturated fatty acid to the \(sn\)-2 position of the GPI lipid \(^{16,17}\). PGAP2 is required for the latter reaction. In \(PGAP2\) mutant cells, the fatty acid is not transferred to the \(sn\)-2 position on the GPI lipid and lyso-forms of GPI-APs are transported to the cell surface \(^{17}\). The lyso-forms of GPI-APs cannot stably associate with the plasma membrane and are released from the cell surface to medium. The released GPI-APs are further cleaved by phospholipase D (PLD) like activity \(^{17,18}\).

The GPI anchor is becoming an increasingly important tool for protein expression and cell membrane engineering \(^{19}\). When a GPI-attachment signal is added to the C-terminus of secretory proteins, the proteins are expressed as GPI-APs. Therefore, it is possible to express a wide range of recombinant proteins on the cell surface through GPI-anchors \(^{20,21}\). In recent years, several studies have focused on using GPI-anchors for tethering proteins to the cell surface and for their incorporation into extracellular vesicles and virus like particles (VLPs) \(^{20,22,23}\). Attempts have been made to use GPI-anchored recombinant proteins in the extracellular vesicles and VLPs for biomedical applications, for example, cancer immunology and vaccination \(^{19}\).

Here, we developed a mammalian protein expression system using GPI-anchoring. In this system, recombinant proteins are expressed as GPI-anchored forms on the cell surface. Therefore, highly expressing cells can be easily enriched with cell sorters by staining of the GPI-anchored proteins on the cell surface. By removing the \(PGAP2\) gene, the GPI-anchored recombinant proteins attached to the membrane are released into the medium. Cells highly expressing recombinant lysosomal acid lipase (LIPA) and \(\alpha\)-galactosidase A (GLA) were realized using this system. The GPI-based protein expression system was found to be efficient for isolating cells producing recombinant proteins.

**Results**

**Generation of PGAP2-KO cells.** One of the issues in recombinant protein production in mammalian cells is to select cell lines that are stably expressing recombinant proteins at high levels. Most biopharmaceutical proteins are soluble secretory proteins and are thus secreted by cells into the medium. Although there are cells...
present in the cell population that are producing high levels of recombinant protein, it is often challenging to select such a high-producing cell line from the bulk cell population. To overcome this issue, we tried to develop a new system to select cells with high protein productivity from a mixture of cells. In this system, we used GPI anchoring of proteins (Fig. 1A). When the secretory target protein is expressed using this system, the proteins are expressed as GPI-anchored forms (Fig. 1B). Therefore, cells expressing high amounts of a target protein can be enriched by sorting the cells for the GPI-anchored form. Finally, the GPI-anchored membrane-bound form can be switched to the secretory form by disruption of the GPI biosynthetic pathway.

To achieve this disruption, the **PGAP2** gene in HEK293 cells was knocked out using CRISPR/Cas9. **PGAP2** is required for the addition of a saturated fatty acid to lyso-GPI-anchors during the GPI fatty acid remodelling reaction in the Golgi (Fig. 1A)17. Deficiency of this protein results in the transport of lyso-GPI-APs, which are sensitive to PLD. Consequently, significant parts of GPI-APs are secreted into the medium17. As expected, when the **PGAP2** gene was knocked out in HEK293 cells, surface expression of CD59, a ubiquitously expressed GPI-AP, was decreased significantly (Fig. 2A). The KO target region was amplified from the genome and sequenced, showing that there was a deletion of 66 nucleotides in **PGAP2**-KO3.

**Rescue of the PGAP2 gene using PB-mediated insertion to the PGAP2-KO cells.** The piggy-Bac (PB) transposon system allows reversible transgenesis and precise re-excision from the genome26, 27. The **PGAP2** gene was rescued by a plasmid carrying the **PB** transposon. The plasmid pPB-FRT-PuroΔTK-PGAP2 construct includes the **PGAP2** gene and the puroΔTK selection cassette flanked by **PB** and **FRT** sequences (Fig. 3A). **PGAP2**-KO cells were co-transfected with pCMV-hyPBase expressing the **PB** transposase (**PB**ase) and pPB-FRT-PuroΔTK-PGAP2 to integrate the **PGAP2** gene into the genome (Fig. 3A). After transfected cells were treated with puromycin, surviving cells were diluted and clonal cell lines were obtained. When the **PB** transposon...
is used for gene integration into the genome, several copies of the construct may be found in a cell. However, a cell line that contains only one PB copy number in the genome was desired for further purposes. Among the clonal cells, we chose one cell line, named HEK293pB-PGAP2 clone P15 (P15), which rescued the expression of CD59 on the cell surface and had only one PB insertion site in the genome (Fig. 3B,C). The PB element containing the PGAP2 gene was inserted at the TTAA sequence of the intron of the SBF2 gene located on chromosome 11 (Fig. 3D).

Since the integrated PGAP2 fragment is flanked by FRT sites and the PB transposon at both ends, the PGAP2 gene can be re-excised by FLP recombinase or PBase (Fig. 4A). In addition, puroΔTK was used for gene integration into the genome, several copies of the construct may be found in a cell. However, a cell line that contains only one PB copy number in the genome was desired for further purposes. Among the clonal cells, we chose one cell line, named HEK293pB-PGAP2 clone P15 (P15), which rescued the expression of CD59 on the cell surface and had only one PB insertion site in the genome (Fig. 3B,C). The PB element containing the PGAP2 gene was inserted at the TTAA sequence of the intron of the SBF2 gene located on chromosome 11 (Fig. 3D).
as the positive and negative selection marker for insertion. The cells excised with the PB element containing PGAP2 and puroΔTK genes are resistant to treatment with the nucleoside analogue, 1-(2-deoxy-2-fluoro-1-D-arabinofuranosyl)-5-iodouracil (FIAU). After the plasmids expressing FLP recombinase or PBase were transfected into P15 cells, followed by FIAU treatment, the surface expression of CD59 on the surviving cells was analysed by flow cytometry. By expression of PBase or FLP recombinase, the P15 phenotype was reverted to that of PGAP2-KO cells, suggesting that the PGAP2 gene was excised correctly (Fig. 4B). These data indicate that the P15 cell line can easily convert from GPI-positive cells to GPI-less cells by expression of PBase or FLP recombinase. By deletion of PGAP2, GPI-APs can be switched from membrane-bound forms to secreted forms.

Expression of recombinant proteins using GPI anchors. Recombinant proteins can be expressed as GPI-APs by simply conferring a GPI-attachment signal sequence to the carboxyl-terminus of the target protein. In the ER, the carboxyl-terminal GPI-attachment signal is exchanged with preformed GPI and this reaction is mediated by GPI transamidase10. As a model protein to express in this system, we chose lysosomal acid lipase (LIPA), which is an enzyme required for breakdown of lipids such as cholesterol esters and triacylglycerols in lysosomes29. Mutations in the LIPA gene cause lysosomal storage diseases such as Wolman disease and cholesteryl ester storage disease30. For treatment of LIPA deficiency, Kanuma (Sebelipase alfa), which is a recombinant LIPA produced by transgenic chicken egg, was approved for use in 2015 in the USA and EU31. We constructed vectors carrying His6-Flag-tagged LIPA (sHF-LIPA) or sHF-LIPA fused with a GPI-attachment signal (sHF-LIPA-GPI), which were stably expressed in cells (Fig. 5A). The sHF-LIPA-GPI was expressed on the cell surface and cleaved by treatment with PI-PLC (Fig. 5B), indicating that LIPA was expressed as a GPI-anchored form.

Selection of cells highly expressing recombinant proteins. The developed over-expression system enabled the sorting of cells expressing high amounts of LIPA. The plasmid for the expression of sHF-LIPA-GPI was transfected into P15 cells and the cells stably expressing sHF-LIPA-GPI were selected with antibiotics. The cells with high levels of expression of sHF-LIPA-GPI on the cell surface were then enriched using a cell sorter (Fig. 6A). After sorting twice, the expression of sHF-LIPA-GPI on the cell surface was increased by 28-fold (with the geometric mean fluorescent intensity) when compared with that of unsorted cells. The PGAP2 gene was then removed from the sorted cells by expression of PBase or FLP recombinase. The surface expression of CD59 and sHF-LIPA-GPI decreased (Fig. 6B). Removal of the PGAP2 gene from cells led to the secretion of sHF-LIPA into the medium (Fig. 6C), whereas P15 cells expressed sHF-LIPA-GPI on the cell surface but did not secrete this protein into the medium. Compared with cells that were sorted once, higher amounts of sHF-LIPA were secreted into the medium for cells that were sorted twice, suggesting that the system requires two sorting steps to ensure maximum selection and thus protein yields.
LIPA activity was detected from the culture media of wild-type HEK293 cells and P15 cells expressing soluble sHF-LIPA (Fig. 7A). In contrast, the activity was not detected in the medium of P15 cells expressing sHF-LIPA-GPI without treatment. Once PGAP2 was removed by either PBase or FLP recombinase, however, a significant level of LIPA activity was detected (Fig. 7A). The activities from the first sorted cells and the second sorted cells were 1.8 and 2.0 times higher than that of P15 cells expressing soluble sHF-LIPA, respectively.

After the second sorting, we isolated clonal cell lines from P15 cells expressing sHF-LIPA-GPI by limiting dilution. We selected 10 clonal cell lines from P15 cells expressing sHF-LIPA and P15 cells expressing sHF-LIPA-GPI that were sorted twice followed by the removal of PGAP2. The sHF-LIPA was expressed in the medium at higher amounts in cells using the GPI-anchoring system when compared with that of the control P15 cells stably expressing soluble sHF-LIPA, respectively.

High expression of α-galactosidase A using GPI-based protein expression system. We finally analysed whether the system can be applied to express other recombinant proteins. As the protein to express, we chose the α-galactosidase A (GLA), which is another lysosomal enzyme. Recombinant GLA is used for the enzyme replacement therapy of the Fabry diseases. Plasmids expressing His6-Flag-tagged GLA (sHF-GLA) and GPI-anchored sHF-GLA (sHF-GLA-GPI) were constructed and transfected into P15 cells (Fig. 8A). After selection by antibiotics, P15 cells expressing sHF-GLA-GPI were stained with anti-Flag antibodies and cells highly expressing GPI-anchored sHF-GLA on the cell surface were enriched twice by cell sorter. The surface level of sHF-GLA was increased 3.2 times (Fig. 8B). When PGAP2 gene was removed from cells by the expression of FLPe recombinase, surface levels of sHF-GLA was decreased. Instead, the proteins started being released into the medium (Fig. 8C). The secreted GLA from cells sorted once and twice were higher than those from unsorted cells and cells expressing soluble sHF-GLA. The activity of GLA was increased in medium prepared from cells sorted twice (Fig. 8D). These results indicate that the system is useful to select cells highly expressing recombinant proteins of interest.

Discussion
In this study, we developed a new system to select cells expressing high amounts of recombinant proteins. The advantage offered by GPI-anchoring of proteins was used to tether proteins to the cell surface. By enrichment...
of cells, in which higher amounts of GPI-anchored recombinant proteins are expressed on the cell surface, we could obtain cells expressing soluble target protein in high amounts. Here, we have established a HEK293 cell

Figure 6. Enrichment of cells highly expressing LIPA. (A) Sorting of cells highly expressing sHF-LIPA-GPI. P15 cells stably expressing sHF-LIPA-GPI were sorted twice. Surface expression of sHF-LIPA-GPI was analysed by flow cytometry. Cells were stained with an anti-Flag antibody followed by a PE-conjugated goat anti-mouse IgG. (B) Sorted cells were transfected with the vector carrying PBase or FLPe recombinase. Expression of CD59 and sHF-LIPA-GPI on the cell surface was analysed. Original, P15 cells expressing sHF-LIPA-GPI after one round of sorting (Sort 1) and after two rounds of sorting (Sort 2). (C) sHF-LIPA in the cell lysate and culture medium was analysed. Culture medium from Sort 1 and Sort 2 cells with or without (−) treatment of PBase or FLPe recombinase were collected and precipitated. Proteins were detected by anti-Flag antibodies. The full image was included in the supplementary information.
line, named P15, in which GPI-APs can be converted from membrane-bound forms to secretory forms. Initially, the PGAP2 gene was knocked out in HEK293 cells to secrete GPI-APs into the medium. Second, the PGAP2 gene was rescued in KO cells by a PB transposon-based plasmid. By introduction of PB transposase or FLP recombinase, the PGAP2 gene can be easily removed from P15 cells, yielding PGAP2-KO cells once more. Using the P15 cells, recombinant proteins, LIPA and GLA, were expressed as the GPI-anchored form. After sorting cells that expressed high levels of GPI-anchored forms of proteins on the cell surface, LIPA-GPI and GLA-GPI were switched from the membrane-bound form to the secretory form by removal of the PGAP2 gene from the cells. The system does not require treatment with any GPI-cleaving enzymes for release. Lyso-forms of GPI-APs are further cleaved by PLD-like activity17, 18. The activity arises from both endogenous expression in cells and the

Figure 7. Secreted LIPA activities from P15 sorted cells. (A) LIPA activity using cell culture medium. The medium was collected after culturing 2.5 × 10^5 cells for 48 h. LIPA activities in the medium were measured by conversion of 4-NPP to nitrophenol. The values shown are means ± SD of triplicate determinants. Results represent one of three comparable experiments. (B) LIPA activities in single clonal cells from P15 cells stably expressing soluble sHF-LIPA and PB-treated P15 cells expressing sHF-LIPA-GPI after two rounds of sorting. The values shown are means ± SD of triplicate determinants. (C) Western blot of LIPA secreted from HEK293 (WT: 1) and P15 (2) cells stably expressing soluble sHF-LIPA and clone No. 5 of the PB-treated P15 (3) cells expressing sHF-LIPA-GPI after two rounds of sorting (left). Culture media were collected and precipitated. Proteins were detected by anti-Flag antibodies. The full image was included in the supplementary information. The relative intensity of sHF-LIPA in the western blot was plotted. The intensity of the sHF-LIPA expressed from WT cells was set as 1. The values shown are mean ± error of two independent experiments.
serum. With this method, we obtained cell lines that expressed recombinant LIPA and GLA at higher levels when compared with conventional methods.

When a GPI-attachment signal is added to the C-terminus of secretory proteins, the proteins are expressed as GPI-APs. Therefore, it has been possible to express a wide range of recombinant proteins on the cell surface.

Figure 8. Selection of cells highly expressing GLA using the molecular switching system. (A) Schematic view of the plasmid constructs of the His6-Flag (HF)-tagged secretory GLA (sHF-GLA) and the GPI anchored GLA (sHF-GLA-GPI). The signal sequence (SS) and GPI attachment signal of human CD59 were used. (B) Sorting of cells highly expressing sHF-LIPA-GPI. P15 cells stably expressing sHF-GLA-GPI were sorted twice. Sorted cells were transfected with or without the vector carrying FLPe recombinase. Expression of sHF-GLA-GPI on the cell surface on the P15 cells expressing sHF-GLA-GPI before (No sort), after one round of sorting (Sort 1) or after two rounds of sorting (Sort 2) was analysed. Cells were stained with an anti-Flag antibody followed by a PE-conjugated goat anti-mouse IgG. (C) sHF-GLA in the cell lysate and culture medium was analysed. Culture medium from Sort 1 and Sort 2 cells with or without (−) treatment of FLPe recombinase were collected and precipitated. Proteins were detected by anti-Flag antibodies. The full image was included in the supplementary information. (D) GLA activity using cell culture medium. The medium was collected after culturing 2.5 × 10⁶ cells for 48 h. GLA activities in the medium were measured by conversion of MU-Gal to 4-methylumbelliferone. The values shown are means ± SD of triplicate determinants. Results represent one of two comparable experiments.
through GPI-anchors. In particular, it would be useful to tether soluble proteins and type-I membrane proteins. GPI-anchor LIPA and GLA were functional, thereby showing that the GPI moiety did not hinder proper protein folding and function. Here, we used His6-Flag-tagged LIPA and GLA as model recombinant proteins to express, because such tags are useful for protein purification and detection. The system can also be used with non-tagged native proteins if an antibody against the target protein is available. In our system, target proteins expressed on the cell surface are stained with antibodies and the high expressing cells are enriched using flow cytometry-based cell sorters or magnetic separators. Therefore, it is a requirement that antibodies can be used with flow cytometry.

In this system, the GPI-glycan part is attached to the C-terminus of proteins, which has both advantages and disadvantages with respect to affecting the function and properties of the target protein. A clear disadvantage is that C-terminal labelling may lead to loss of protein function, and thus our system cannot be used for proteins that suffer loss of activity upon C-terminal modification. In addition, because proteins are modified with the GPI-glycan, they are not identical to their native forms. Since soluble GPI-APs, which are cleaved by GPI-cleaving enzymes, exist in our body, cleaved GPI itself would not become the antigen. When the proteins are used for clinical purpose, however, attention should be paid to immunogenicity, because attachment of the GPI-glycan yields non-native target proteins. These negative points need to be addressed and improved in future studies.

Advantages of the expression system include protection from carboxypeptidases degradation because of C-terminal modification with a glycan moiety. Second, there are several proteins that recognize GPI-glycans such as α-toxin and aerolysin, which can be used for enrichment and purification of target proteins. Third, GPI-anchors have unique structures and are chemically and enzymatically processed. GPI moieties are also chemically modified in vivo. Therefore, the GPI-anchor recombinant proteins could be labelled or co-crosslinked with reagents such as fluorescent probes and nanoparticles. Further research would help to expand the applications of the system.

Plasmids expressing target proteins with monitoring proteins such as GFP are used for the selection of cells expressing recombinant proteins in high levels. In these methods, it is assumed that cells highly expressing monitoring proteins are also able to secrete large amounts of recombinant proteins. Since our method monitors the recombinant protein on the cell surface, it is a more direct approach to monitor protein expression levels. The dihydrofolate reductase (DHFR)-methotrexate (MTX) system is frequently used for increasing the expression of recombinant pharmaceutical proteins. In this system, plasmids carrying a target gene of interest with the DHFR region together with target gene are amplified in the genome. Our system can be combined with the DHFR system to improve protein productivity. The advantage of our system is that cells expressing high levels of the target recombinant protein are enriched by cell sorting. Our findings show that the GPI-based protein expression system is more efficient when compared with other methods, as it is faster and highly productive. The system should be useful for large-scale production of recombinant proteins.

Material and Methods

Cells, antibodies and reagents. Human embryonic kidney 293 (HEK293) cells were cultured in Dulbecco’s Modified Eagle medium (DMEM) supplemented with 10% fetal calf serum. The appropriate antibiotic concentrations were used where necessary: puromycin (1 μg/ml), hygromycin (400 μg/ml), and streptomycin (100 μg/ml)/penicillin (100 μg/ml). Mouse monoclonal anti-CD59 (clone 5H8), which was kindly provided from Taroh Kinoshita (Osaka University), and anti-M2 Flag antibodies (Sigma, St. Louis, MO, USA) were used as primary antibodies, whereas phycoerythrin (PE)-conjugated goat anti-mouse IgG (Biolegend, San Diego, CA, USA) was used as the secondary antibody. 1-(2-Deoxy-2-fluoro-β-D-arabinofuranosyl)-5-iodo-2,4-(1H,3H)-pyrimidinedione (FIAU) and 4-NPP were purchased from Sigma.

Knockout of PGAP2 in HEK293 cells. The PGAP2 gene was knocked out by the CRISPR/Cas9 system. The pX330-EGFP plasmid vector was digested with BbsI. The PGAP2 KO target 5′-AGAAGCCAGGCGCGGATTCG-3′ and 5′-GCGGGCGATAAGGACACATCG-3′ were designed using the E-CRISP website and were ligated into digested pX330-EGFP to generate pX330-EGFP-PGAP2-1 and pX330-EGFP-PGAP2-2, respectively. After transfection of knockout constructs into HEK293 cells, GFP positive cells were sorted using an S3e Cell Sorter (BioRad, Hercules, CA, USA). Sorted cells were further cultured for 10 d and limiting diluted to obtain the clonal cells. The knockout of PGAP2 was confirmed by PCR.

Plasmid construction. The plasmid pME-Puro-sHF-GPI containing a signal sequence, a His6 tag, a Flag tag and a GPI attachment signal of human CD59 was used to construct a plasmid for expression of GPI-anchor LIPA and GLA. LIPA and GLA fragments were obtained from human cDNA amplified using the primer sets 5′-CGATGAGCAAGCTCGAGGGGAAACTGACAGCTTTG-3′/5′-TCCACACATTCTCAGAGGTATTTCTCCTATTGATTTAATATT-3′ and 5′-CGATGAGCAAGCTCGAGGGGAAACTGACAGCTTTG-3′/5′-TCCACACATTCTCAGAGGTATTTCTCCTATTGATTTAATATT-3′. The fragments were then ligated into the XhoI site of pME-Puro-sHF-GPI by In-fusion cloning (Takara, Shiga, Japan). The resulting constructs, pME-Puro-sHF-LIPA-GPI and pME-Puro-sHF-GLA-GPI, were confirmed by sequencing. The shF-LIPA-GPI and shF-GLA-GPI fragments were digested and ligated into the pHyg vector to yield the pHyg-shF-LIPA-GPI and pHyg-shF-GLA-GPI plasmids, respectively.

For the plasmid expressing the soluble form of LIPA or GLA, a DNA fragment corresponding to the mature LIPA or GLA sequence after the ER insertion site was amplified and ligated into XhoI and NolI sites of pME-Phe-shF-LIPA-GPI to generate pME-Phe-shF-LIPA and pME-Phe-shF-GLA. For rescue mediated

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by the PB system, pPB-FRT-PGk-PuroΔTK was constructed and contained a PGk promoter, a multiple cloning site, a bovine growth hormone (GH) polyadenylation signal, SV40 promoter and puroΔTK flanked by both PB terminal repeat sequences and flippase recognition target (FRT) at both ends. The Flag-tagged rat PAG2 gene was amplified and cloned into EcoRI and NotI of pPB-FRT-PGk-PuroΔTK to give pPB-FRT-PuroΔTK-PAG2. The pCMV-byPBase was kindly provided from Kosuke Yusa (Sanger Institute) and pCAG-FLPe-IRES-puro was purchased from Addgene.

**Flow cytometry and cell sorting.** Cells were harvested by a trypsin/EDTA solution for CD59 staining or by PBS containing 2 mM EDTA and 0.5% BSA for Flag staining. Cells (5 × 10⁶)/sample were stained with anti-CD59 or anti-Flag (10 μg/ml) as the first antibody, and PE-conjugated goat anti-mouse IgG was used as the second antibody. After staining, cells were analysed using Accuri C6 (BD, San Jose, CA, USA). Where necessary, cells were treated with phosphatidylinositol-specific phospholipase C (PI-PLC; Thermo Scientific, Carlsbad, CA, USA) for 1.5 h at 37 °C prior to antibody staining. For cell sorting, the cell sorting solution (PBS, 2 mM EDTA and 0.5% BSA) was used for resuspension of antibodies and washing instead of the FACS solution. Stained cells were sorted by an S3e Cell Sorter.

**PB copy number determination.** To rescue PGAP2-KO cells, pPB-FRT-PuroΔTK-PAG2 was co-transfected with pCMV-byPBase. Two days after transfection, the cell medium was replaced by medium containing puromycin to select positive cells. After the limiting dilution, inserted copy number and insert sites of the P-B-FRT-PuroΔTK-PAG2 fragment were analysed for the obtained clones. The genomic DNAs from each clone were isolated using the Wizard genomic DNA purification kit (Promega, Madison, WI, USA). Genomic DNA was digested by HaeIII, followed by ligation of adaptors. Splinkerette-PCR was performed to determine PB insertion sites50, 51. Flanking sequences were amplified using primer sets Spl-P1, 5′-CGAATCTGAACCGTTCGTACGAGAA-3′ and 3PB-1st, 5′-TATACAGACCGTAAACACATCGCTT-3′ for the 1st PCR, and Spl-P1 5′-TCGTAGCAGAATCGCTGCTCC-3′ and 3PB-2nd, 5′-CGCATGATATCCTTTAAGTACGTCACAA-3′ for the second nested PCR. To determine PB copy number, amplified DNA was applied on 2% agarose gel and checked by electrophoresis. The insertion sites were determined by sequencing. The cell line, named P15, with a single PB copy in the genome was chosen for further experiments.

**Recombinant mammalian protein expression.** To make stable cell lines expressing soluble forms shF-LIPA or shF-GLA and membrane-bound forms shF-LIPA-GPI or shF-GLA-GPI, linearized DNA plasmids were transfected into cells. Two days after transfection, the cell medium was replaced by medium containing hygromycin (400 μg/ml). To remove PB transposons from cells expressing GPI-fused proteins, sorted cells in a 6-well plate were transfected with 5 μg DNA of pCMV-byPBase or pCAG-FLPe-IRES-puro for PBase or Flippase, respectively. After 2 d, medium containing 1 μM FIAU was applied, and the medium was changed every day for 7 d.

**Western blotting of shF-LIPA and shF-GLA prepared from medium and cell lysates.** Cells (5 × 10⁶) were plated and cultured in 2 ml of medium. After 48 h, 1.4 ml medium was collected. The medium was centrifuged at 10,000 × g for 5 min and 1000 μl of the supernatant was collected in a new tube. Then, 20 μl of prewashed anti-Flag beads (MBL, Aichi, Japan) for DDĐDK-tagged proteins were added. The tube was rotated at 4 °C for 2 h, and then centrifuged at 10,000 × g for 1 min. The supernatant was removed and the beads with tagged proteins were washed 3 times with cold PBS. The proteins were eluted with the Flag peptide. For the cell lysates, after removing the medium, 5 × 10⁶ cells were harvested with the cell sorting solution and washed using cold PBS at 4 °C. One hundred microliters of lysis buffer (50 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1% Triton-X100, 1 mM EDTA, protein inhibitor, 1 mM PMSF) was added to the cell pellets and the mixture was incubated on ice for 30 min. After incubation, the tube was centrifuged at 10,000 × g for 15 min at 4 °C. The supernatant was collected and mixed with sample buffer and boiled at 95 °C for 5 min. The solution was kept at –20 °C until western blotting. The recombinant LIPA and GLA were detected using the anti-Flag antibody and horseradish peroxidase (HRP)-conjugated anti-mouse IgG as the primary antibody and secondary antibody, respectively. Proteins were visualized using ImageQuant™ LAS 4000 (GE Healthcare, Little Chalfont, UK).

**LIPA activity.** The medium was collected after culturing 2.5 × 10⁵ cells in a 12-well plate for 48 h in 1 ml medium without any antibiotics. After centrifugation at 10,000 × g, 0.8 ml of medium was collected and then stored at −20 °C until use. LIPA activity was measured by an optimized assay for lipase activity using cell medium52, 53. Briefly, 100 μl assay buffer (100 mM citrate buffer (pH 5.0), 0.5% Triton X-100, 1 mM CaCl₂) and 20 μM 4-nitrophenyl-palmitate (4-NPP) dissolved in isopropanol/acetoneitrile (4/1 v/v) were prepared before the assay. The assay mixture was obtained by adding 4-NPP to the assay buffer to give a final concentration of 4-NPP of 1 μM. The mixture was then placed in a 60 °C water bath for 5 min, mixing continuously until the solution was transparent. An appropriate amount of medium was centrifuged at 10,000 × g at 4 °C for 5 min, and the supernatant was collected in a new tube. Ten microliters of cell medium in transparent 96-well plates, which was immediately covered by a dark paraffin paper before placing it in the incubator at 30 °C for 45 min. Subsequently, 120 μl of 1 M Tris–HCl (pH 8.0) was added to the assay mixture to terminate the reaction. The absorbance was measured at 405 nm, using an Enspire 2300 Multilabel Reader (Perkin Elmer, Waltham, MA, USA). One unit of lipase activity was defined as the amount of 4-nitrophenol nmol per min released from 4-NPP at 30 °C.

**GLA activity.** The medium for GLA activity was prepared the same as described above for LIPA activity. For measuring GLA activity, an optimized method using 4-methyllumbellsifer-α-D-galactopyranoside (MU-Gal,
Santa Cruz Biotechnology, Santa Cruz, CA, USA) as a substrate was described previously\(^{54–56}\). Briefly, 100 μl of assay buffer (60 mM citrate-phosphate buffer (pH 4.6), 1 mM MgCl\(_2\)) and 20 mM MU-Gal dissolved distilled water were prepared before the assay. The assay mixture was obtained by adding MU-Gal to the assay buffer to make 2 mM MU-Gal solution. Ten μl of cell medium were added to the MU-Gal assay mixture in a dark 96-well plate, which was immediately covered by a dark paraffin paper before placing it in the incubator at 37 °C for 60 min. Afterwards, 100 μl of 0.4 M glycine-NaOH buffer (pH 10.6) were added to the assay mixture to terminate the reaction. The fluorescence was recorded using Enspire 2300 Multilabel Reader (Perkin Elmer, Waltham, MA, USA) at 350 nm and 465 nm for excitation and emission wavelengths, respectively. The 4-methylumbelliferone (Sigma) was used to create the standard curve. One unit of galactosidase activity was defined as the amount of 4-methylumbelliferone pmol per min released from MU-Gal at 37 °C.

References

1. Zhu, J. Mammalian cell protein expression for biopharmaceutical production. Biotechnol Adv 30, 1158–1170 (2012).
2. Bandaranayake, A. D. & Almo, S. C. Recent advances in mammalian protein production. FEBS Lett 588, 253–260 (2014).
3. Hacker, D. L. & Balasubramanian, S. Recombinant protein production from stable mammalian cell lines and pools. Curr Opin Struct Biol 38, 129–136 (2016).
4. Mattanovich, D. et al. Recombinant protein production in yeasts. Methods Mol Biol 824, 329–358 (2012).
5. Swiec, K., de Freitas, M. C., Covas, D. T. & Piccano-Castro, V. Recombinant glycoprotein production in human cell lines. Methods Mol Biol 1258, 223–240 (2015).
6. Dalton, A. C. & Barton, W. A. Over-expression of secreted proteins from mammalian cell lines. Protein Sci 23, 517–525 (2014).
7. Ho, S. C. & Yang, Y. Identifying and engineering promoters for high level and sustainable therapeutic recombinant protein production in cultured mammalian cells. Biotechnol Lett 36. 1569–1579 (2014).
8. Dietmair, S., Nielsen, L. K. & Timmins, N. E. Mammalian cells as biopharmaceutical production hosts in the age of omics. Biotechnol J 7, 75–89 (2012).
9. Xu, L. et al. A mammalian expression system for high throughput antibody screening. J Immunol Methods 395, 45–53 (2013).
10. Kinosita, T., Fujita, M. & Maeda, Y. Biosynthesis, remodeling and functions of mammalian GPI-anchored proteins: recent progress. J Biochem 144, 287–294 (2008).
11. Kinosita, T. & Fujita, M. Biosynthesis of GPI-anchored proteins: special emphasis on GPI lipid remodeling. J Lipid Res 57, 6–24 (2016).
12. Ohashi, K. et al. Gaalp and gp88 are components of a glycosylphosphatidylinositol (GPI) transamidase that mediates attachment of GPI to proteins. Mol Biol Cell 11, 1523–1533 (2000).
13. Fujita, M. et al. GPI glycan remodeling by PGAP5 regulates transport of GPI-anchored proteins from the ER to the Golgi. Cell 139, 352–365 (2009).
14. Fujita, M. & Kinosita, T. Structural remodeling of GPI anchors during biosynthesis and after attachment to proteins. FEBS Lett 584, 1670–1677 (2010).
15. Fujita, M. & Kinosita, T. GPI-anchor remodeling: potential functions of GPI-anchors in intracellular trafficking and membrane dynamics. Biochim Biophys Acta 1821, 1050–1058 (2012).
16. Maeda, Y. et al. Fatty acid remodeling of GPI-anchored proteins is required for their raft association. Mol Biol Cell 18, 1497–1506 (2007).
17. Tashima, Y. et al. PGAP2 is essential for correct processing and stable expression of GPI-anchored proteins. Mol Biol Cell 17, 1410–1420 (2006).
18. Lee, G. H. et al. A GPI processing phospholipase A2, PGAP6, modulates Nodal signaling in embryos by shedding CRIPTO. J Cell Biol 215, 705–718 (2016).
19. Heider, S., Dangerfield, J. A. & Metzner, C. Biomedical applications of glycosylphosphatidylinositol-anchored proteins. J Lipid Res 57, 1778–1788 (2016).
20. Feng, H. et al. Incorporation of a GPI-anchored engineered cytokine as a molecular adjuvant enhances the immunogenicity of HIV VLPs. Sci Rep 5, 11856 (2015).
21. Zhang, L. et al. Screening for glycosylphosphatidylinositol-modified cell wall proteins in Pichia pastoris and their recombinant expression on the cell surface. Appl Environ Microbiol 79, 5519–5526 (2013).
22. Patel, J. M. et al. Protein transfer-mediated surface engineering to adjuvantage virus-like nanoparticles for enhanced anti-viral immune responses. Nanomedicine 11, 1097–1107 (2015).
23. Patel, J. M. et al. Influenza virus-like particles engineered by protein transfer with tumor-associated antigens induces protective antitumor immunity. Biotechnol Bioeng 112, 1102–1110 (2015).
24. Hansen, L. et al. Hypomorphic mutations in PGAP2, encoding a GPI-anchor-remodeling protein, cause autosomal-recessive intellectual disability. Am J Hum Genet 92, 573–583 (2013).
25. Krawitz, P. M. et al. PGAP2 mutations, affecting the GPI-anchor-synthesis pathway, cause hyperphosphatasia with mental retardation syndrome. Am J Hum Genet 92, 584–589 (2013).
26. Li, X. et al. PiggyBac transposase tools for genome engineering. Proc Natl Acad Sci USA 110, E2279–E2287 (2013).
27. Yusa, K. PiggyBac Transposon. Microbiol Spectr 3, MDNA3-0028-2014 (2015).
28. Kühnlig, K. M. et al. Multiplexed transposon-mediated stable gene transfer in human cells. Proc Natl Acad Sci USA 107, 1343–1348 (2010).
29. Grumet, L. et al. Lysosomal Acid Lipase Hydrolyzes Retinyl Ester and Affects Retinoid Turnover. J Biol Chem 291, 17977–17987 (2016).
30. Desai, N. K. & Md, D. P. W. Lysosomal Acid Lipase Deficiency. In Endotext (eds De Groot, L., J. et al.), South Dartmouth (MA) pp (2000).
31. Frampton, J. E. Scheliples Alfa: A Review in Lysosomal Acid Lipase Deficiency. Am J Cardiovasc Drugs 16, 461–468 (2016).
32. Thomas, A. S. & Hughes, D. A. Fabry disease. Pediatr Endocr Rev 12(Suppl 1), 88–101 (2014).
33. Yu, S. et al. Recent progress in synthetic and biological studies of GPI anchors and GPI-anchored proteins. Curr Opin Chem Biol 17, 1006–1013 (2013).
34. Abe, H., Shimma, Y. & Jigami, Y. In vitro oligosaccharide synthesis using intact yeast cells that display glycosyltransferases as the cell surface through cell wall anchored protein Pt. Glycobiology 13, 87–95 (2003).
35. Sharma, D. K., Vidugiriene, J., Bangs, J. D. & Menon, A. K. A cell-free assay for glycosylphosphatidylinositol anchoring in African trypanosomes. Demonstration of a transamidation reaction mechanism. J Biol Chem 274, 16479–16486 (1999).
36. Abrami, L. et al. A pore-forming toxin interacts with a GPI-anchored protein and causes vacuolation of the endoplasmic reticulum. J Cell Biol 140, 525–540 (1998).
37. Gordon, V. M. et al. Clostridium septicum alpha toxin uses glycosylphosphatidylinositol-anchored protein receptors. J Biol Chem 274, 27274–27280 (1999).
38. Zhao, P. et al. Proteomic identification of glycosylphosphatidylinositol anchor-dependent membrane proteins elevated in breast carcinoma. J Biol Chem 287, 25230–25240 (2012).
39. Ferguson, M. A. J., Kinoshita, T. & Hart, G. W. Glycosylphosphatidylinositol Anchors. In Essentials of Glycobiology (eds J. Varki, A. et al.) 2nd Ed., Cold Spring Harbor (NY), pp (2009).
40. Zitzmann, N. & Ferguson, M. A. Analysis of the carbohydrate components of glycosylphosphatidylinositol structures using fluorescent labeling. Methods Mol Biol 116, 73–89 (1999).
41. Cortes, L. K. et al. Proteomic identification of mammalian cell surface derived glycosylphosphatidylinositol-anchored proteins through selective glycan enrichment. Proteomics 14, 2471–2484 (2014).
42. Lu, L., Gao, J. & Guo, Z. Labeling Cell Surface GPIs and GPI-Anchored Proteins through Metabolic Engineering with Artificial Inositol Derivatives. Angew Chem Int Ed Engl 54, 9679–9682 (2015).
43. Vainauskas, S., Cortes, L. K. & Taron, C. H. In vivo incorporation of an azide-labeled sugar analog to detect mammalian glycosylphosphatidylinositol molecules isolated from the cell surface. Carbohydr Res 362, 62–69 (2012).
44. Browne, S. M. & Al-Rubeai, M. Selection methods for high-producing mammalian cell lines. Trends Biotechnol 25, 425–432 (2007).
45. Helman, D. et al. Novel membrane-bound reporter molecule for sorting high producer cells by flow cytometry. Cytometry A 85, 162–168 (2014).
46. Ng, S. K. Generation of high-expressing cells by methotrexate amplification of destabilized dihydrofolate reductase selection marker. Methods Mol Biol 801, 161–172 (2012).
47. Mali, P., Esvelt, K. M. & Church, G. M. Cas9 as a versatile tool for engineering biology. Nat Methods 10, 957–963 (2013).
48. Hirata, T. et al. Post-Golgi anterograde transport requires GARP-dependent endosome-to-TGN retrograde transport. Mol Biol Cell 26, 3071–3084 (2015).
49. Heigwer, F., Kerr, G. & Boutros, M. E-CRISP: fast CRISPR target site identification. Nat Methods 11, 122–123 (2014).
50. Horie, K. et al. A homozygous mutant embryonic stem cell bank applicable for phenotype-driven genetic screening. Nat Methods 8, 1071–1077 (2011).
51. Rong, Y. et al. Genome-Wide Screening of Genes Required for Glycosylphosphatidylinositol Biosynthesis. PLoS One 10, e0138553 (2015).
52. Guo, J., Chen, C. P., Wang, S. G. & Huang, X. J. A convenient test for lipase activity in aqueous-based solutions. Enzyme Microb Technol 71, 8–12 (2015).
53. Gupta, N., Ratni, F. & Gupta, R. Simplified para-nitrophenyl palmitate assay for lipases and esterases. Anal Biochem 311, 98–99 (2002).
54. Chiba, Y. et al. Production in yeast of alpha-galactosidase A, a lysosomal enzyme applicable to enzyme replacement therapy for Fabry disease. Glycobiology 12, 821–828 (2002).
55. Lukas, J. et al. Enzyme enhancers for the treatment of Fabry and Pompe disease. Mol Ther 23, 456–464 (2015).
56. Shi, Z. D. et al. Synthesis and characterization of a new fluorogenic substrate for alpha-galactosidase. Anal Bioanal Chem 394, 1903–1909 (2009).

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Author Contributions
E. Matabaro performed the main experiments and wrote an original draft; Z. He performed piggyBac transposon experiments; Y. S. Liu designed cell biological experiments and edited a paper; H. J. Zhang and X. D. Gao proposed the project and edited a paper; and M. Fujita proposed and supervised the project and wrote a paper.

Additional Information
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