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A versatile fluorescence-quenched substrate for quantitative measurement of glucocerebrosidase activity within live cells

Matthew C. Deen[a,1], Yanping Zhu[b,1], Christina Gros[a], Na Na[a], Pierre-André Gilormini[a,b], David L. Shen[a], Sandeep Bhosale[a], Nadia Anastasi[c], RuiQi Wang[a], Xiaoyang Shan[b], Eva Harde[c], Ravi Jagasia[c], Francis C. Lynn[d], and David J. Vocadlo[a,b,2]

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Loss of activity of the lysosomal glycosidase β-glucocerebrosidase (GCase) causes the lysosomal storage disease Gaucher disease (GD) and has emerged as the greatest genetic risk factor for the development of both Parkinson disease (PD) and dementia with Lewy bodies. There is significant interest into how GCase dysfunction contributes to these diseases, however, progress toward a full understanding is complicated by presence of endogenous cellular factors that influence lysosomal GCase activity. Indeed, such factors are thought to contribute to the high degree of variable penetrance of *GBA* mutations among patients. Robust methods to quantitatively measure GCase activity within lysosomes are therefore needed to advance research in this area, as well as to develop clinical assays to monitor disease progression and assess GCase-directed therapeutics. Here, we report a selective fluorescence-quenched substrate, LysoFQ-GBA, which enables measuring endogenous levels of lysosomal GCase activity within living cells. LysoFQ-GBA is a sensitive tool for studying chemical or genetic perturbations of GCase activity using either fluorescence microscopy or flow cytometry. We validate the quantitative nature of measurements made with LysoFQ-GBA using various cell types and demonstrate that it accurately reports on both target engagement by GCase inhibitors and the *GBA* allele status of cells. Furthermore, through comparisons of GD, PD, and control patient-derived tissues, we show there is a close correlation in the lysosomal GCase activity within monocytes, neuronal progenitor cells, and neurons. Accordingly, analysis of clinical blood samples using LysoFQ-GBA may provide a surrogate marker of lysosomal GCase activity in neuronal tissue.

Parkinson disease | glycoside hydrolase | enzyme kinetics | fluorescence-quenched substrate | flow cytometry

Significance

Loss of lysosomal glucocerebrosidase (GCase) activity contributes to the development of both Gaucher disease and Parkinson disease. GCase is of high interest due to its potential as both a therapeutic target and diagnostic marker. However, an inability to accurately measure GCase activity within lysosomes is hindering progress. We report a fluorescence-quenched substrate, LysoFQ-GBA, which enables quantitative measures of lysosomal GCase activity within patient derived tissues. A strong correlation between GCase activity in induced pluripotent stem cell-derived neurons and monocytes suggests that easily accessed blood samples may serve as a surrogate for activity within patient neurons. LysoFQ-GBA should therefore enable the community and, more generally, its development provides a blueprint on creating related substrates for other disease-associated lysosomal enzymes.

The principle substrate of the lysosomal glycoside hydrolase β-glucocerebrosidase (GCase, CAZY family GH30) is the sphingolipid glucosylceramide (GlcCer) (1). Biallelic loss of function mutations in the gene *GBA*, which encodes GCase, lead to the accumulation of GlcCer and its downstream metabolite glucosyl sphingosine (GlcSph) within lysosomes of various tissues. The accumulation of these substrates is thought to drive development of the lysosomal storage disease known as Gaucher disease (GD) (2). Notably, GD patients show considerable phenotypic variability, particularly in the presence and severity of neuropathic symptoms, which are only weakly correlated to patient genotype (3–5). Underscoring the importance of GCase function within brain, its dysfunction has also emerged as the most prevalent genetic risk factor for Parkinson disease (PD), with 5–10% of PD patients carrying a mutation in *GBA* (6, 7). A mechanistic role for loss of GCase activity contributing to PD pathogenesis is supported by observations that either chemical inhibition or loss of function mutations in GCase accelerates PD phenotypes (8, 9). Nevertheless, the precise molecular mechanism by which loss of GCase activity within lysosomes contributes to these diseases remains a topic of intense interest (10, 11).

Studying GCase activity within lysosomes is complicated by cellular factors that regulate the activity of this enzyme. Most notably, these factors include lysosomal integral membrane protein 2 (LIMP-2), which aids in trafficking GCase from the endoplasmic reticulum (ER) to lysosomes, and Saposin C, which modulates its lysosomal activity (12–14). These proteins, and likely several others, act as modifiers that influence lysosomal GCase activity and in doing so influence the severity and penetrance of GD and PD (15, 16). The regulation of lysosomal activity of GCase is accordingly not fully understood (16–19). The influence that these modifiers have on lysosomal GCase activity is typically characterized by using standard assays that use simple fluorogenic glucosides to measure its activity in tissue homogenates where lysosomes have been broken open. This approach, however, disrupts the native environment of the lysosome and also leads to a great dilution of lysosomal proteins, which can lead to a loss of

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[1]M.C.D. and Y.Z. contributed equally to this work.

[2]To whom correspondence may be addressed. Email: dvocadlo@sfu.ca.

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important protein-protein interactions. A further problem with such tissue lysate assays is that they report on the total cellular activity of GCase activity, which includes the activity of immature GCase localized within the ER and Golgi. This last point is of particular importance in studying the mutant forms of this enzyme, which are known to be inefficiently trafficked to the lysosome, as well as in characterizing the effects of putative modifiers of GCase activity that might influence its localization (20, 21). Highlighting the difficulties of using such lysate assays to measure the endogenous function of lysosomal GCase is a study that examined the effects of mutant α-synuclein on cellular GCase activity (9). While α-synuclein did not influence total cellular GCase activity, assays of purified lysosomal fractions from mutant cells revealed a significant reduction in GCase activity that was attributed to defects in its trafficking to lysosomes. Given the importance of GCase in brain in both GD and PD, coupled with the complex regulation of GCase activity within lysosomes, robust methods to enable measuring lysosomal GCase activity would accelerate understanding its relationship to GD and PD and could eventually help identify factors that influence its activity.

Methods to accurately measure the activity of enzymes within living cells are a topic of high interest (22), yet creating suitable substrates for many enzymes has proven to be a major challenge. For GCase, one previous approach to studying its activity in live cells has been to use 5-(pentafluorobenzoylamino)-fluorescein di-β-D-glucopyranoside (PFB-FDGlu) (23–25). The electrophilic pentafluorophenyl substituent is thought to covalently label intracellular thiols and result in cellular retention of both the substrate and product. This substrate should therefore lead to stable fluorescence intensities that reflect GCase activity, which contrasts with commonly used fluorogenic substrates including, for example, fluorescein β-D-glucopyranoside (FDGlu) that is known to readily diffuse out of cells (26). Unfortunately, the quantitative use of PFB-FDGlu in live cells is complicated by several factors. The first factor is that because it is functionalized with two glucose residues, it produces two distinct enzymatic products that have different photophysical properties, leading to complex kinetics (27, 28). The second factor is that the fluorescence intensity of fluorescein is highly pH-dependent due its ionization within a physiological range ($pK_a = 6.4$), such that variations in lysosomal acidity impact the accuracy of measurements (29). Finally, and perhaps most problematic, is that PFB-FDGlu is not selective for GCase and in cell lines expressing other β-glucosidases, including neurons, complicated subtractive assays are needed (25, 30). Accordingly, new strategies and substrates are needed to enable precise and accurate measurement of GCase activity within live cells.

Fluorescence-quenched substrates are one promising approach to studying enzymatic activities in living cells. This class of substrate, which undergoes a dark-to-light transition upon enzymatic processing, has been used to report on the activity of disease-related enzymes, most notably within cells for various proteases (31–33) and on the cell surface for a mycolyl transferase (34, 35). Most studies, however, report on the use of fluorescence-quenched substrates in a qualitative rather than a quantitative manner. While their qualitative use is valuable, the development of fluorescence-quenched substrate as quantitative tools to study enzyme activity within tissues would enable their wider adoption and their more ready application to both fundamental and translational research. Given the important roles played by GCase in both GD and PD, a fluorescence-quenched substrate that could enable convenient and accurate measurements of lysosomal GCase activity would be useful in helping understand the molecular mechanisms through which loss of GCase activity contributes to the development of these diseases. From a clinical perspective, there is interest in measuring Gcase activity in patient tissue samples to study its involvement in PD (36–38). Assays of GCase activity in dried blood spots and lysates of peripheral blood mononuclear cells (PBMCs), in particular, have been widely used due to the ease of collecting samples but also because of the high levels of GCase activity in these tissues (24, 39–41). Notably, characterization of PBMCs has suggested that they recapitulate some of the biochemical changes associated with PD, which may make them suitable for use as a surrogate marker that can report on GCase activity within brain (42). The ability to make such surrogate measures would be valuable, not only for studying the pathology of GBA-associated PD, but also to help advance GCase targeted therapeutics of which a handful of currently entering clinical trials (43).

Here, we detail the design and synthesis of a simple to use fluorescence-quenched substrate that enables accurate measurements of lysosomal GCase activity. We illustrate its value in making quantitative measurements, using both widefield fluorescence microscopy and flow cytometry. We demonstrate the versatility of this substrate by analyzing GCase activity in diverse cell lines including those obtained by differentiation of PD and GD patient-derived inducible pluripotent stem cells (iPSCs). Finally, we show that GCase activities measured using this approach in patient-derived monocytes and neurons are correlated and match *GBA* allele status, suggesting lysosomal GCase activity within live patient PBMC may be a useful surrogate measurement of lysosomal GCase activity within neurons of the brain.

Results

Synthesis and In Vitro Properties of Fluorescence-Quenched GCase Substrates. Previous effort toward the creation of a fluorescence-quenched GCase substrate yielded up substrate 9b (44) which, though offering a conceptual advance, suffered from poor aqueous solubility and delivered only weak signal intensity relative to background fluorescence. We noted from analysis of the in vitro enzyme kinetic data, however, that the core glucoside component of substrate 9b was recognized as a substrate and hydrolyzed by GCase with reasonable efficiency. Since that work, we pursued lines of research that ultimately led us to focus on variation of the pendant groups as a means to deliver to a GCase probe that would overcome these limitations. Specifically, we expected that inclusion of a small polar group could greatly improve the solubility of a candidate substrate and that an alternative, less hydrophobic, fluorophore would also aid in this regard. As a fluorophore-quencher pair, we selected BODIPY-FL and BHQ-1 due to the pH insensitivity of this fluorophore, which is important to enable accurate measurement of GCase activity within lysosomes that are well known to having varying pH. Moreover, because the BODIPY-FL fluorophore has excitation and emission bands similar to those of fluorescein, we reasoned it should be compatible with commonly used instruments including those with argon ion lasers. To improve substrate solubility we introduced an lysosomotropic N^{ε},N^{ε}-dimethyl-L-lysine (dML) residue within the arm of the substrate extending from the 6-hydroxyl of the core glucose moiety. We reasoned this basic tertiary amine would not only improve aqueous solubility of the substrate but would also function as a lysosomotropic group that would promote retention of both the substrate and product within lysosomes. We expected these collective features would deliver a substrate for quantitative analysis of lysosomal GCase activity (Fig. 1).

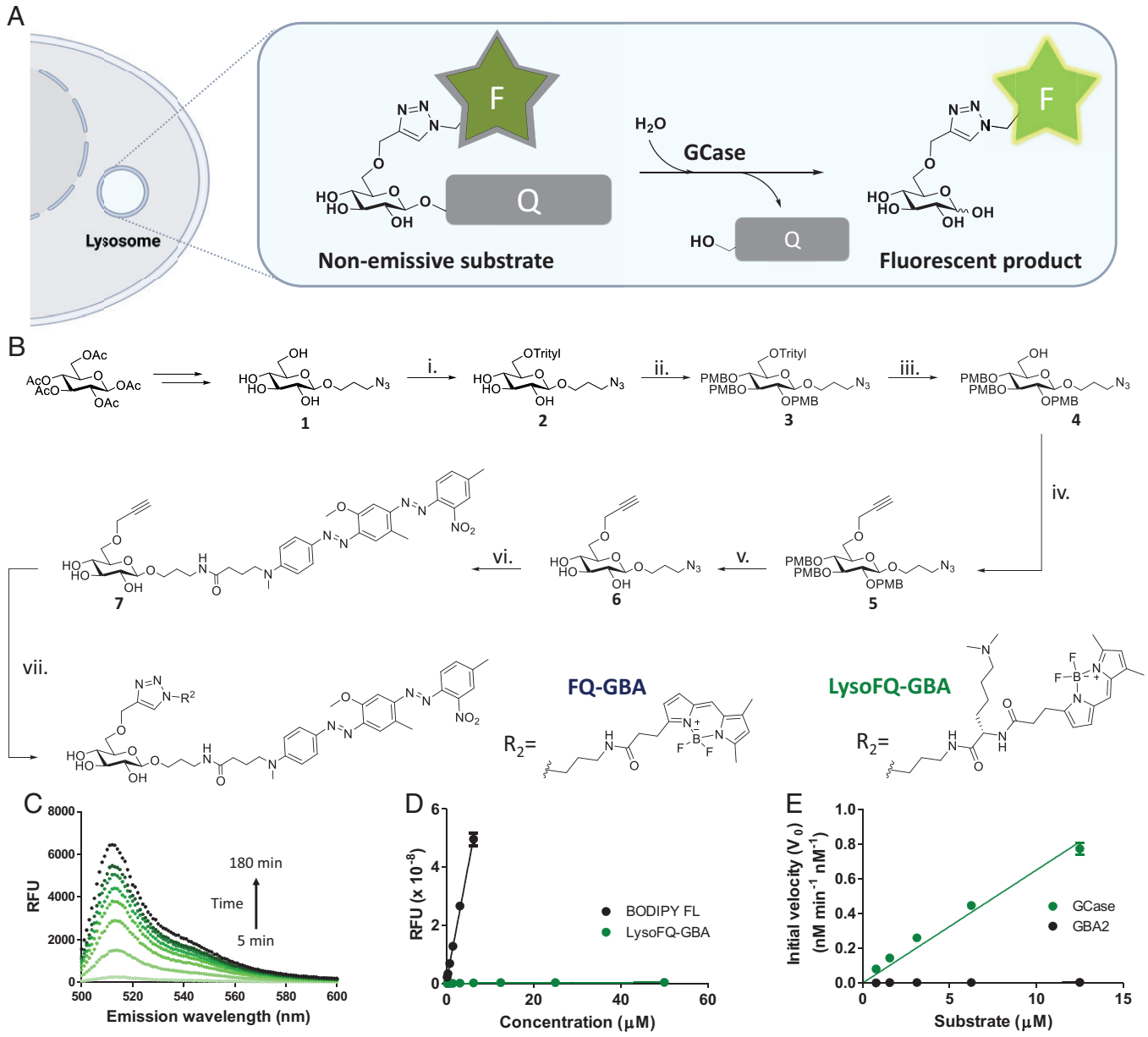

Fig. 1. Design and in vitro characterization of fluorescence-quenched substrates for GCase. (*A*) GCase catalyzed cleavage of fluorescence-quenched substrates described in this work. (*B*) The synthetic route used to access FQ-GBA and LysoFQ-GBA: i. Trityl chloride (1.5 equiv.), pyridine, room temperature (RT), 71% ii. Paramethoxybenzyl chloride (4 equiv.), NaH (5 equiv.), DMF, 0 °C, 78%, iii. Formic acid, Et_2O, RT, 63%, iv. Propargyl bromide (1.5 equiv.), NaH (2 equiv.), DMF, 0 °C, 93% v. DDQ (4.5 equiv.), DCM:H_2O (2:1), RT, 79% vi. a) PMe_3 (3 equiv.), THF, RT, b) BHQ-2 acid (1.2 equiv.), HBTU (1.2 equiv.), DIPEA (3 equiv.), DMF, RT, 67% yield over two steps; vii. Azide (1.1 equiv.), $CuSO_4$ (0.3 equiv.) sodium ascorbate (0.6 equiv.), DCM:H_2O, RT, 82% MDFL1, 31% LysoFQ-GBA. (*C*) Time-dependent uncaging of 250 nM LysoFQ-GBA by GCase, where the black curve is a standard solution of 250 nM BODIPY-FL. (*D*) Determination of fluorescence-quenching efficiency through comparison of dose-dependent fluorescence of BODIPY-FL and LysoFQ-GBA. (*E*) Comparison of the Michaelis-Menten kinetics of LysoFQ-GBA with recombinant GCase (20 nM) and GBA2 (20 nM). (Error bars: SD [n = 3].).

Moreover, this substrate would be expected to have excellent signal-to-noise and would also be simple to use and compatible with a range of analytical approaches.

We developed a reliable divergent synthetic route to prepare two candidate substrates. One of these, lysoFQ-GBA, incorporated the lysosomotropic dML residue, and a comparative control compound, FQ-GBA, lacking this group. To synthesize these two substrates, we started by accessing tetrol (**1**) through previously established methods (44). We then orthogonally protected the 6-hydroxyl group by tritylation and installed *para*-methoxybenzyl (PMB) groups to protect the secondary hydroxyl groups. Selective detritylation using acetic acid gave the free 6-hydroxyl which we then alkylated using propargyl bromide to give (**5**) in good yield. Oxidative removal of the PMB groups using DDQ delivered the bifunctional intermediate (**6**) bearing an azide and an alkyne. Following Staudinger reduction of the azide with trimethyl phosphine, the resulting amine was used directly in an amide coupling reaction to install the fluorescence-quencher BHQ-1. From the resulting central intermediate (**7**) we used copper-catalyzed azide-alkyne cycloaddition (CuAAC) chemistry to install either BODIPY-FL to generate FQ-GBA (Fig. 1*B*), or a preformed BODIPY-FL-dML conjugate (*SI Appendix*, Scheme S1) to generate LysoFQ-GBA (Fig. 1*B*).

With these substrates in hand, we immediately noted the greater aqueous solubility of LysoFQ-GBA as compared to FQ-GBA, which we attributed to the presence of the tertiary amine. We next turned to determining the suitability of these substrates for use in live cells by assessing their in vitro properties

including their enzymatic uncaging (Fig. 1*C*), quenching efficiency (Fig. 1*D*), and GCase catalyzed turnover (Fig. 1*D*). After careful purification of both FQ-GBA and LysoFQ-GBA, we found they both showed exceptional quenching efficiencies of 99.8% and >99.9%, respectively (*SI Appendix*, Fig. S1*B* and Fig. 1*C*). This remarkably effective quenching suggested to us that these substrates would possess the sensitivity necessary for application in live cells. Finally, we found that LysoFQ-GBA was cleaved by GCase with a second order rate constant (k_{cat}/K_M = 65 M^{-1} min^{-1}) (Fig. 1*D*) that is comparable to the GCase-catalyzed turnover of both FQ-GBA (k_{cat}/K_M = 35 M^{-1} min^{-1}) and 9b (k_{cat}/K_M = 60 M^{-1} min^{-1}) (*SI Appendix*, Fig. S1) (44). Finally, by comparing k_{cat}/K_M values, we found that FQ-GBA was 32-fold selective for GCase over the functionally related β-glucosidase GBA2 (k_{cat}/K_M = 1.1 M^{-1} min^{-1}) (Fig. 1*D*). Notably, LysoFQ-GBA was even more selective, with GCase turning over this substrate 220-fold more efficiently than GBA2 (k_{cat}/K_M = 0.29 M^{-1} min^{-1}). These collective observations indicate that LysoFQ-GBA has desirable in vitro properties that make it a promising fluorescence-quenched substrate for use in monitoring lysosomal GCase activity within cells.

LysoFQ-GBA Is a Selective Substrate for Lysosomal GCase Activity within Cells. We next evaluated if FQ-GBA and LysoFQ-GBA selectively and quantitatively reported on GCase activity within living cells. As a first step, we examined GCase activity measured using FQ-GBA and LysoFQ-GBA in SK-*N*-SH neuroblastoma cells. The fluorescent signal arising from turnover of FQ-GBA and LysoFQ-GBA displayed regions of linear dose and time dependence (Fig. 2*A* and *SI Appendix*, Fig. S2). These data suggest that, when using suitable conditions, these substrates can be used to conduct quantitative live cell measures of GCase activity. We next compared LysoFQ-GBA and FQ-GBA within live SK-*N*-SH cells to determine if inclusion of the lysosomotropic group improved signal intensity associated with lysosomal GCase activity. Comparing the fluorescence intensities arising from their turnover, we found that the lysosomotropic dML moiety within LysoFQ-GBA resulted in a 35-fold improvement in signal intensity as compared to FQ-GBA (Fig. 2*B*). Importantly, for both substrates, pretreatment of cells with the highly selective GCase inhibitor AT3375 (Fig. 2*B*) resulted in a complete loss of signal, indicating both substrates were being selectively turned over by GCase. To determine if LysoFQ-GBA is indeed processed within lysosomes, we performed colocalization studies by confocal microscopy using LysoTracker Red (RND-99) as a lysosomal marker. We observed extensive colocalization of signal puncta as measured using Pearson's correlation coefficient (PCC = 0.72 ± 0.06) (Fig. 2*C*). Collectively, these data support the idea that FQ-GBA and LysoFQ-GBA might be useful for quantitative measures of the activity of mature lysosomal GCase.

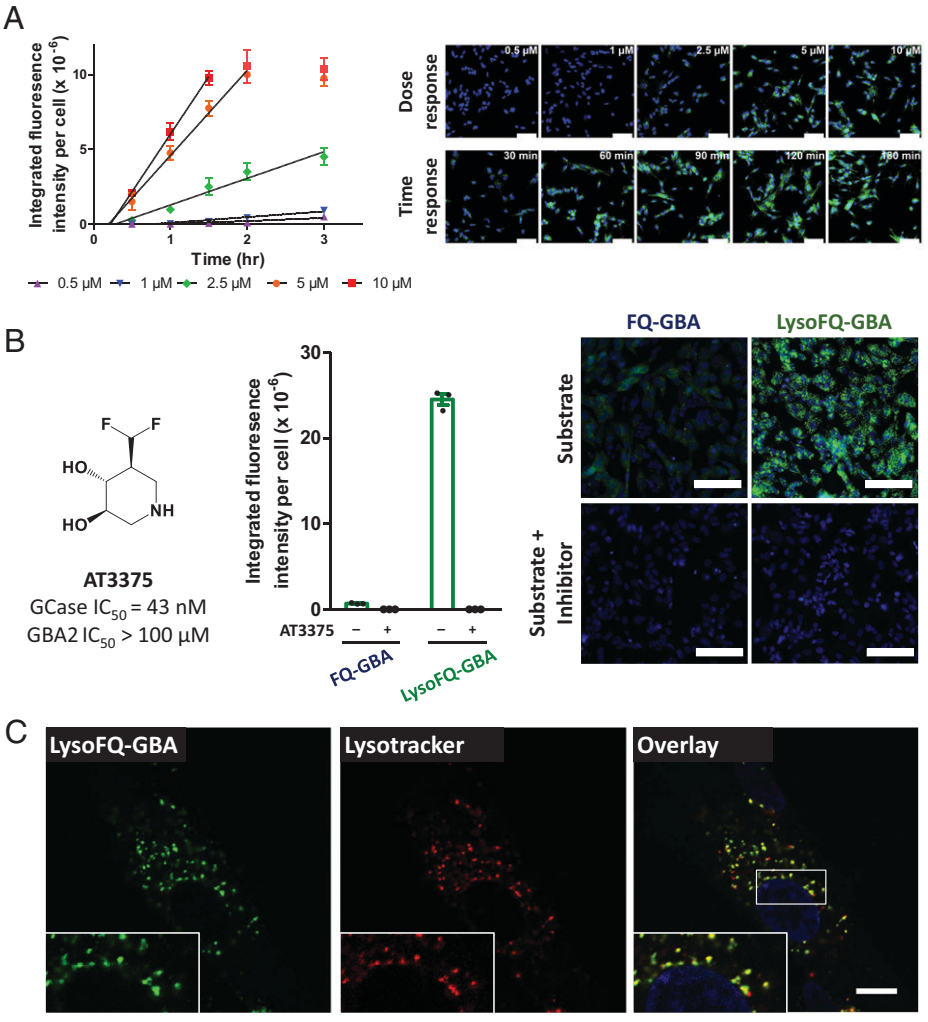

Fig. 2. Determination of the localization and quantitative nature of in situ processing of LysoFQ-GBA in live SK-*N*-SH cells. (*A*) Dose and time response of LysoFQ-GBA in live SK-*N*-SH cells (▲ = 0.5 μM, ▼ = 1 μM, ♦ = 2.5 μM, ● = 5 μM, ■ = 10 μM). (*B*) Comparison of signal intensity of FQ-GBA and LysoFQ-GBA in live SK-*N*-SH cells. (Scale bars: 100 μm.) (*C*) Colocalization of LysoFQ-GBA and the lysosomal marker LysoTracker Red, DND-99. (Scale bar: 10 μm.).

One major factor limiting the utility of fluorescence-quenched substrates is, as noted above, the tendency of their fluorescent products to diffuse within and, ultimately, out of cells (45). This problem leads to instability in signal intensity and inaccurate localization, causing challenges in using such live cell substrates in a reliable quantitative manner (45). As noted above, the lysosomotropic dML residue within LysoFQ-GBA improved fluorescence signal intensity during live cell assays (Fig. 2*B*). To determine whether this difference might arise from retention of the fluorescent product within cells, we compared the stability of fluorescence signals for FQ-GBA and LysoFQ-GBA using a stopped time-course experiment (Fig. 3*A*). After a 2-h incubation with each substrate, cells were washed to remove excess substrate and media supplemented with a high concentration of AT3375 was added to rapidly inhibit GCase within cells. After stopping the enzymatic reaction in this way, the signal intensity was monitored as a function of time. Strikingly, the fluorescent signal within FQ-GBA-treated cells rapidly decreased in an exponential manner with $t_{1/2}$ = 19 min (Fig. 3*B*), leading to a near complete loss of fluorescent signal after 3 h. In contrast, the fluorescent signal within LysoFQ-GBA-treated cells decreased only slightly over the same time frame (Fig. 3*B*), showing that the lysosomotropic dML residue greatly improves retention of the fluorescent product within lysosomes. We were curious to test how well-retained the signal arising from turnover of LysoFQ-GBA was as compared to the signal arising from turnover of PFB-FDGlu. Surprisingly, using PFB-FDGlu, we observed rapid loss of fluorescent signal with a $t_{1/2}$ = 20 min (*SI Appendix*, Fig. S3), suggesting that the majority of fluorescent product was rapidly lost from cells. Moreover, analysis of the distribution of fluorescence signal arising from turnover of PFB-FDGlu showed it was not retained within lysosomes (*SI Appendix*, Fig. S3). Given these observations, we conclude that measurements of GCase activity using FQ-GBA and PFB-FDGlu are complicated by signal diffusion, which leads to time-dependent loss of fluorescent product from the lysosomes of cells. In contrast, the high selectivity of LysoFQ-GBA for GCase, the linear time and dose dependence of its turnover in cells, coupled with excellent retention of the resulting fluorescent product within lysosomes, all point to LysoFQ-GBA being well suited to quantitatively measure GCase activity within living cells using fluorescence microscopy.

LysoFQ-GBA Reports Quantitatively on Lysosomal GCase Activity. We next set out to show that LysoFQ-GBA could be used in a quantitative manner in live cells. We measured the effects of various chemical and genetic perturbations to GCase activity. We first quantified engagement of GCase by the potent and highly selective competitive GCase inhibitor AT3375 (IC_{50} = 43 nM) within SK-*N*-SH cells. We obtained a cellular IC_{50}^{app} value of 170 nM (Fig. 4*A*) with the data exhibiting a hill slope of −1.0, supporting AT3375 acting as a competitive inhibitor of GCase

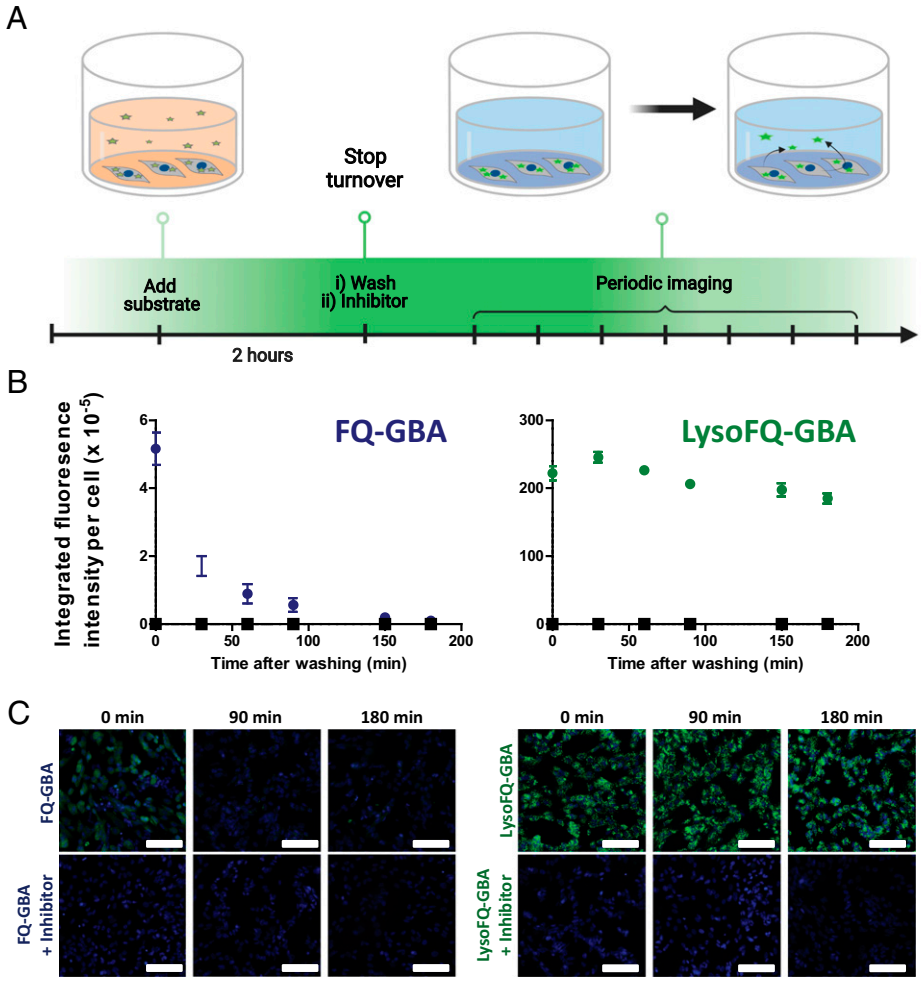

Fig. 3. Quantifying the impact of the lysosomotropic di-methyllysine group on in situ signal stability of LysoFQ-GBA. (*A*) Schematic showing the protocol to determine signal stability in live cells. (*B*) Quantification of fluorescent signal loss of FQ-GBA and LysoFQ-GBA in SK-*N*-SH cells. (Error bars: SEM [*n* = 3].) (*C*) Representative images of the substrate diffusion assay with FQ-GBA and LysoFQ-GBA (inhibitor = 10 μM AT 3375). (Scale bars: 100 μm.).

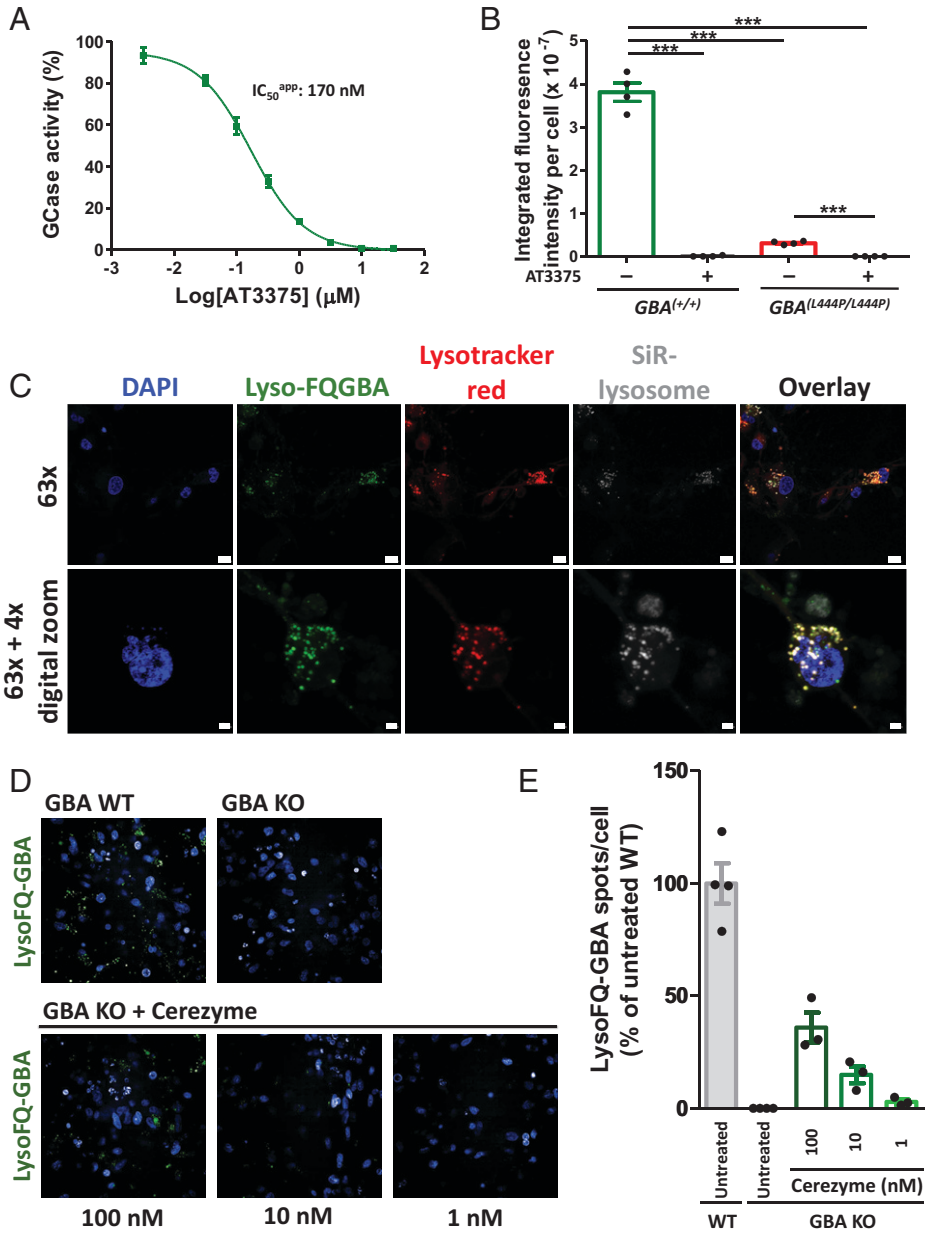

Fig. 4. LysoFQ-GBA quantitatively reports on lysosomal GCase activity in response to chemical and genetic perturbations. (*A*) Determinations of the IC_{50}^{app} of AT3375 in live SK-*N*-SH cells. (Error bars: SEM [$n = 3$].) (*B*) Measurement of residual lysosomal GCase activity in patient fibroblasts (Error bars: SEM [$n = 4$], ***$P < 0.001$.) (*C*) Representative high-resolution confocal images showing colocalization of LysoFQ-GBA with the lysosomal markers LysoTracker Red, DND-99, and SiR-lysosome in live mature forebrain neurons. (Scale bars: 10 μm in 63× images and 2 μm in 63× + 4× digital zoom images.) (*D*) Representative high-resolution confocal images of human neurons showing response in LysoFQ-GBA signal to enzyme replacement with Cerezyme. (*E*) Measurements of puncta with GCase activity, as measured by LysoFQ-GBA in human neurons. (Error bars: SEM [$n = 3$].).

within cells. We next evaluated the ability of LysoFQ-GBA to accurately report on level of active GCase within lysosomes by using fibroblasts harboring either two alleles of wild-type GBA (GM0049) or two mutant alleles of the deleterious L444P mutation (L444P/L444P, GM10915) (Fig. 4*B*). We measured the residual GCase activity within lysosomes of the L444P/L444P to be 8 ± 1%. This value is in reasonable agreement with the 12% residual GCase activity previously determined when assaying GCase activity within whole cell lysates obtained from these patient cells, particularly considering that this value from lysates includes enzyme that is known to be retained in the ER (46). We then set out to explore the potential of LysoFQ-GBA for use in forebrain neurons. As a first step toward this goal, we confirmed LysoFQ-GBA turnover in neurons was localized to lysosomes by measuring colocalization of its signal with LysoTracker Red and SiR-Lysosome, two established lysosomal markers, in iPSC-derived neurons from the well characterized PGPC1 ($GBA^{+/+}$) cell line (Fig. 4*C*). Having established lysosomal turnover of LysoFQ-GBA within neurons, we showed GCase was responsible for this activity by noting no turnover of substrate could be observed in human neurons in which *GBA* had been deleted ($GBA^{-/-}$), but lysosomal activity within this neuronal line could be rescued in a dose-dependent manner by addition of a GCase replacement therapeutic (Cerezyme) (Fig. 4 *D* and *E*). Additionally, we demonstrated that a similar dose-dependent rescue of GCase activity with Cerezyme in mouse neurons was observed using LysoFQ-GBA (*SI Appendix*, Fig. S4). These collective data show that LysoFQ-GBA, with its lysosomotropic dML group, can be used to quantitatively measure lysosomal GCase activity within various types of live cells including

neurons, without complications arising from adventitious turnover by cytosolic GBA2.

We recognized that one broadly useful application of LysoFQ-GBA would be to measure the activity of GCase within suspension cells by flow cytometry. Such an assay could ultimately be applied to clinical assays using patient tissues like PBMCs. To investigate this possibility, we started with SK-*N*-SH cells to allow us to compare observations with our microscopy-based results obtained using these cells. Time- and dose-dependent experiments revealed similar linear regions for both flow cytometry and fluorescence microscopy data (*SI Appendix*, Fig. S5). We next measured target engagement of AT3375 by flow cytometry and established an IC_{50}^{app} of 90 nM (*SI Appendix*, Fig. S6), which was in good agreement with the value of 170 nM we measured by microscopy. These experiments together established that LysoFQ-GBA is well-suited to analysis of GCase activity using different fluorescence-based analytical methods.

Lysosomal GCase Activity in Monocytes May Serve as a Surrogate Measurement of Activity in Neuronal Tissue. Given the interest in measuring lysosomal GCase activity in peripheral tissue from PD or GD patients (24, 36, 39), we next evaluated whether LysoFQ-GBA could be used in monocytes, which are readily obtained from patient blood. Moreover, given the inability to measure GCase activity directly in patient brain, we reasoned that GCase activity in monocytes could eventually prove useful as a surrogate measure of GCase activity in neurons. We therefore set out to test whether such a correlation could be observed by using LysoFQ-GBA to measure GCase activity in monocytes, neural progenitor cells (NPCs), and neurons obtained by differentiation from the same set of patient-derived iPSCs. We used established wild-type (PGPC1, PGPC17, $GBA^{+/+}$) cell lines (47), two previously described heterozygous (UOXFi001B, UOXFi003A, $GBA^{N370S/+}$) PD lines (48), as well as a compound heterozygous GD line (GD1260, $GBA^{L444P/P415R}$) that we established by reprogramming fibroblasts using the method involving nonintegrating Sendai virus (49). For this newly established GD1260 line (*SI Appendix*, Fig. S7), we confirmed morphology by light microscopy, chromosomal integrity using karyotype analysis, phenotype by examining the expression of stemness marker OCT4 and TRA-1-60 using immunocytochemistry and flow cytometry analysis respectively, and confirmed its capacity to differentiate into three germ layers and grow stably over 30 passages. With these patient cell lines in hand, we set out to assess the relative activity in neuronal and monocyte lineages.

Toward this goal, we first differentiated wild-type PGPC1 iPSCs into monocytes and NPCs, after which we then showed that turnover of LysoFQ-GBA by GCase was linear over regions in both the time- and dose-response in these cell lineages (*SI Appendix*, Fig. S8). These observations allowed us to identify suitable assay conditions that we could use to make quantitative measurements of lysosomal GCase activity (Fig. 5). We therefore subsequently differentiated the other cell lines harboring mutant alleles of *GBA* and measured GCase activity in monocytes (Fig. 5*A* and *D*), NPCs (Fig. 5*B* and *E*), and forebrain neurons (Fig. 5*C* and *G*). Unfortunately, despite repeated efforts, the 003A heterozygote PD patient iPSC line could not be cleanly differentiated to neurons, and a significant fraction of astrocytes were always observed to contaminate neurons (*SI Appendix*, Fig. S9), ruling out analysis of GCase activity within neurons from this line. Nevertheless, relative to the wild-type PGPC1 line, the wild-type line PGPC17 had 90 ± 3% activity in NPCs and 96 ± 14% activity in neurons, the two GBA-PD heterozygotes lines (001B and 003A) displayed 53 ± 6% and 56 ± 7% activity in NPCs and 40 ± 3% activity in 001B neurons, and the compound heterozygote line showed just 2 ± 1% residual GCase activity in NPCs and 12 ± 5% in neurons. Encouragingly, LysoFQ-GBA therefore revealed a clear correlation between relative GCase activity and *GBA* allele status of each cleanly differentiated patient cell lines. Notably, when we compared these live cell GCase activity measurements to those we made using cell lysates, we observed that the cell lysate assay reported higher residual activity in mutant cell lines (*SI Appendix*, Fig. S10). These observations are in line with previous reports that in vitro assays function to measure the total cellular GCase activity including GCase localized to the ER and Golgi, which is known to be elevated due to trafficking defects seen for disease-relevant point mutants of GCase (20). Notably, we observed a similar correlation between allele status and GCase activity when selectively assaying GCase activity in iPSC-derived monocytes (Fig. 5*A*). In monocytes, the wild-type line PGPC17 had 113 ± 11% activity, the two GBA-PD heterozygotes lines displayed 49 ± 7% and 55 ± 7% activity, and the compound heterozygote line had 3.9 ± 0.2% residual GCase activity as compared to the wild-type PGPC1 line. Comparing activity measurements between each lineage for each cell line, we found a strong correlation between GCase activities in monocytes as compared to activity in both NPCs ($m = 1.1$, $R^2 = 0.95$) (Fig. 5 *E* and *F*) and neurons ($m = 1.1$, $R^2 = 0.97$) (Fig. 5 *G* and *H*). These data suggest that measuring lysosomal GCase activity within monocytes using LysoFQ-GBA may provide a useful surrogate measure for GCase activity within neurons of patients.

Discussion

The discovery that mutations in *GBA* are the most common genetic risk factor for PD has spawned growing interest in the mechanisms by which this enzyme is regulated within cells and how its function goes awry in PD (11). Although no clear consensus has emerged in the literature, GCase activity appears to be decreased in the sporadic PD patient population both within brain (39) and blood (24). In addition, the modest penetrance of *GBA* mutations has prompted searching for genetic modifiers of GCase activity, which has already led to identification of a few genes that influence GCase function (12, 23, 50). Given these and other emerging lines of research into both PD and GD, chemical biology tools for the accurate and precise measurement of lysosomal GCase activity are of great value. The fluorescence-quenched substrate LysoFQ-GBA that we describe here fills this need. We detail a robust synthetic route and show LysoFQ-GBA is a versatile tool to measure GCase activity within lysosomes of live cells.

Emphasizing the versatility of LysoFQ-GBA is its ability to report on the activity of GCase in cell lines expressing endogenous levels of enzyme activity. Fluorescence-quenched substrates often depend on the use of cancer lines or genetically manipulated cell lines that over-express a target enzyme (51, 52). Moreover, such substrates have rarely been deployed in a rigorous manner to define linear regions of response and thereby enable quantitative measurements. In contrast, the excellent sensitivity of LysoFQ-GBA enables accurately measuring enzymatic turnover in patient cell lines having greatly diminished GCase activity. We illustrate this ability to measure relative lysosomal GCase activity within patient tissues using five different iPSC patient cell lines differentiated to monocytes, NPCs, and neurons. Through these studies, we defined a strong correlation between the lysosomal GCase activities measured among these different cell lineages,

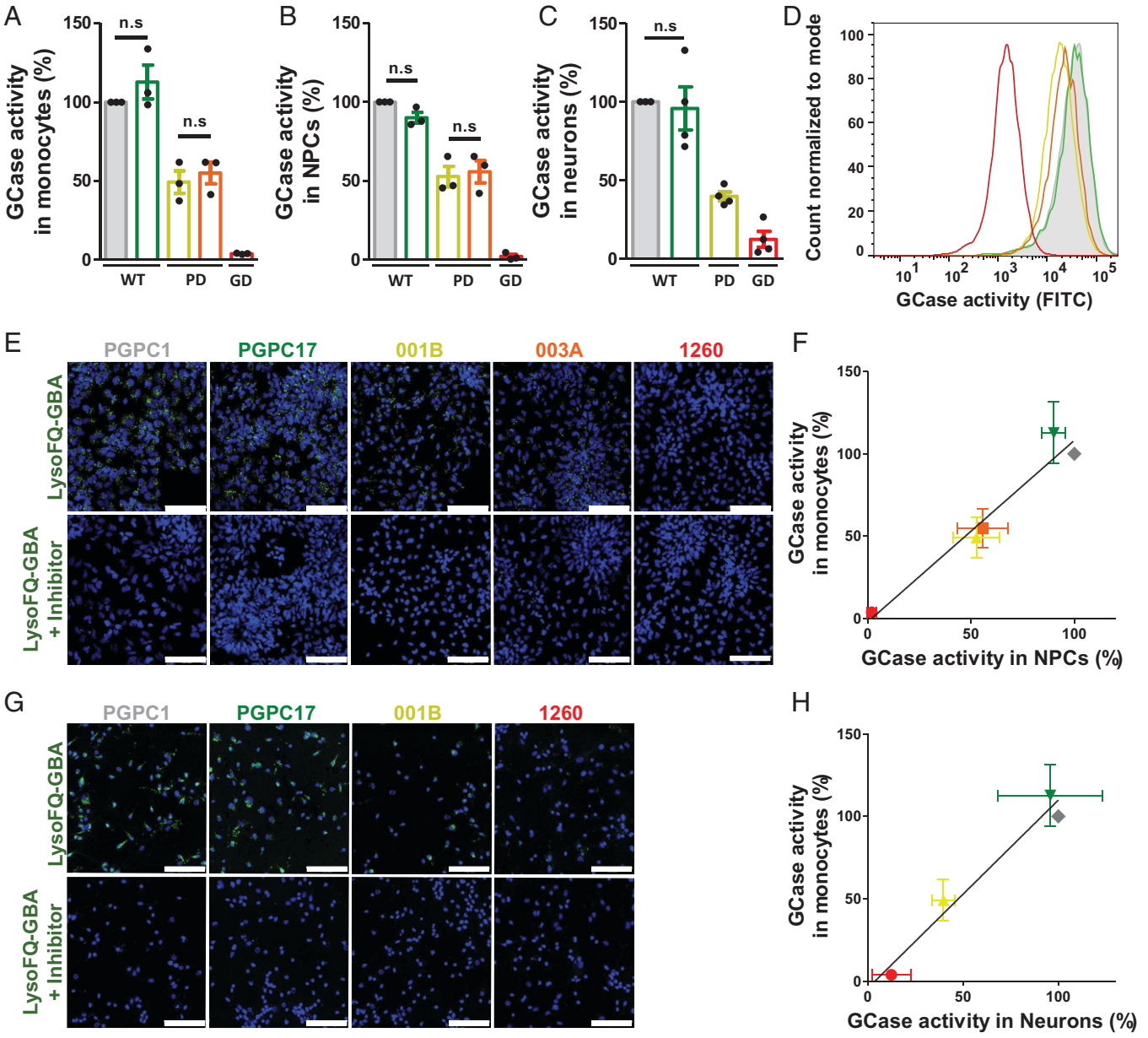

Fig. 5. LysoFQ-GBA reports on lysosomal GCase activity in different lineages of iPSC derived cells (■ = PGPC1 [$GBA^{+/+}$], ■ = PGPC17 [$GBA^{+/+}$], ■ = 001B [$GBA^{N370S/+}$], ■ = 003A [$GBA^{N370S/+}$], ■ = 1260 [$GBA^{L444P/P415R}$]). (*A*) Measurement of residual lysosomal GCase activity in patient derived patient derived monocytes using flow cytometry. (Error bars: SEM [n = 3].) (*B*) Measurement of residual lysosomal GCase activity in neural progenitor cells (NPCs) using microscopy. (Error bars: SEM [n = 3].) (*C*) Measurement of residual lysosomal GCase activity in iPSC-derived human forebrain neurons using microscopy. (Error bars: SEM [n = 4].) (*D*) Representative histograms for flow cytometry analyses of different lines of patient iPSC-derived monocytes. (*E*) Representative images of lysosomal GCase activity in different lines of patient iPSC-derived neural progenitor cells (NPCs). (Scale bars: 100 μm.) (*F*) GCase activities in NPCs and monocytes using LysoFQ-GBA show a linear correlation. (Error bars: SD [n = 3].) (*G*) Representative images of lysosomal GCase activity in different lines of patient iPSC-derived forebrain neurons. (Scale bars: 100 μm.) (*H*) GCase activities in IPSC-derived forebrain neurons and monocytes show a linear correlation. (Error bars: SD [n = 4].).

supporting the idea that monocytes could serve as a useful surrogate tissue for the study of GCase activity within neurons. Notably, our observations are in agreement with a recent report that monocytes are a useful model for detecting the biochemical changes characteristic of PD patients including, most notably, α-synuclein accumulation (42). LysoFQ-GBA should therefore enable a range of diverse experiments involving assessing lysosomal GCase activity in a variety of different cell types. We foresee, for example, that measuring lysosomal GCase activity will aid in identifying and studying cellular factors that influence GD disease phenotype and contribute to the variable penetrance of *GBA* mutations in both GD and PD. We also anticipate that LysoFQ-GBA and associated GCase substrates may aid in clinical studies of PD, particularly because GCase activity may be lower in the general PD patient population (24, 39). Indeed, emerging research suggests that GCase activity in peripheral tissue may serve as a potential biomarker for the development of PD (24). Finally, the advancement of gene therapies targeting GBA for PD could benefit from analysis of lysosomal GCase activity to monitor efficacy and durability of treatments. Therefore, we expect that the precise and accurate measures of lysosomal GCase activity afforded by LysoFQ-GBA will enable a range of useful clinical assays.

As noted above, few fluorogenic enzyme substrates have been shown to serve in an accurate quantitative manner (44). This issue may stem from poor signal to noise due to rapid diffusion

of the fluorescent cleavage product (45). Previous approaches to improve signal stability of live cell enzyme substrates have used latent amine groups that are released upon enzymatic cleavage (31), however, these appear less general and accessible. We addressed this diffusion problem through inclusion of a simple lysosomotropic dML residue within the substrate. Lysosomal retention of the resulting fluorescent product greatly increased signal stability and intensity, leading to cellular assays that showed both linear dose and time dependence in various cell types using both microscopy and flow cytometry. Notable in this regard is that these measurements of activity in wild-type and mutant cell lines are as precise as those performed using cell lysates but, unlike such lysate assays, report only on physiologically relevant lysosomal enzyme. Furthermore, several features of LysoFQ-GBA make this probe well-suited for a wide range of applications. The synthetic accessibility of LysoFQ-GBA and its amenability to late-stage derivatization should allow developing related fluorescence-quenched substrates with photophysical properties compatible for use with other live cell assays including bio-orthogonal (53) or nanobody-based labeling of proteins (54). More generally, we anticipate that the approach to the design and validation of LysoFQ-GBA for quantitative measurement of lysosomal GCase activity provides a useful blueprint for the development of new enzyme substrates for live-cell imaging of enzyme activities that will open new possible experimental lines of inquiry.

Materials and Methods

A detailed description of the materials and methods used in these studies can be found in the *SI Appendix*. This *SI Appendix* specifically includes a details regarding the (i) synthetic procedures used in making BODIPY-FL, BHQ-1, FQ-GBA, and LysoFQ-GBA including characterization of the intermediates and products; (ii) experimental methods used in the generation of GD1260 iPSC and characterization of this cell line; (iii) culture and differentiation methods used to generate monocytes, NPCs, and neurons from the iPSC lines used in these studies; (iv) methods for the culture of fibroblasts and SK-*N*-SH cells; (v) GCase lysate enzyme assays; and (vi) methods for live cell assays and colocalization studies performed by microscopy and flow cytometry using the various cell lines and lineages.

Data Availability. All study data are included in the article and/or *SI Appendix*.

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Author affiliations: [a]Department of Chemistry, Simon Fraser University, Burnaby, BC V5A 1S6, Canada; [b]Department of Molecular Biology and Biochemistry, Simon Fraser University, Burnaby, BC V5A 1S6, Canada; [c]Roche Pharma Research and Early Development, Neuroscience and Rare Diseases Discovery and Translational Area, Roche Innovation Center Basel, F. Hoffmann-La Roche Ltd, Basel, Switzerland; and [d]Diabetes Research Group, BC Children's Hospital Research Institute, Vancouver, BC V5Z 4H4, Canada

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