Engineering intracellularly retained Gaussia Luciferase reporters for improved biosensing and molecular imaging applications

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SUPPLEMENTARY MATERIALS

MATERIAL AND METHODS

Materials

Chemicals, enzymes, and reagents

We purchased cell culture plates, fetal bovine serum (FBS), penicillin, streptomycin, and cell culture media from GIBCO BRL (Frederick, MD); lipofectamine 2000 transfection reagent and pcDNA3.1(+) eukaryotic expression vector for constructing different plasmids from Invitrogen (Carlsbad, CA); ampicillin for bacterial culture and dimethyl sulfoxide (DMSO) from Sigma (St. Louis, MO); bacterial culture media from BD Diagnostic Systems (Sparks, MD); and all restriction and modification enzymes, and T4-DNA ligase from New England Biolabs (Beverly, MA). We constructed the plasmid vectors encoding the variants of FLUC (FLUC1, FLUC2 and TFLUC), sGLUC (sGLUC and tGLUC), CBLUC, and RLUC using DNA fragments that were PCR-amplified using the templates available from our plasmid bank. We also purchased 5X passive lysis buffer and Luciferase assay reagent II (LARII) from Promega Corp. (Madison, WI); the plasmid extraction kit and DNA gel elution kit from Qiagen (Valencia, CA) and Epoch life sciences (Missouri city, TX); coelenterazine from Nanolight (Pinetop, AZ); Taq-DNA Polymerase for PCR amplification from 5-Prime (Gaithersburg, MD); GLUC antibody from Nanolight (Pinetop, AZ); and GAPDH antibody from Cell signaling (Danvers, MA). The primers for PCR amplifications were synthesized by the Stanford Protein and Nucleic Acid (PAN) facility. Sequencing of plasmid vectors was carried out by Sequetech DNA sequencing service (Mountain View, CA). We purchased drugs such as rapamycin, estradiol, tunicamycin, doxorubicin, carboplatin, 4-hydroxytamoxifen, diethylstilbestrol, genistein, raloxifene, methyl-piperidino-pyrazole hydrate, TBHQ (tertiary butyl hydroquinone), and MITO Tempo from Sigma Aldrich (St Louis, MO).

Methods

Plasmids and constructs

We constructed different optical reporters such as, FLUC1, FLUC2 (human codon optimized FLUC1), TFLUC (thermostable-FLUC; containing single amino acid changes at five different positions of FLUC1 protein)[1], CBLUC (Click beetle luciferase-Green), hRLUC (human codon optimized Renilla luciferase), sGLUC, and tGLUC (N-terminal 16-amino acids deleted version) by PCR amplification of respective cDNAs using forward primer with NheI restriction enzyme site and reverse primer with XhoI restriction enzyme site, and cloning into a respective enzyme (*NheI*/*XhoI)* digested pcDNA 3.1(+) plasmid backbone. The sequence confirmed clones were used for further experiments. We constructed sGLUC, sGLUC-KDEL, sGLUC-$(KDEL)_2$, sGLUC-$(KDEL)_3$, and sGLUC-$(KDEL)_4$ by inserting the wild-type GLUC sequence and additional DNA sequences coding for KDEL into pcDNA, replacing the MCS sequence between the *NheI* and *XhoI* restriction enzyme sites. The KDEL-coding DNA sequences were amplified by PCR using primers referenced in the supplementary materials (SI-Table). Similarly, we cloned the N-terminal engineered clones of sGLUC (N-DGEN-7-GLUC, N-DGEN-40-GLUC, TK-GLUC, BNLS-GLUC) into a pcDNA3.1 (+) backbone under a CMV promoter at the NheI/XhoI restriction enzyme sites. We transformed plasmids into competent *E. coli* (Top10) cells, and screened colonies for GLUC activity. We sequence-confirmed the constructs before using them for further experiments.

Construction of Split-tGLUC complementation sensor systems

We generated vector constructs needed for two different imaging sensor systems based on split-tGLUC complementation, namely: (1) a rapamycin mediated protein-protein interaction system (FRB-FKBP12 interaction)[2-4], and (2) an estrogen receptor (ER) intramolecular folding sensor system[5-7]. We constructed vectors expressing N-tGLUC-FRB, FKBP12-C-tGLUC, and N-

tGLUC-ER(LBD)-C-tGLUC fusion proteins by using a PCR based cloning strategy in pcDNA 3.1(+) vector backbone under a constitutive CMV promoter. The sequence-confirmed vectors were used for various *in vitro* transfection experiments.

Transient transfection and GLUC assay using luminometry

To compare luciferase activity from various engineered GLUC constructs and split-luciferase based complementation sensor systems, we performed transient transfections in HEK-293T cells. HEK-293T cells plated to 80% confluence (100,000 cells/well in 12-well culture plates) were transfected using 1 µg of respective plasmids and Lipofectamine 2000 transfection reagent, following the manufacturer protocol. We assessed the cells after 48 h post transfection for secreted luciferase activity in the medium, as well as intracellular luciferase activity after lysing the cells in 1X Passive Lysis Buffer (PLB, Promega). We normalized the activity by estimating protein from the cell lysates.

Lentiviral vector construction, virus production, and stable cell generation by viral transduction

GLUC and GLUC with C-terminal KDEL peptide in different repeat number were constructed in a lentiviral backbone (pHAGE-UBI-dTomato-CMV-GLUC). Respective GLUC constructs cloned in a pcDNA 3.1 (+) backbone were released by *NheI/PmeI* restriction enzymes and cloned into respective enzyme digested pHAGE-UBI-dTomato-CMV-MCS vector backbone. The clones were confirmed by sequencing before using them for viral production. We used three-vector transfection systems (pHAGE-UBI-dTomato-CMV-GLUC, VPR, and VSVG) adopting the calcium phosphate transfection method. The concentrated pure virus after titration was used to generate stable Ln229 cells by transduction. After three continuous passages, the cells of uniform expression were FACS sorted and used for various further experiments.

Cell culture and generation of stable cell clones expressing equal levels of sGLUC variants using lentiviral transduction

293T, MDA-MB231, and Ln229 (ATCC, Manassas, VA) cells were grown in Dulbecco's Modified Eagle's Medium (Cat #: 11995) supplemented with 10% FBS and 1% penicillin/streptomycin. Stable MDA-MB231 cells expressing GLUC variants with different numbers of KDEL repeats and the sGLUC-DEVD-(KDEL) caspase sensor were produced by lentiviral transduction. We followed our previously published procedure. In brief, the cells plated 24 h before transduction to a 80% confluence in 10 cm plates were washed once with PBS, and then 2 ml of ($1X10^8$ PFU) virus mixed with 3 ml of serum free Opti-MEM and 5 µl of 1 mg/mL of Polybrene were added. The plate was incubated for 4 hr at 37 °C and 5% CO_2 with intermittent mixing. Four hours later the medium was supplemented with 5 ml of complete medium containing 20% FBS. The cells were sub-cultured thrice before we FACS sorted (using co-expressed dTomato) for uniformly expressing clones to be used in further experiments.

Luciferase assay

Cells were harvested and lysed in 1X Passive Lysis Buffer (Promega, Madison, WI), by placing the tubes on an orbital shaker for 10 min. We then collected cell lysates and centrifuged them at 14,800 rpm at 4 °C for 5 min to remove cell debris. A 20/20n Luminometer (Turner Biosystems, Sunnyvale, CA) was used for GLUC bioluminescence measurements. In brief, we assayed 5 µL samples of cleared supernatant for GLUC activity by the addition of 100 µL of (10 µg/mL) coelenterazine (Nanolight, Pinetop, AZ) in PBS, with an integration time of 3 sec. Similarly, the GLUC assay for the secreted protein was performed by adding 5 µL medium with 100 µL of (10 µg/mL) coelenterazine. The results were calculated by measuring the total volume of cell lysate or medium.

In-plate GLUC assay using IVIS optical CCD camera imaging and calibration for activity decay to measure the absolute level of signal

Stable MDA-MB231 cell lines expressing GLUC constructs were plated for various experiments. After 24 h, we washed and changed the media in these experiments. Where indicated, cells were also treated with corresponding drugs in respective doses. After 48 h, we removed 5 µL of media and transferred this to black wall 96-well plates, which were then assayed for GLUC activity by the addition of 50 µL coelenterazine (10µg/ml, Nanolight, Pinetop, AZ) into each well. We also assayed the cells in each well for GLUC activity by the addition of 100 µL coelenterazine into each well. We acquired measurements by bioluminescence imaging using the IVIS-Lumina imaging system (Perkin Elmer, Waltham, MA). We normalized GLUC bioluminescence with the decay rate of the signal from the time of first adding substrate to each row until the time imaging began. We used these values to normalize decay corrections in all other experiments.

Immunoblot analysis

Six million cells of each stable MDA-MB231 cell line expressing GLUC constructs were harvested by trypsinization, and spun at 5,000 rpm for 5 min, and the cell pellets were lysed in RIPA buffer containing protease inhibitor cocktail and EDTA by sonication at 40% amplitude for 15 sec three times. The cell lysates were centrifuged at 15,000 rpm for 5 min at 4 °C to remove the membrane proteins before they were used for protein estimation using the Nanodrop. We prepared protein samples, containing 200 µg of total protein in 1X Lamelli loading buffer with β-mercaptoethanol (Life Technologies, Carlsbad, CA), which were denatured at 95 °C for 5 min, resolved on 4–12% SDS-polyacrylamide pre-cast gels (Life Technologies, Carlsbad, CA) and electroblotted onto a polyvinylidene difluoride nylon membrane (Bio-Rad, Hercules, CA). We used the SeeBlue Pre-Stained Standard (Life Technologies, Carlsbad, CA) to enable molecular weight estimations. Membranes were

blocked in 5% non-fat dry milk in Tris-buffered saline containing 0.01% Tween-20 (TBST) followed by incubation with a primary antibody. As appropriate, we used antibodies suitable for the detection of GLUC (#E8023S, New England Biolabs, Ipswich, MA). Following three TBST washes, we incubated membranes with the appropriate horseradish-peroxidase (HRP) conjugated secondary antibody (Sigma Aldrich). After three additional TBST washes, we incubated immunoblots with the Pierce ECL Western Blotting Substrate (Thermo Scientific, Waltham, MA) for 1 min, and then used the IVIS-Lumina imaging system (Caliper Life Sciences, Hopkinton, MA) to detect and measure chemiluminescent signals and bands respectively. A GAPDH primary antibody (B-5-1-2, Santa Cruz Biotechnology, Santa Cruz, CA) was used as a loading control antibody. Similarly, for measuring the caspase sensor cleaved sGLUC proteins, we used samples from MDA-MB231 cells stably expressing caspase sensor sGLUC (GLUC-DEVD-$(KDEL)_3$) after treating with chemotherapeutic drugs (paclitaxel, doxorubicin, and carboplatin). We followed the similar procedure mentioned above for detecting GLUC protein.

Treatment and assay conditions to measure caspase and endoplasmic reticulum stress sensors.

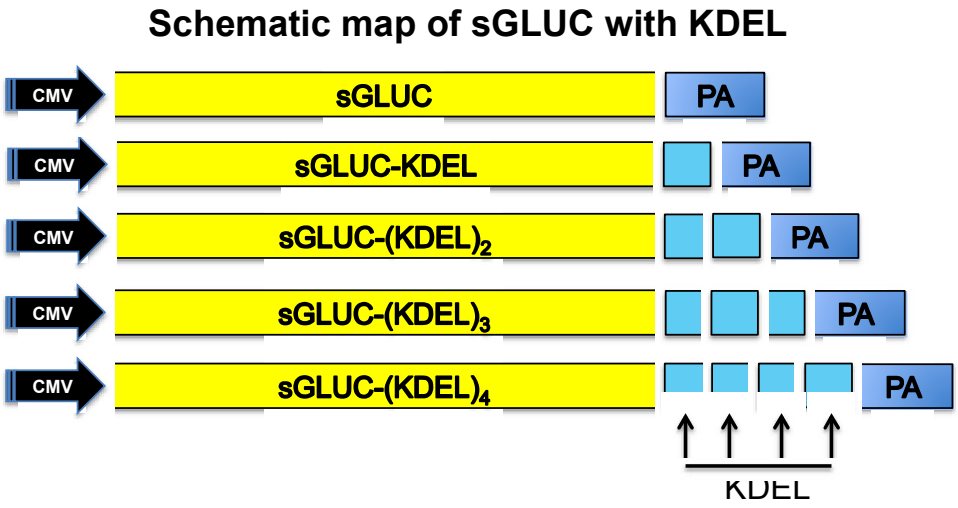
MDA-MB231 cells stably expressing sGLUC with different KDEL repeats and with DEVD caspase cleavage peptide were used for evaluating secretion of GLUC protein in response to treatment using an endoplasmic reticulum stressor (tunicamycin), and a chemotherapeutic drug. The cells were treated with different concentrations of tunicamycin (0-10 μg/ml), doxorubicin (0-10 μM), paclitaxel (0-25 nM) and carboplatin (0-25 μM). The GLUC signal in medium and cells (with and without lysis) were evaluated over time for several days. The results were calculated as relative fold in signal with and without treatment.

Statistical analyses.

Data were presented as the means ± SEM. Differences were analyzed by unpaired two-tailed *t*-test between two groups. Statistical analysis was performed by one-way ANOVA and $p<0.05$ was taken to indicate a significant difference between groups.

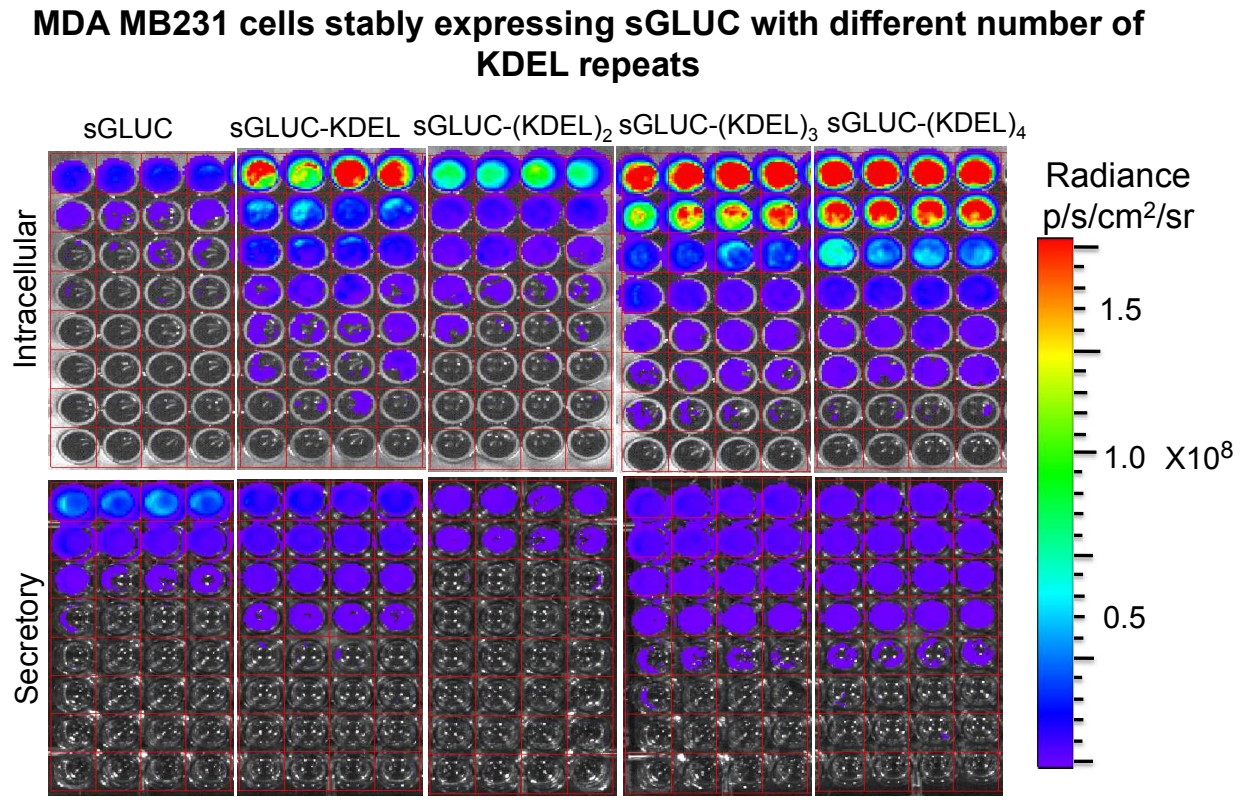
SUPPLEMENTARY FIGURES

Supplementary Figure 1



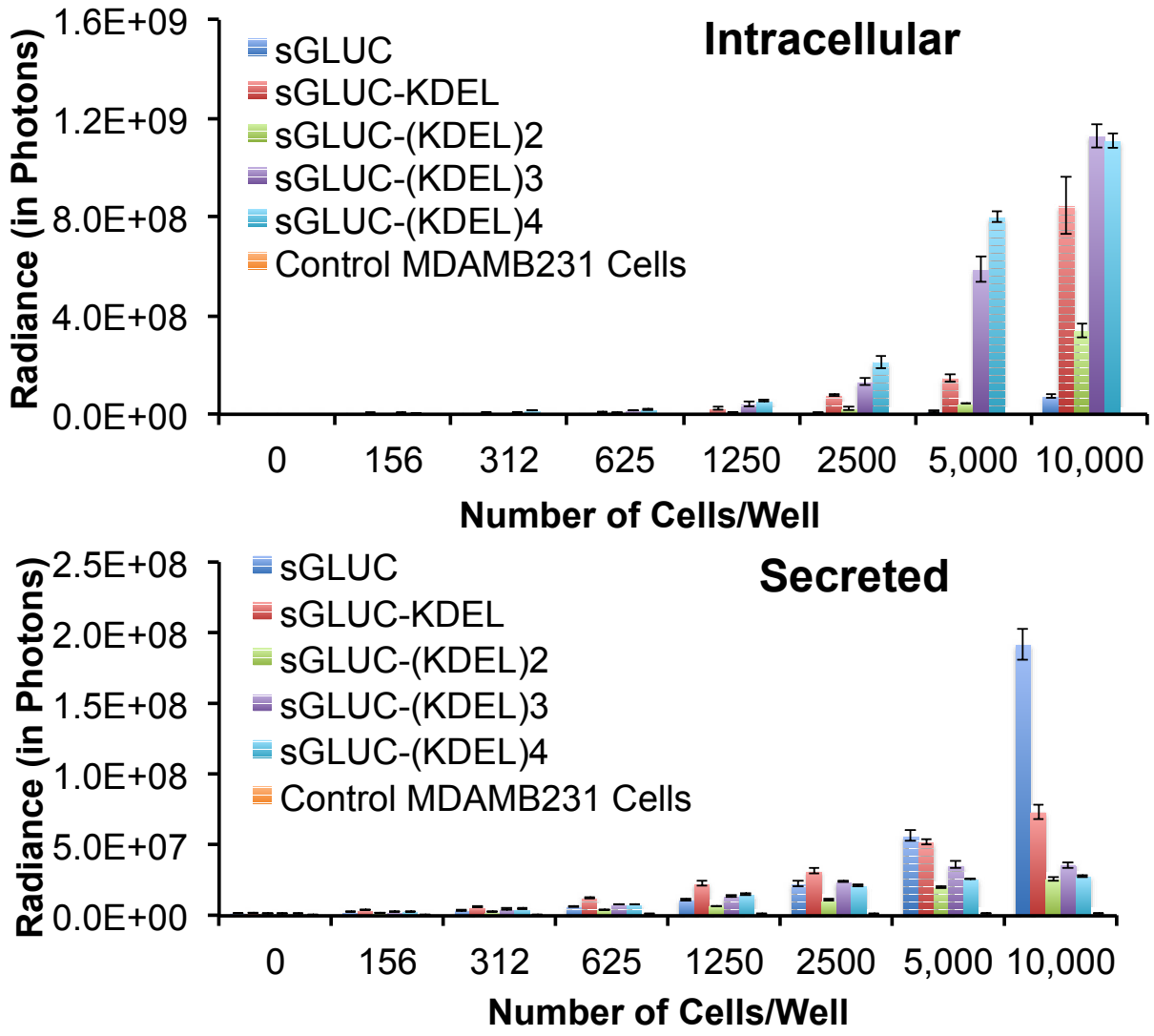
Supplementary Figure 1. Schematic map of sGLUC with different numbers of KDEL repeats. CMV: promoter, sGLUC: coding sequence for sGLUC protein, KDEL: four amino acid endoplasmic reticulum-targeting peptide, PA: Poly A sequence of SV40 virus.

Supplementary Figure 2



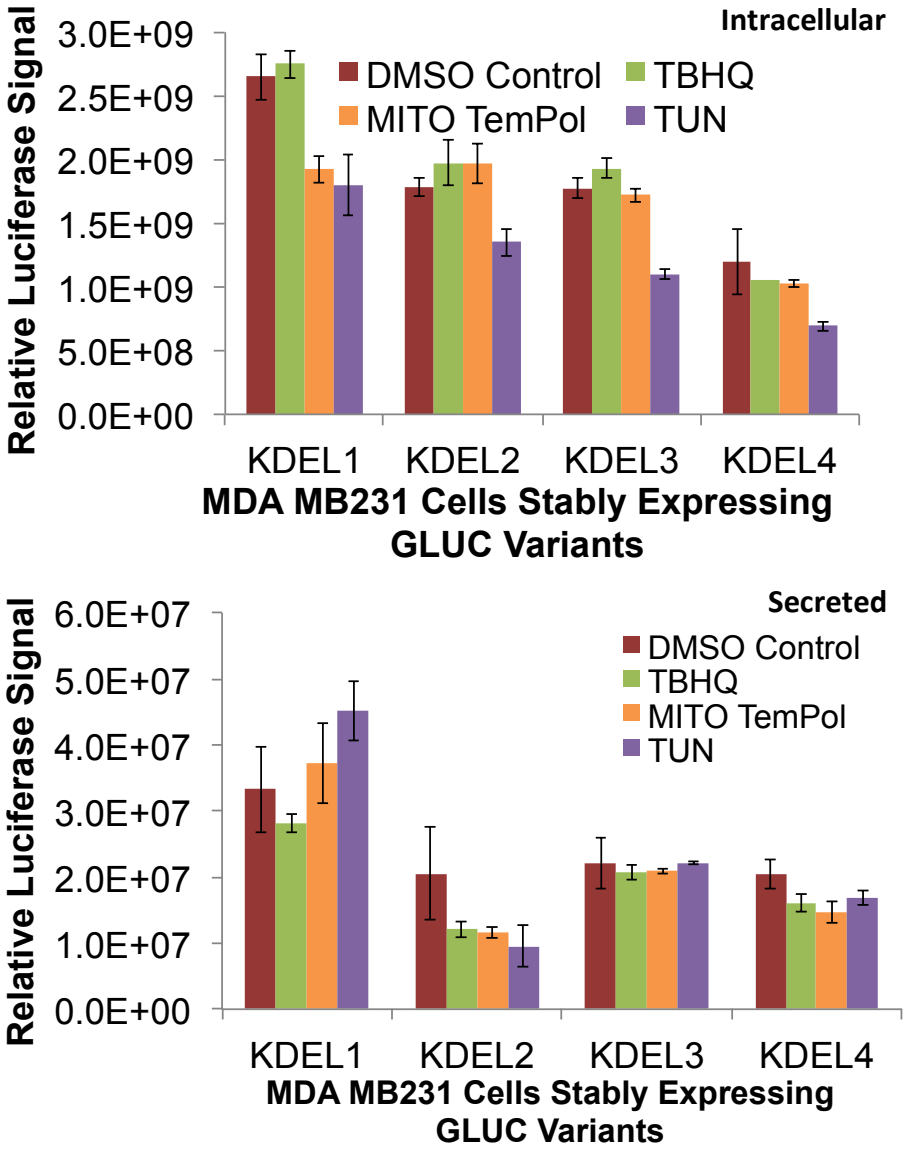
Supplementary Figure 2. MDA-MB231 cells stably expressing sGLUC with different numbers of KDEL repeats measured for GLUC signal from cells (intracellular) and in the medium (secretory) after plating different numbers of cells (10,000, 5,000, 2,500, 1,250, 625, 312, 166) by optical CCD camera imaging.

Supplementary Figure 3



Supplementary Figure 3. Intracellular and secretary GLUC protein levels measured in MDA-MB231 cells stably expressing an equal number of GLUC constructs with different numbers of KDEL repeats. (**a**). GLUC enzyme activity measured from cell lysate (Intracellular). (**b**). GLUC enzyme activity measured from medium (Secreted).

Supplementary Figure 4



Supplementary Figure 4. Intracellular and secretory GLUC signal measured from MDA-MB231 cells stably expressing GLUC variants with different numbers of KDEL repeats in response to the treatment of different stressors (TBHQ: an inducer of oxidative stress (20 μM), MITO-TemPol: an inducer of oxidative stress in mitochondria (10 μM), and tunicamycin: an inducer of endoplasmic reticulum stress (2.5 μg/ml)).

SUPPLEMENTARY TABLE

Supplementary Table 1

PCR-Primers Names	Primer Sequence (5'-3')
sGLUC-F	5'-CTAGCTAGCATGGGAGTCAAAGTTCTGT-3'
sGLUC-(KDEL)-R	5'-CCGCTCGAGCTACAGTTCGTCTTTGTCACCACCGGCCCCT TGAT-3'
sGLUC-$(KDEL)_3$-R	5'-CCGCTCGAGCTATAGTTCGTCTTTGAGCTCATCCTTTAACTC ATC-3'
sGLUC-$(KDEL)_4$-R	5'-CCGCTCGAGCTATAGTTCGTCTTTTAGTTCGTCTTTGAGCTC ATCCTTTAACTCATC-3'
sGLUC-(KDEL)-DEVD-R	5' CCGCTCGAGCTATAGTTCGTCTTTGAGCTCATCCTTTAACTC ATC-3'
sGLUC-$(KDEL)_3$-DEVD-R	5'-CCGCTCGAGCTATAGTTCGTCTTTGAGCTCATCCTTTAACT CATCCTTGTCAACTTCATCGTCACCACCGGCCCCCTTGAT-3'
NDGEN-GLUC-CL7	5'-CTAGCTAGCATGGGATCAAAAGCCAGACACGGATGAGTTC TATTGGA-3'

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