

TARGETED *IN VIVO* O-GLCNAC SENSORS REVEAL DISCRETE COMPARTMENT-SPECIFIC DYNAMICS DURING SIGNAL TRANSDUCTION

Luz D. Carrillo¹, Joshua A. Froemming¹ and Lara K. Mahal^{*1,2}

Supplemental Procedure 1

Splicing by overlap extension PCR was used to construct a hybrid insert containing eCFP (Clontech, Mountain View, CA) and GafD (Timo Korhonen, University of Helsinki). This insert was cloned into the bacterial expression vector pRSET A at the 5' *Bam*H I and 3' *Nco* I sites to create the CFP/GafD vector (construct pLKM2; ref 18). To append the casein kinase peptide to the construct OS2 a 5' PCR primer was designed to insert an *Nco* I site followed by two linker regions at the beginning and end of the casein kinase II peptide before the coding sequence of Venus. A 3' primer was designed to anneal downstream of Venus and introduce a stop codon and a unique site *Hind* III. The primers used were pLKM4.Fwd 5'-CAT GCC ATG GGG CGG CAG CGG CGG CCC GGG CGG CAG CAC CCC GGT GAG CAG CGC GAA CAT GAT GGG CGG CAG CGG CGG CAT GGT GAG CAA GGG CGA GGA G-3' and pLKM4.Rev 5'-GGG AAG CTT TTA CTT GTA CAG CTC GTC CAT GCC-3'. The PCR reactions were performed using the Taq DNA Polymerase Kit (New England Biolabs) in a final reaction volume of 50 μ L. The reaction consisted of 41.5 μ L ddH₂O, 5 μ L 10X buffer, 1 μ L dNTPs, 1 μ L pLKM3 (1ng/ μ L), 0.5 μ L pLKM4.Fwd primer (20 μ M), 0.5 μ L pLKM4.Rev primer (20 μ M) and 0.5 μ L Taq polymerase. The reaction was denatured at 94°C for 10 minutes, followed by 30 cycles of 30 seconds at 94°C, 30 seconds at 55 °C and 1 minute at 72 °C, and finally a 4 °C hold after all the cycles were completed. PCR product and pLKM2 were digested with *Nco* I and *Hind* III at 37°C for 3 hours. Restriction digest reactions were gel purified using the QIAquick Gel Extraction Kit (Qiagen). The PCR product and pLKM2 were ligated using the T4 DNA Ligase (New England Biolabs), resulting in the OS2 sensor. This construct was transformed into *E. coli* DH5 α cells and plated on selective media. A 5 mL culture of transformed *E. coli* DH5 α cells containing the construct was grown to saturation and the plasmid was purified using a QIAprep Spin Miniprep Kit (Qiagen).

A control sensor was also developed. OS2 control sensor had an active GafD domain and all of the serine or threonine residues in the casein kinase II domain were changed to alanine or glycine. To develop this reporter a 5' PCR primer was designed to insert an *Nco* I site, the two linker regions identical to the ones in OS2, and a mutated casein kinase II peptide before the coding sequence of Venus. A 3' primer was designed to anneal downstream of Venus and introduce a stop codon and a unique *Hind* III site. For OS2 control sensor the primers used were pLDC5.Fwd 5'-CAT GCC ATG GGG CGG CAG CGG CGG CCC GGG CGG CGC CGG CCC GGT GGG CGG CGC GAA CAT GAT GGG CGG CAG CGG CGG CAT GGT GAG CAA GGG CGA GGA G-3' and pLKM4.Rev 5'-GGG AAG CTT TTA CTT GTA CAG CTC GTC CAT GCC-3'. The PCR reaction conditions, purification, ligation to pLKM2 and transformation were identical to the cloning of OS2 sensor. Sequence for all the constructs prepared was confirmed by the University of Texas DNA sequencing facility.

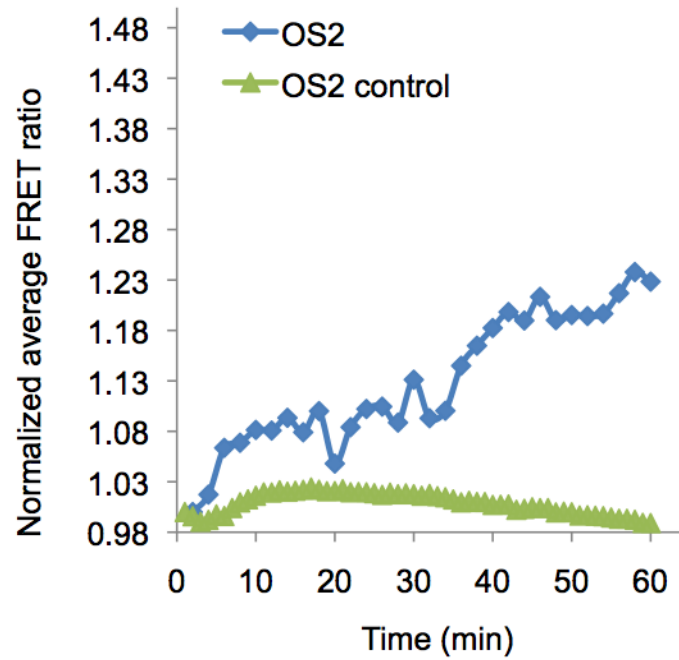
The nuclear localization sequence (NLS) from simian virus 40 large T-antigen (SV40), PKKRKVEDA, was attached to the construct Nuc-OS2 at the 3'-end before the stop codon. The 5' PCR primer designed for the cloning of OS2 sensor was used to incorporate an *Nco* I site, the linkers, the substrate domain and the beginning sequence of Venus. A 3' primer was constructed to anneal downstream of Venus and introduce the NLS, a stop codon and a unique site *Hind* III. The primers used were pLKM4.Fwd 5'-CAT GCC ATG GGG CGG CAG CGG CGG CCC GGG CGG CAG CAC CCC GGT GAG CAG CGC GAA CAT GAT GGG CGG CAG CGG CGG CAT GGT GAG CAA GGG CGA GGA G-3' and pLDC28.Rev 5'-GGG AAG CTT TTA GGC GTC CTC CAC TTT GCG TTT TTT TGG CTT GTA CAG CTC GTC CAT GCC -3'

The cytoplasmic localized sensor (Cyto-OS2) was tether with the nuclear exclusion sequence, LPPLERLTL, from HIV1 Rev-protein. The 5' PCR primer designed for the cloning of OS2 sensor was used on these PCR reactions to incorporate an *Nco* I site, the linkers, the substrate domain and the beginning sequence of Venus. A 3' primer was designed for each construct bearing their corresponding NES between the annealing sequence downstream of Venus and the stop codon followed by the unique site *Hind* III. The primers used were pLKM4.Fwd 5'-CAT GCC ATG GGG CGG CAG CGG CGG CCC GGG CGG CAG CAC CCC GGT GAG CAG CGC GAA CAT GAT GGG CGG CAG CGG CGG CAT GGT GAG CAA GGG CGA GGA G-3' and pLDC29.Rev 5'-GGG AAG CTT TTA GAT GTC CAG GCC GGC CAG TTT CAG GGC CAG CTT GTA CAG CTC GTC CAT GCC -3' and pLDC42.Rev 5'-GGG AAG CTT TTA CAG GGT CAG GCG TTC CAG TGG TGG CAG CTT GTA CAG CTC GTC CAT GCC -3'.

The PCR reactions for the Nuc-OS2 and Cyto-OS2 were performed using the Taq DNA Polymerase Kit (New England Biolabs) in a final reaction volume of 50 μ L. The reaction consisted of 41.5 μ L ddH₂O, 5 μ L 10X buffer, 1 μ L dNTPs, 1 μ L pLKM3 (500pg/ μ L), 0.5 μ L Fwd primer (20 μ M), 0.5 μ L Rev primer (20 μ M) and 0.5 μ L Taq polymerase. The reaction was denatured at 95°C for 1 minute, followed by 30 cycles of 30 seconds at 95°C, 30 seconds at 55 °C and 1 minute at 72 °C, 1 cycle of 1 minute at 95°C, 30 seconds at 55 °C and 1 minute at 72 °C and finally a 4 °C hold after all the cycles were completed. PCR product and pLKM2 were digested with *Nco* I and *Hind* III at 37°C for 3 hours. Restriction digest reactions were gel purified using the QIAquick Gel Extraction Kit (Qiagen). Each PCR product and pLKM2 were ligated using the T4 DNA Ligase (New England Biolabs), resulting in the Nuc-OS2 sensor and Cyto-OS2. These constructs were transformed into *E. coli* DH5 α cells and plated on selective media. A 5 mL culture of pLKM4 in pRSET A transformed *E. coli* DH5 α cells was grown to saturation and the plasmid was purified using a QIAprep Spin Miniprep Kit (Qiagen).

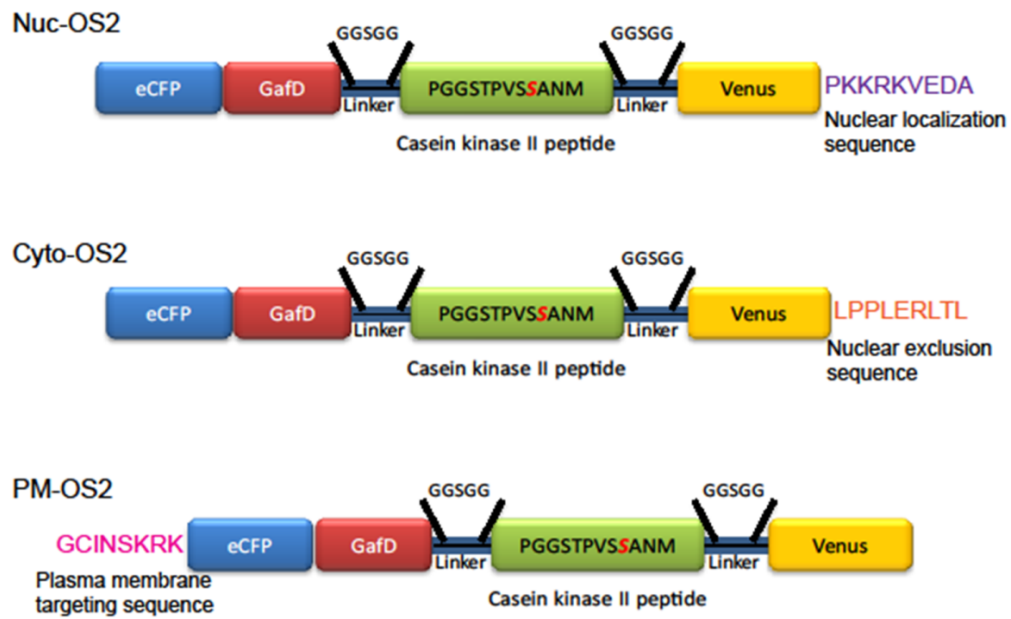
To target the OS2 sensor to the plasma membrane, the myristoylation and palmitoylation sequence, GCINSKRK, from the NH₂-terminus of Lyn kinase was utilized. A 5' PCR primer was designed to incorporate a *Bam*H I site, the plasma membrane sequence, and the start of the coding sequence of CFP. A 3' primer was constructed to anneal downstream of GafD and a unique site *Nco* I. The primers used were pLDC26.Fwd 5'-CGC GGA TCC GCG ATG ATG GGC TGC ATC AAC AGC AAA CGC AAA GAC ATG GTG AGC AAG GGC GAG GAG -3' and pLDC26.Rev 5'-CAT GCC ATG GTG TTA GTG TGT CAT TCA GCG TAA ATG-3'. The PCR conditions were identical to one used for the Nuc-OS2 sensor, but the construct pLKM2 was used as the template DNA. The PCR products and pLDC18 (vector containing the CKII peptide that can be modified by O-GlcNAc, linker regions and Venus domain) or pLDC5 (vector containing the CKII peptide with no serine or threonine residues, linker regions and Venus domain) were ligated using the T4 DNA Ligase (New England Biolabs), resulting in the PM-OS2 and control sensor respectively. These constructs were transformed and purified using the same procedures described earlier. Sequence for all the constructs created was confirmed by the University of Texas DNA sequencing facility.

Supplemental Figure S1



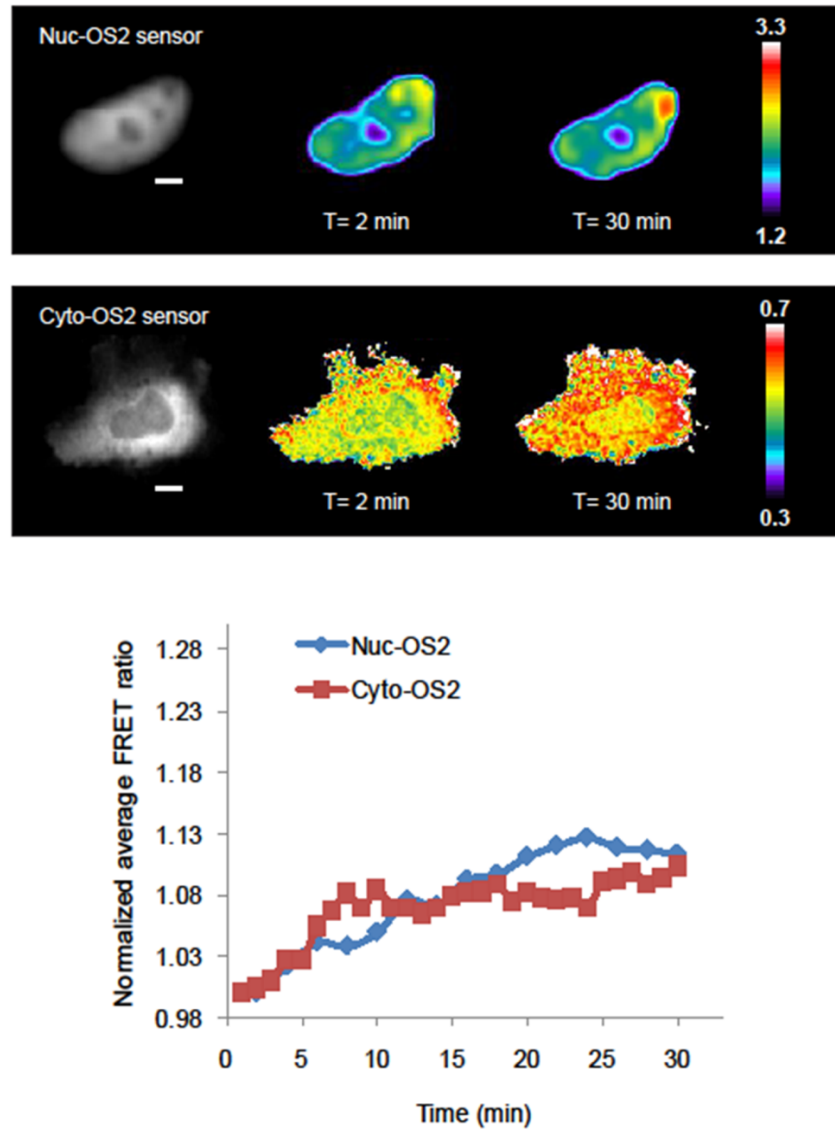
Supplemental Figure S1. Graphical representation of the O-GlcNAc response to PUGNAc and glucosamine of the OS2 and OS2 control sensors. HeLa cells were transfected with OS2 and OS2 control sensors and ratiometric FRET measurements were quantified for 60 min following the addition of PUGNAc (100 μ M) and glucosamine (4 mM). The OS2 sensor shows an increase in FRET, thus a increase in the activity of O-GlcNAc. The OS2 control sensor does not shown changes in FRET over time. This is a representative graph of three independent experiments.

Supplemental Figure S2



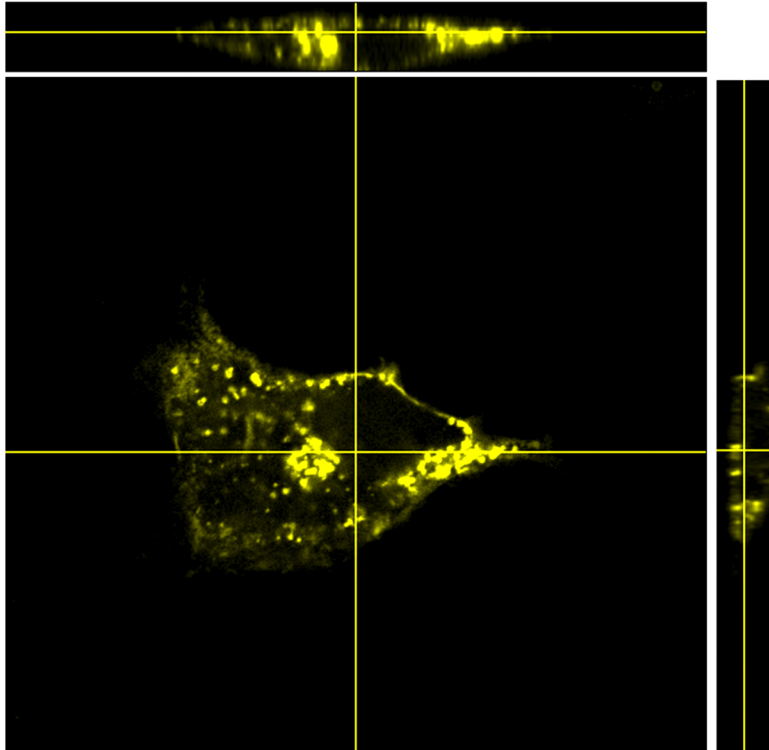
Supplemental Figure S2. Domain structures and localization sequences of the targeted OS2 sensors. The nuclear-localized OS2 sensor (Nuc-OS2) was created by fusing the C-terminus of OS2 with the nuclear localization sequence, PKKRKVEDA, derived from the simian virus 40 large T-antigen. Cyto-OS2 was generated by fusion of a nuclear exclusion peptide LPPLERLTL from the HIV-1 Rev protein to the C-terminus of OS2. To create the plasma membrane sensor (PM-OS2), we fused the peptide sequence GCINSKRK from Lyn kinase containing both a myristoylation and palmitoylation site to the N-terminus of the OS2 sensor.

Supplemental Figure S3



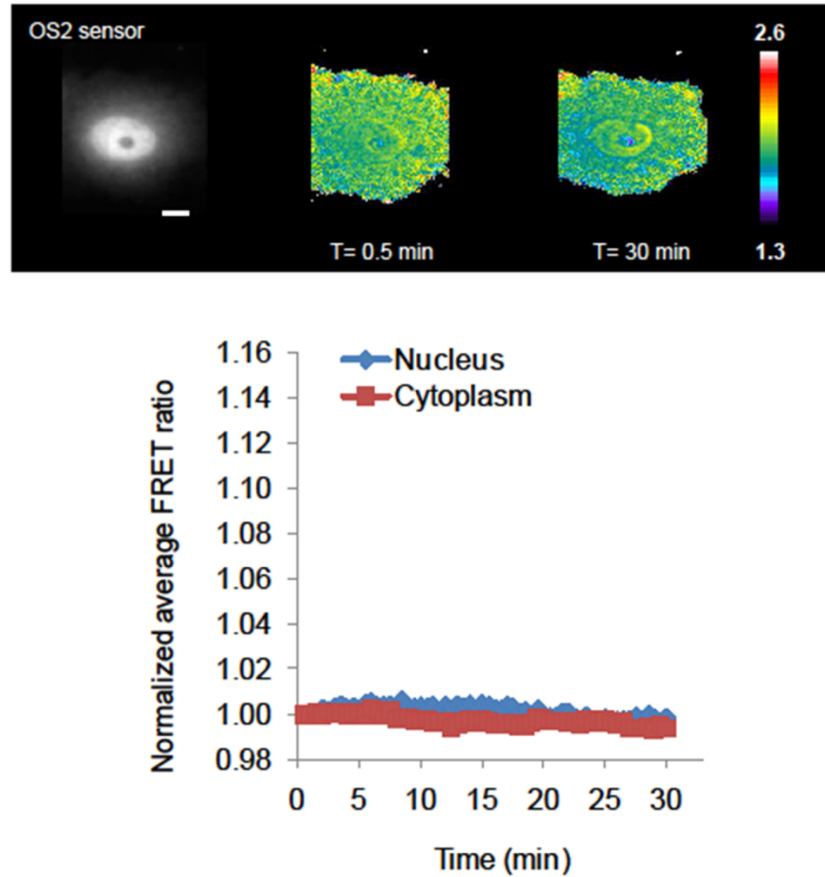
Supplemental Figure S3. Cellular response of the nucleus and cytoplasm localized OS2 sensors to PUGNAc and glucosamine treatment. HeLa cells were transfected with Nuc-OS2 and Cyto-OS2 sensors and ratiometric FRET measurements were quantified for 30 min following the addition of PUGNAc (100 μ M) and glucosamine (4 mM). Pseudocolor images of the Nuc-OS2 and Cyto-OS2 sensors show an increase in FRET, thus an increase in the activity of O-GlcNAc in the nucleus and in the cytoplasm. The normalized average FRET ratio for this cell was plotted in a time course. The color bar represents the FRET ratio values. Nuc-OS2 data is shown in blue and the Cyto-OS2 data is visualized in red. Data is representative for a minimum of 3 cells from independent experiments.

Supplemental Figure S4



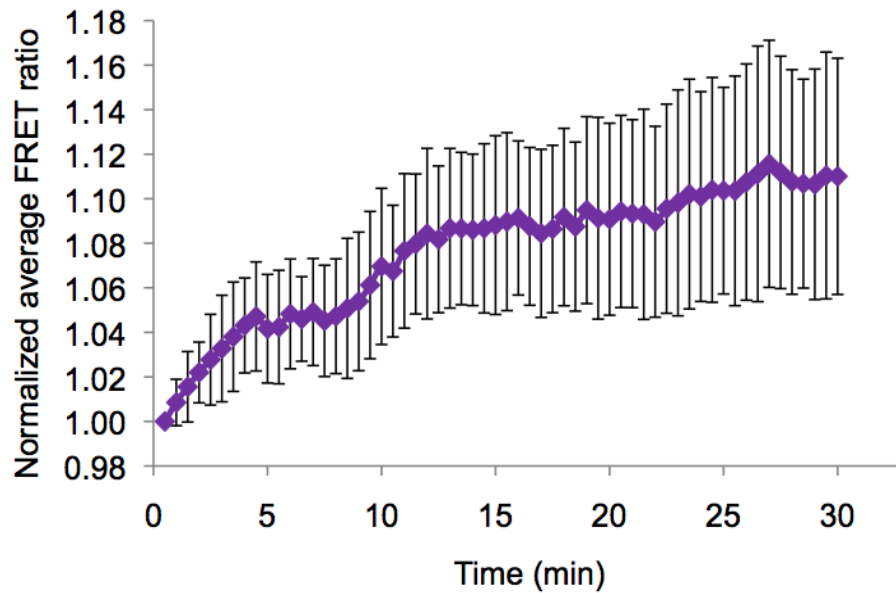
Supplemental Figure S4. Confocal microscopy image of a Cos7 cell transfected with PM-OS2. Images were obtained in the YFP channel on a Zeiss LSM710 NLO using a 63x oil immersion lens. 14 images were obtained in all. Image 7 of the series is shown. Localization of the sensor occurs at both the cell surface and in an intercellular compartment, as has been seen previously for Lyn-kinase localization sequence localized sensors in Cos7 cells.

Supplemental Figure S5



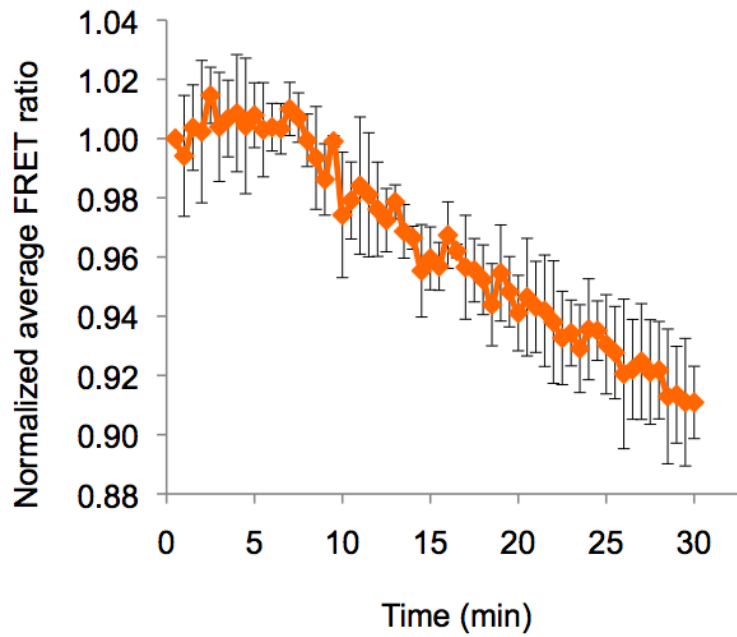
Supplemental Figure S5. The OS2 sensor shows no basal O-GlcNAc response in serum-starved Cos7 cells. Serum-starved Cos7 cells overexpressing OS2 sensor were monitored for 30 min after no treatment. The monochrome image represents the YFP channel and it shows the distribution of the reporter in the cell. No change in FRET was observed in either the nuclear or cytoplasmic compartment in the absence of serum stimulation. The graph on the right represents the normalized average FRET ratio over time. The scale bar represents 10 μ m. The color bar represents the ratiometric FRET ratio values.

Supplemental Figure S6



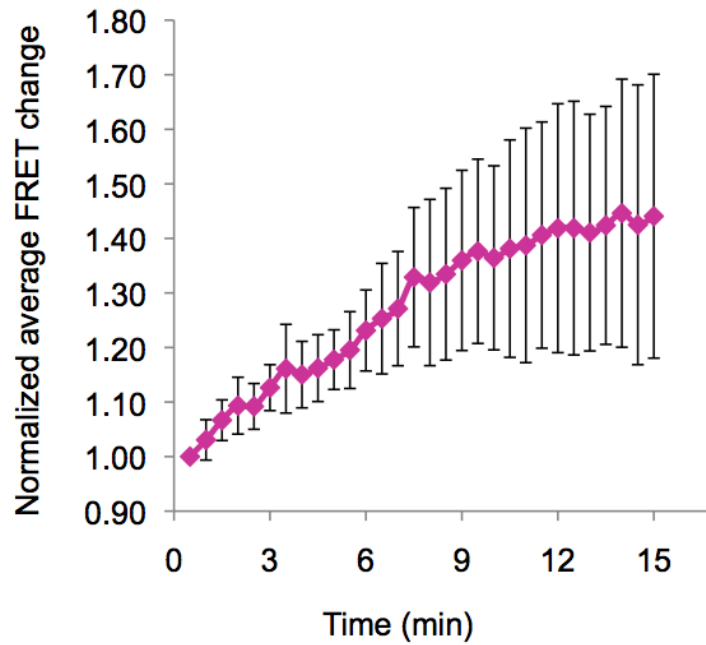
Supplemental Figure S6. Average FRET response of the Nuc-OS2 sensor during serum stimulation. Graphical representation of the normalized average FRET ratio timecourse. Overall, serum- starved Cos7 cells overexpressing the nucleus localized OS2 sensor (Nuc-OS2) show an increase in FRET response following serum treatment in 30 min. On average, the FRET response in the nucleus began to plateau at ~22 min. These results represent the average response including +/- SD per time point. Data represents 9 cells from three independent experiments.

Supplemental Figure S7



Supplemental Figure S7. Average FRET response of the cytoplasm localized OS2 sensor (Cyto-OS2) in response to serum. Graphical representation of the normalized average FRET ratio timecourse after serum stimulation. O-GlcNAc activity detected in the cytoplasm by the Cyto-OS2 sensor does not begin to decrease until ~ 8 min after the addition of serum and does not plateau in the 30 min timeframe. These results represent the average response including \pm SD per time point for 3 cells. Data represents 3 cells from three independent experiments.

Supplemental Figure S8



Supplemental Figure S8. Average FRET response of the PM-OS2 sensor during serum treatment. Graphical representation of the normalized average FRET ratio timecourse. Examination of the average response of the plasma membrane localized OS2 sensor (PM-OS2) revealed that the signal begins to plateau at ~12 min. These results represent the average response including +/- SD per time point. Data represents 5 regions from 3 cells from three independent experiments.