Running title: intracellular processing of group X $sPLA_2$ in HEK293 cells

SUPPLEMENTARY DATA

Experimental procedures

Confocal Laser Fluorescence Microscopy. Cells were plated at 4×10^5 cells/mL on 4 cover slips (Electron Microscopy Sciences) in 6-well plates (3 mL medium/well), incubated for 48 h (cells reached 80% confluence) and treated as follows. The cells were washed once with PBS containing 1 mM $CaCl_2$ and 1 mM $MgCl_2$, and covered with 3.7% formaldehyde (w/v prepared by dilution of 37% formaldehyde with PBS). After 30 min at room temperature, the cells were washed twice with PBS. Then cells were stored in PBS at 4°C for up to 2 weeks. PBS was removed by aspiration, and the cells were covered with quenching solution (50 mM ammonium chloride in PBS). After 10 min incubation at room temperature, the cells were washed twice with PBS. For permeabilization and blocking, PBS-0.1% Triton X-100-3% BSA was added, and the cells were incubated at room temperature for 15 min. After removing the solution, the cells were incubated for 30 min at room temperature in PBS-1% BSA containing anti-hGX $sPLA_2$ rabbit polyclonal IgG (1) or normal Rabbit IgG (Jackson ImmunoResearch Laboratories ChromPure Rabbit IgG #011-000-003), both at 20 μg/mL. For double staining with ER staining, anti-protein disulphide isomerase antibody (Abcam # ab27925, 5 μg/mL) was added in the same solution. Cells were then washed twice with PBS. Cy3-conjugated goat anti-rabbit IgG (Jackson ImmunoResearch Laboratories #111-165-003) was added in PBS-1% BSA (1 μg/mL). For double staining with ER staining, alexa fluor 647 goat anti-mouse IgG (H+L) (Invitrogen # A-20990) was added in the same solution (2.5 μg/mL). After 30 min at room temperature, the cells were washed twice with PBS-0.02% Tween. For double staining with nucleus staining, TO-PRO-3 iodide (Molecular Probes, 400-fold dilution in PBS-1% BSA) was added. After 15 min incubation, the cells were washed with PBS, and cover slips were mounted on glass slides with mounting solution (Electron Microscopy Sciences # 17984-2s). For double staining with Golgi or triple staining with ER and Golgi, the cells were washed three times with Hank's balanced salt solution (HBSS) and treated with 5 mM NBD-C6 ceramide (Invitrogen # N-22651) in HBSS for 30 min at 4°C. The cells were washed twice with 2 mg/mL BSA in HBSS for 60 min at 4°C to enhance Golgi staining. The cells were washed twice with HBSS, and cover slips were mounted on glass slides using mounting solution. The slides were viewed with a Leica SL confocal microscope equipped with a 65 X oil immersion objective.

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Results

In HEK293 transfected cells, both ProhGX (Fig. S1) and PromGX (Fig. S2A) $sPLA_2$s co-localized with ER and partially with Golgi markers, indicating that they are targeted to the secretory pathway, as expected for actively secreted proteins harboring a signal peptide. This pattern of immunocytolocalization was identical to that previously observed for the SphGIIA-hGX $sPLA_2$ construct coding for mature hGX $sPLA_2$, ie without the propeptide sequence (2). The expression of the AA mutant of PromGX $sPLA_2$ was identical to that of wild-type PromGX $sPLA_2$ (Fig. S2B).

Figure legends

Supplemental Fig. 1. Immunofluorescence microscopy of HEK293 cells transfected with ProhGX $sPLA_2$. **(A) Nucleus/ProhGX $sPLA_2$ co-staining.** Non-transfected (control, left column) or ProhGX $sPLA_2$-transfected cells (right column) were fixed at room temperature and permeabilized with Triton X-100. After treating with quenching solution, the cells were labeled with control IgG or antibody to hGX $sPLA_2$ as described under "Experimental Procedures". The top row shows the DIC image. Cy3 goat anti-rabbit IgG was used as the secondary antibody to detect ProhGX $sPLA_2$ (second row). TO-PRO-3 (Alexa 647) staining showed the nucleus (third row). The merge is shown in the bottom row. **(B) Golgi/ProhGX $sPLA_2$ double staining.** Non-transfected cells (control, first column) and ProhGX $sPLA_2$-transfected (middle and right columns). The cells in the first and third columns were treated with anti-hGX $sPLA_2$ antibody, and the cells in the second column were treated with rabbit IgG control serum. The top row is the DIC images, the Cy3 row is the hGX $sPLA_2$ image, the NBD row is the Golgi marker and the bottom row is the merge. The red arrows show colocalization of hGX $sPLA_2$ and Golgi. **(C) ER/ ProhGX $sPLA_2$ double staining.** Same layout as for Fig. 1B. Top row are the DIC images, the Cy3 row is the hGX $sPLA_2$ image, the Alexa647 row is ER marker, and the bottom row is the merge. The colocalization of hGX $sPLA_2$ and ER is shown in pink**. (D) Triple staining for ProhGX $sPLA_2$, ER and Golgi in ProhGX $sPLA_2$-transfected HEK293 cells.** The yellow arrow shows colocalization of ProhGX $sPLA_2$ and the Golgi marker, and the red arrow shows colocalization of ProhGX $sPLA_2$ and the ER marker.

Supplemental Fig. 2. Immunofluorescence microscopy of HEK293 cells transfected with

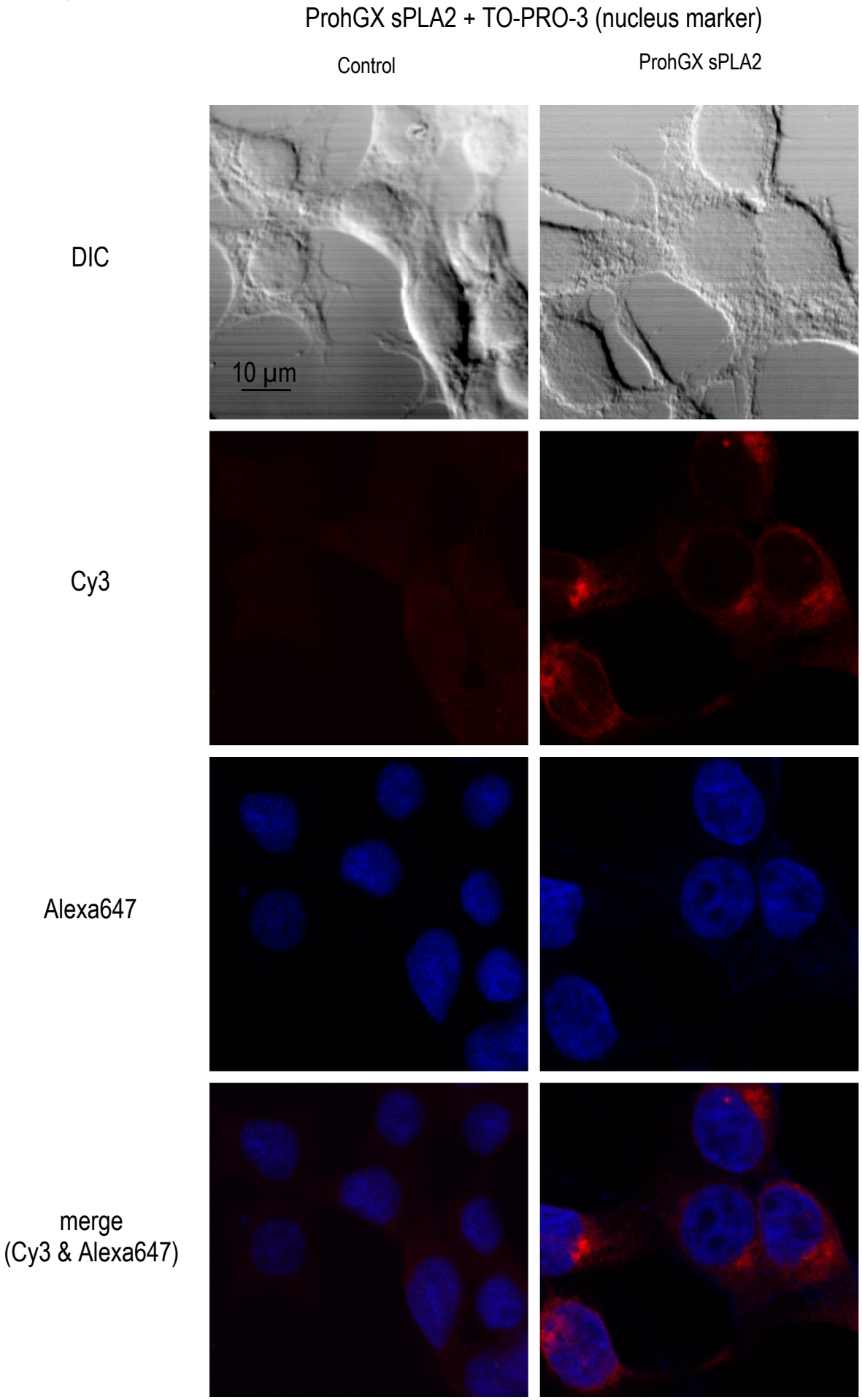
wild-type and the AA mutant of PromGX $sPLA_2$. (A) The first column is non-transfected cells (control), the 2nd and 3rd columns are PromGX wild-type $sPLA_2$-transfected cells treated with control rabbit IgG serum (2nd column) or antibody to mGX $sPLA_2$ (3rd column). The top rows are the DIC images, the Cy3 row is the PromGX staining, the Alexa647 row is the ER staining, and the bottom row is the merge. **(B)** Same as for Fig. 2A, except that HEK293 cells were transfected with the AA mutant of PromGX $sPLA_2$.

References

1. Nevalainen, T. J., Eerola, L. I., Rintala, E., Laine, V. J., Lambeau, G., and Gelb, M. H. (2005) *Biochim. Biophys. Acta* **1733**, 210-223
2. Mounier, C. M., Ghomashchi, F., Lindsay, M. R., James, S., Singer, A. G., Parton, R. G., and Gelb, M. H. (2004) *J. Biol. Chem.* **279**, 25024-25038

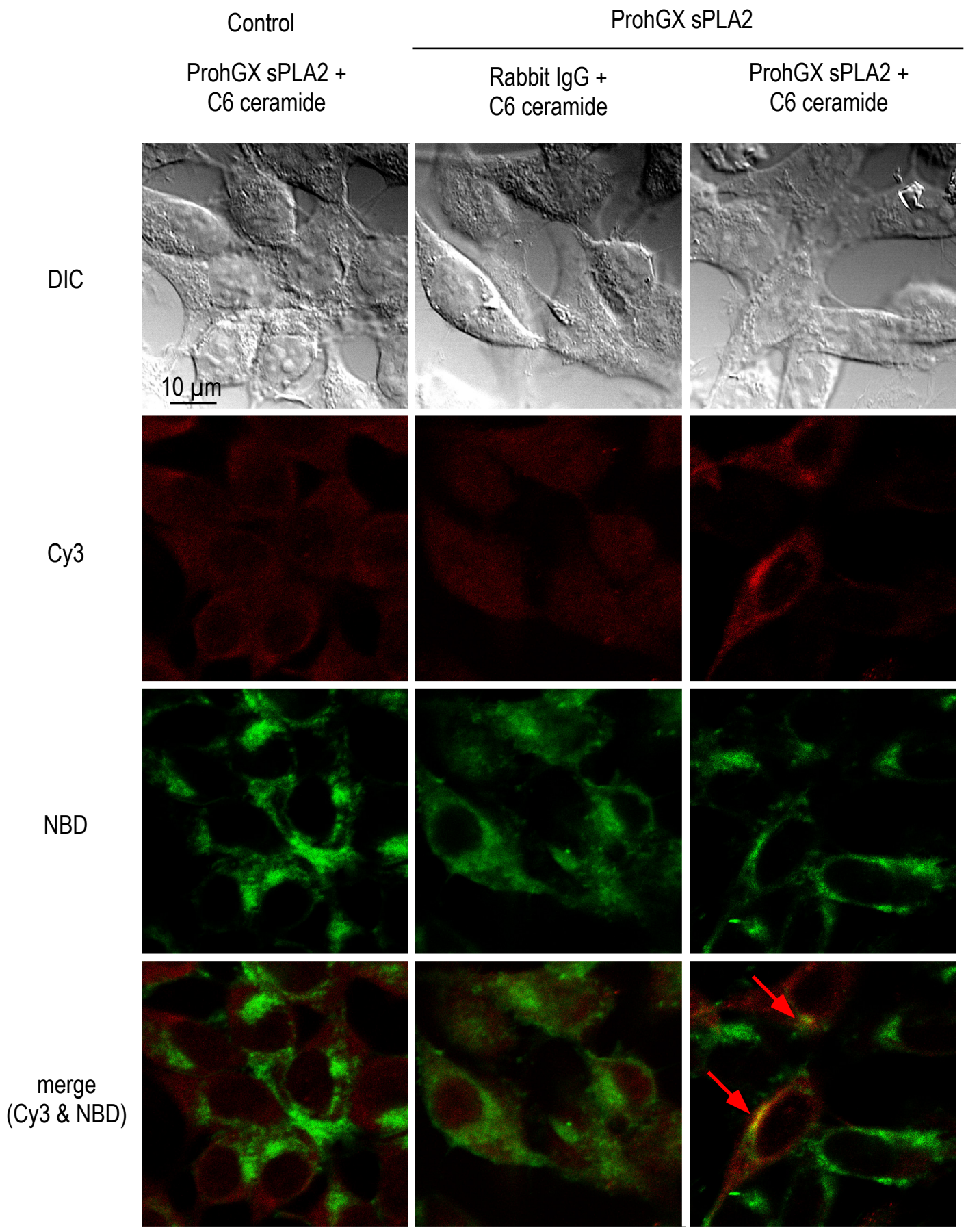
Supplementary data

Fig 1A



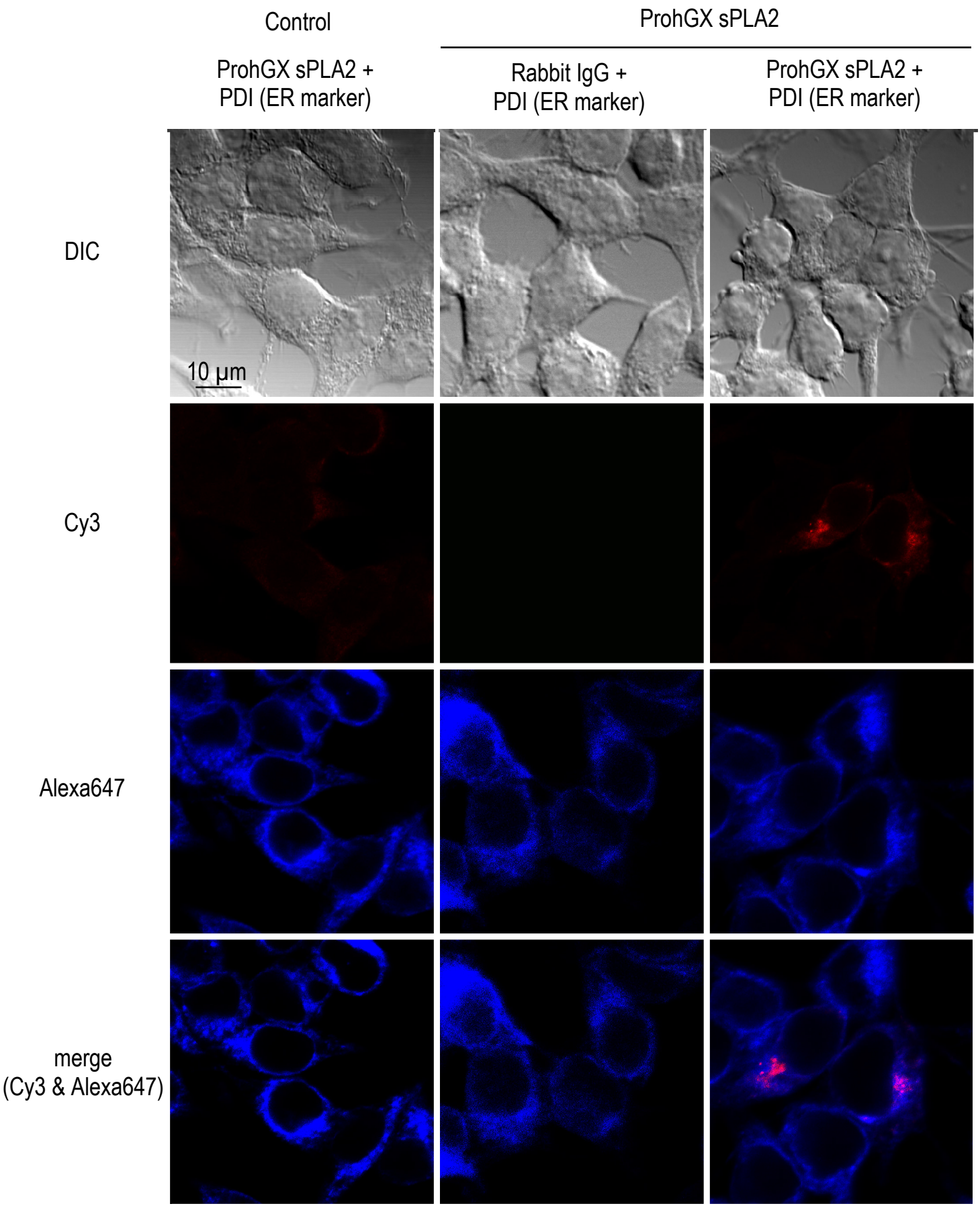
Supplementary data

Fig 1B



Supplementary data

Fig 1C

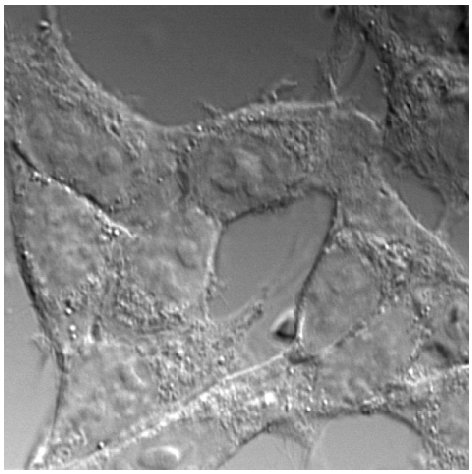


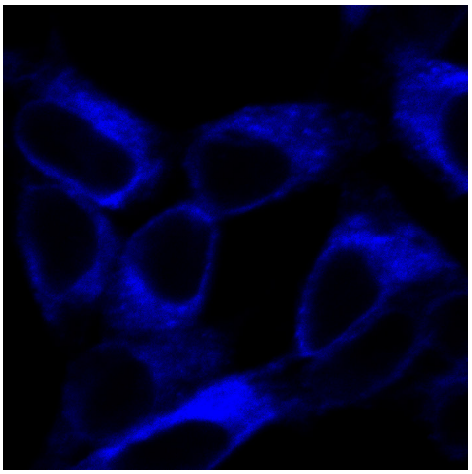
Supplemental data

Fig 1D

ProhGX sPLA2 + PDI (ER marker) + C6 ceramide (Golgi marker)

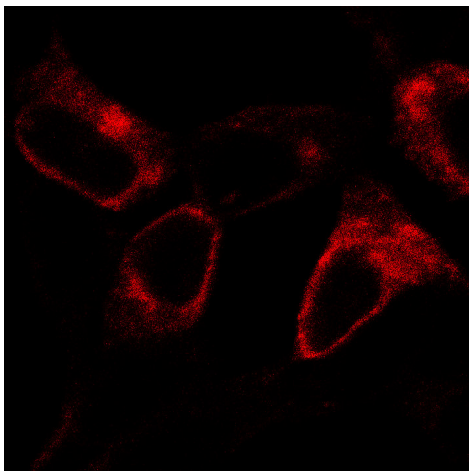
DIC

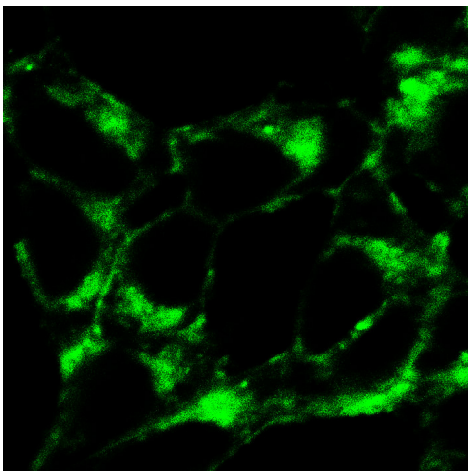




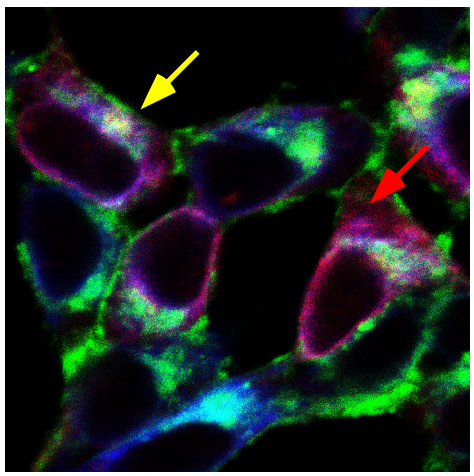
Alexa647
(ER)

Cy3
(ProhGX sPLA2)





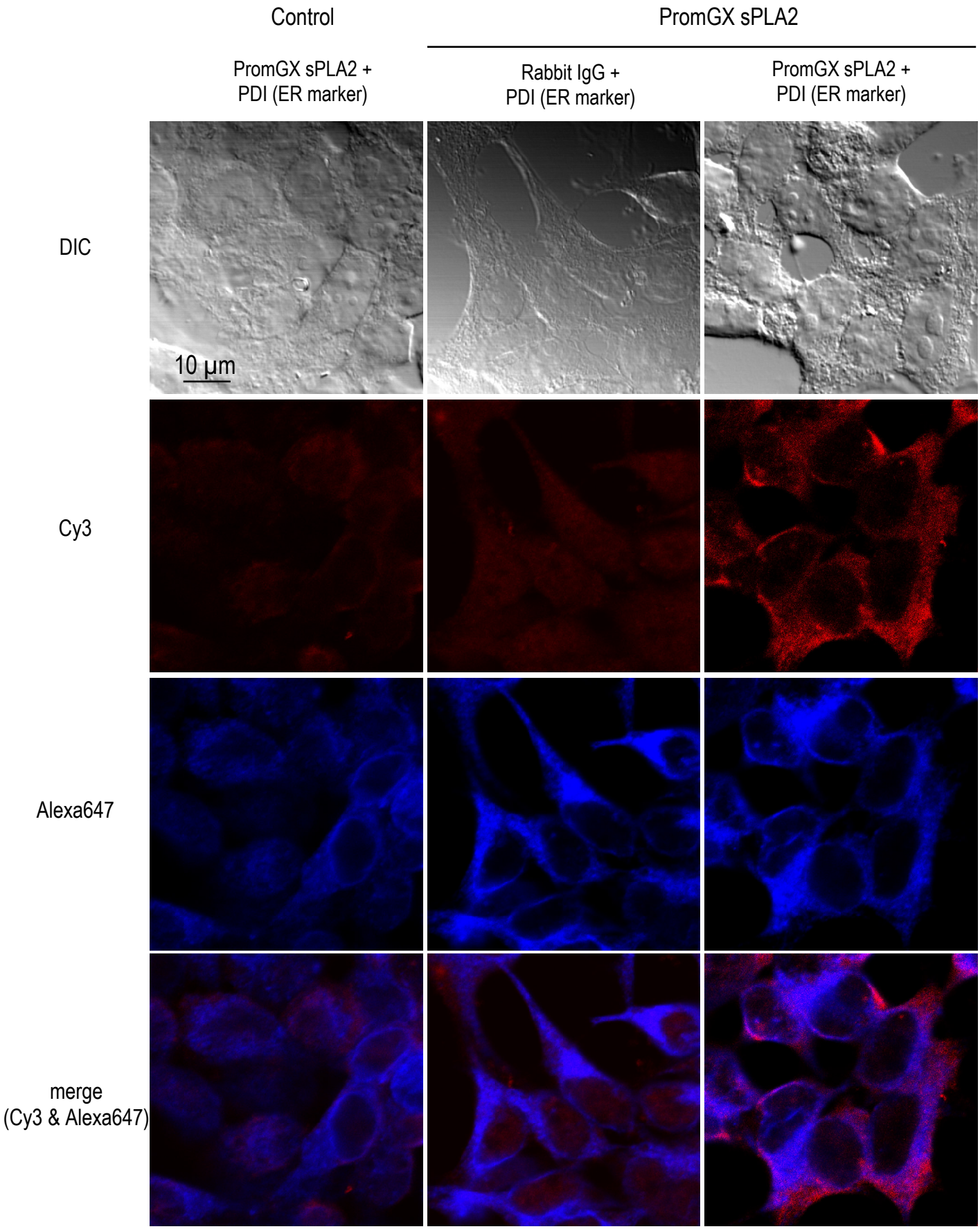
NBD
(Golgi)



Merge
(Cy3 & Alexa647 & NBD)

Supplementary data

Fig 2A



Supplementary data

Fig 2B