Supplemental data

Fig. S1. Schematic representation of the two RBC membrane:cytoskeleton anchorage complexes. Left, 4.1R complex; right, ankyrin-based complex. Adapted from (1) where abbreviations are explained. In red, components used in this study. Although very important, cholesterol is not represented, for the sake of simplicity. For further details on membrane lipid:protein interactions, please refer to Discussion.

Fig. S2. Characterization of normal untreated (A), PMA/CalA-treated (B,C) and spherocytotic RBCs (D-G). (A) Smooth membrane of fully and partially spread RBCs. Cells were attached onto PLK-coverslips and labelled with SM* as at Fig. 1A prior to fixation and processing for scanning electron microscopy. Notice reduced electron back-scattering in fully spread RBCs (arrows) as compared with minimally spread cells (here a loosely attached aggregate; arrowhead at a). Scale bars, 5µm. **(B) Phosphorylation of adducin.** RBCs were left untreated (first lane; 0min) or treated with PMA+CalA for the indicated times to uncouple membrane:cytoskeleton at 4.1R complexes. Western blotting of ghost lysates for phosphorylation at serine 726 of α-adducin, a protein of 4.1R complexes, with α-spectrin as loading control (representative of 3 experiments). **(C) Surface deformation upon PKC activation.** Normal RBCs attached onto PLK-coverslips were kept untreated (left) or incubated with PMA+CalA, then processed as at panel A. Notice that PMA/CalA-treated RBCs retain a flat surface but retract at the periphery (arrowhead) and bulge in the center (arrow), suggesting decreased membrane tension. Scale bar, 5µm. **(D,E) Ankyrin status.** RBC ghost lysates from two healthy (C#1, splenectomized; C#2, non-splenectomized) and two splenectomized spherocytotic patients (P#1, P#2) were resolved by SDS-PAGE and stained by Coomassie blue (D), or analyzed by western blotting using

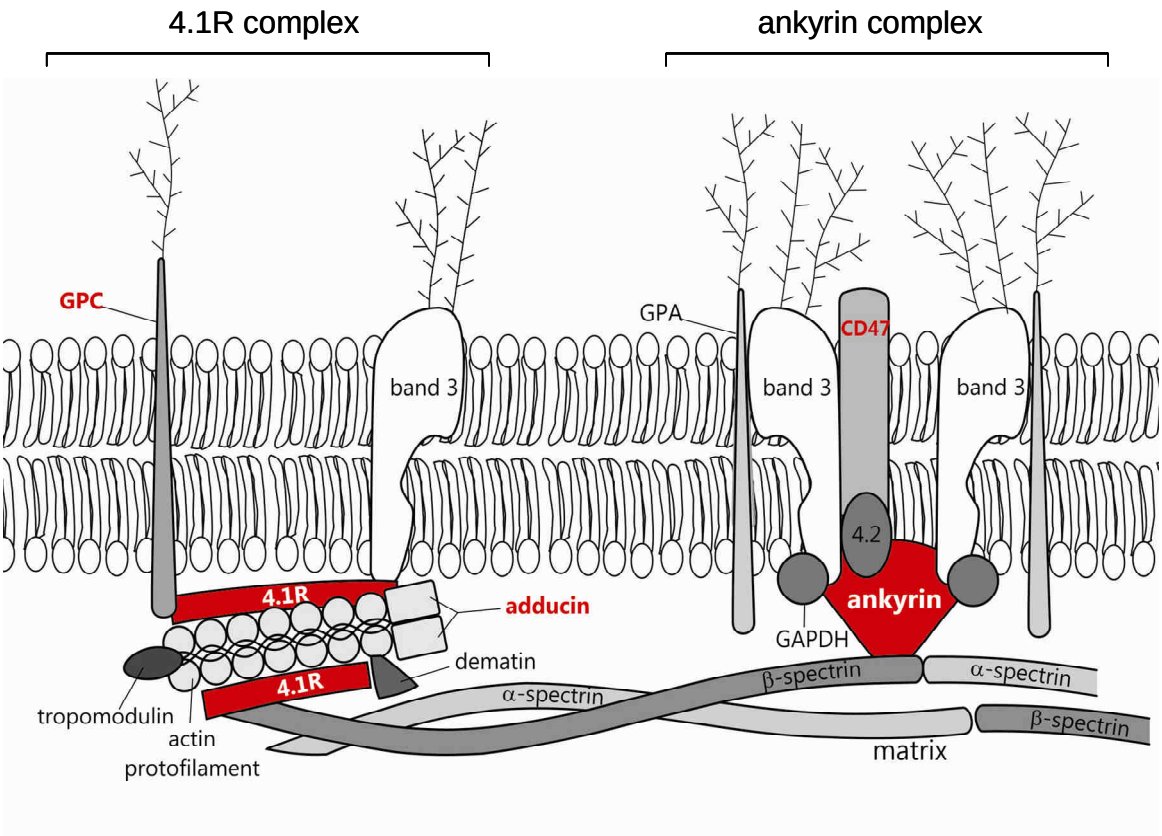
ankyrin-R and pan-spectrin antibodies, with aquaporin-1 (AQP1) as loading control (E, representative blots of 3 experiments). **(F) Sequencing of the ankyrin-1 exons.** Note the single point mutation in exon 28 of P#2 (c.3157C>T). No mutation was found in any exon of P#1. Whether P#1 suffers from a mutation in the ankyrin promoter region or in another gene coding for another component of ankyrin complex remains to be clarified. **(G) Osmotic resistance.** RBCs freshly isolated from splenectomized (C#1, open circles) and non-splenectomized (C#2, open squares) normal donors and from patients P#1 (filled circles) and P#2 (filled squares) were evaluated for hemolysis. Upon transfer into hypotonic media, 50% hemolysis (dotted line) occurs at ~200mOsm for P#2 and ~175mOsm for P#1 *versus* ~ 135mOsm for C#1 and C#2.

Fig. S3. Cholesterol level controls SM* and PC* but not GlcCer* domains. (A) Efficacy and innocuity of mβCD on normal RBCs. Normal RBCs were treated in suspension with the indicated concentrations of mβCD, then evaluated for residual cholesterol level (a), membrane lysis [b, evaluated by haemoglobin release (2), normalized to 100% under 0.2% Triton X-100] and insertion level of GlcCer* (open columns), SM* (filled columns) and PC* (hatched columns) [c, for assay, see (3)]. All results are expressed as percentages of control. **(B) Large field views of Fig. 3.** Normal RBCs were either kept untreated (a, c, e) or preincubated with 0.25mM mβCD (b, d, f). Notice the overall resistance of GlcCer* domains to cholesterol depletion, contrasting with disappearance of SM* and PC* domains. Scale bar, 5µm. **(C) Segregation between SM* and DiIC18 domains upon marginal cholesterol depletion.** Normal RBCs were treated with 0.06mM mβCD, attached onto PLK-coverslips then sequentially labelled with SM* and DiIC18, washed and imaged. Scale bar, 5µm.

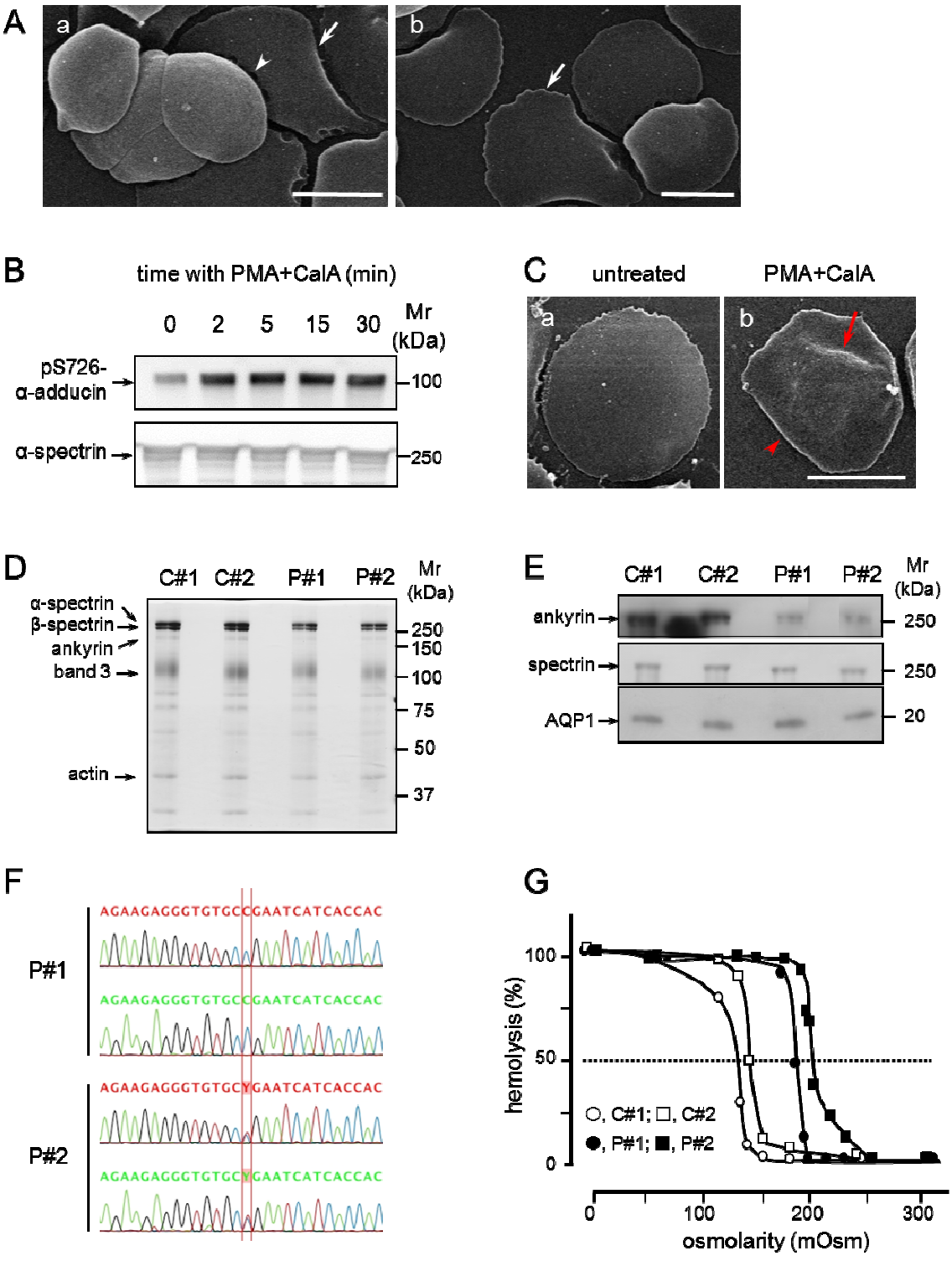
Fig. S4. Differential restriction of micrometric lipid* domains by membrane:cytoskeleton anchorage via 4.1R and ankyrin complexes - general views. RBCs from normal splenectomized donor C#1 (normal) were attached onto PLK-coverslips and either kept untreated (a,d,g) or treated with PMA+CalA (b,e,h) and compared with untreated RBCs from the splenectomized spherocytotic patient P#1 (c,f,i) for labelling with GlcCer* (a-c), SM* (d-f) and PC* (g-i). Scale bar, 5µm.

Fig. S5. Differential association of micrometric lipid* domains with patched glycophorin C (4.1R complex) and CD47 (ankyrin complex): gallery of domains presented at Fig. 8. RBCs from a normal donor were attached onto PLK-coverslips, labelled with GlcCer* (a-e, green), SM* (f-j, green) or PC* (k-o, green) and glycophorin C (GPC, 4.1R complex; red) and CD47 (ankyrin complex; blue) were patched by antibodies. Notice that (i) SM* domains (green at f-j) overlapped GPC patches (red at f-j); (ii) PC* domains (green at k-o) were instead preferentially circumscribed by GPC (red at k-o); (iii) CD47 patches were never as tightly linked to lipid* domains, but frequently seemed in closer proximity to GlcCer* and SM* (blue at a-j) than to PC* domains. Scale bars, 1µm. Dotted circles indicate lipid* domain boundaries.

D’Auria et al., Figure S1



D'Auria et al., Figure S2



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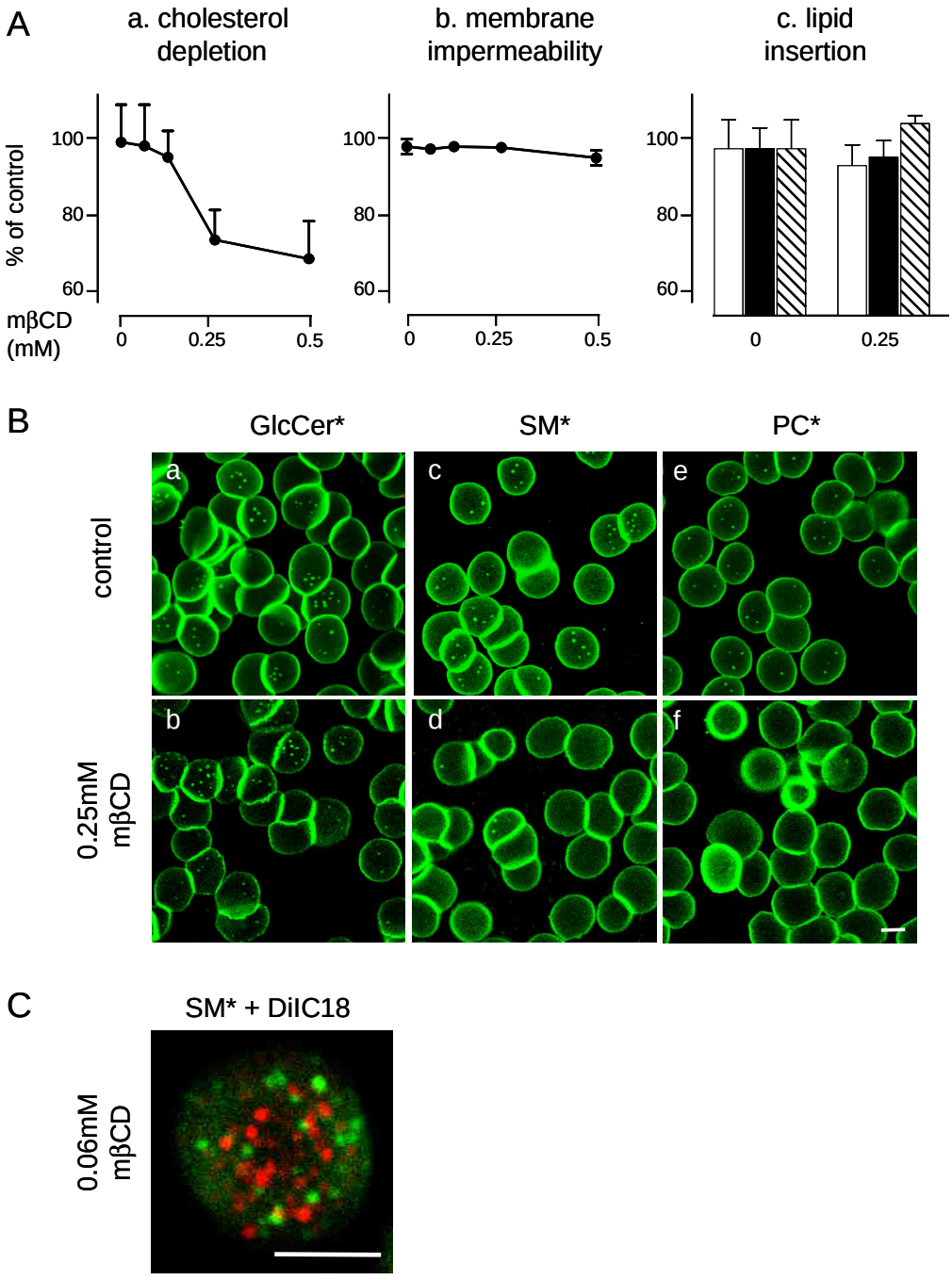

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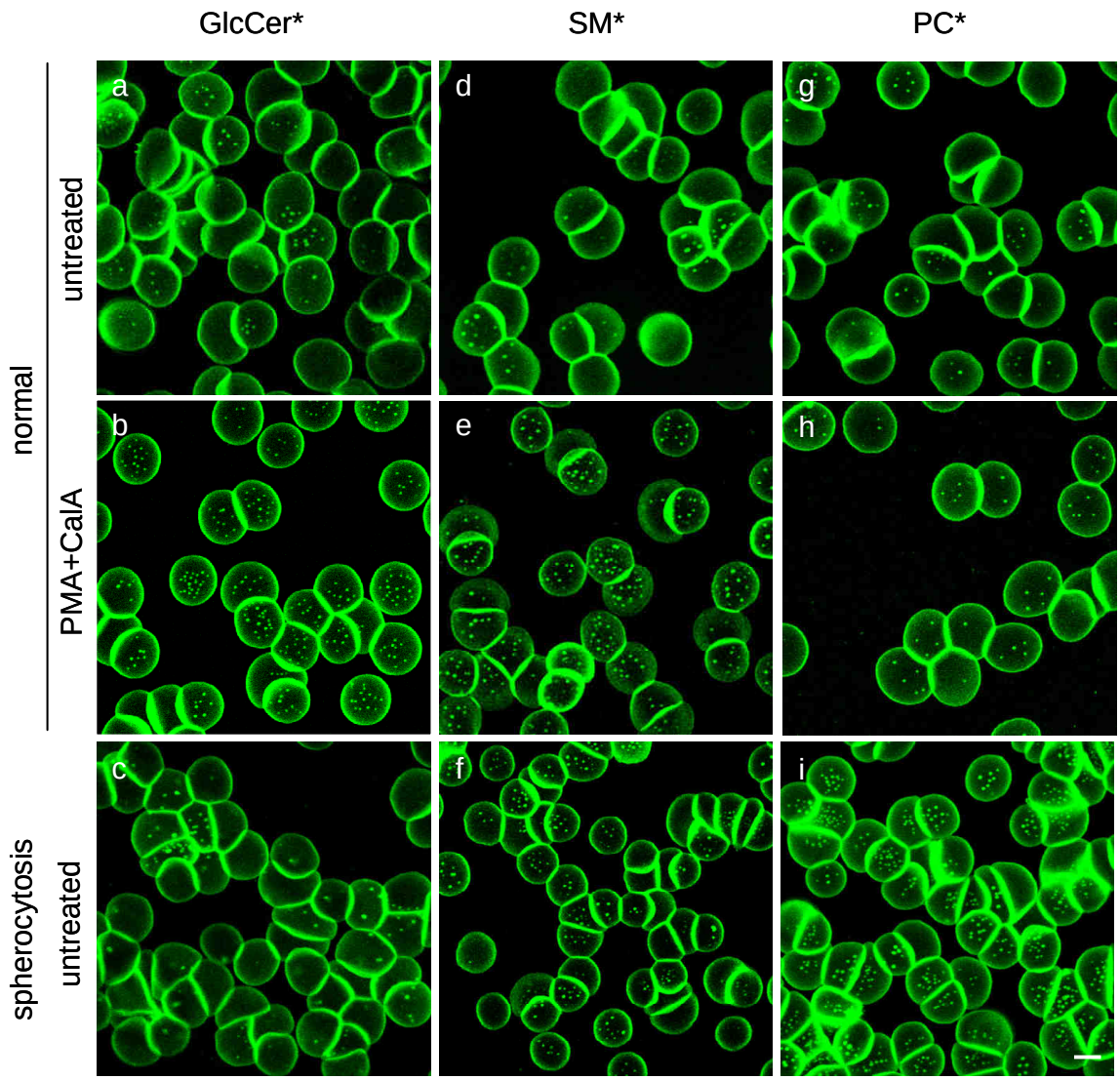

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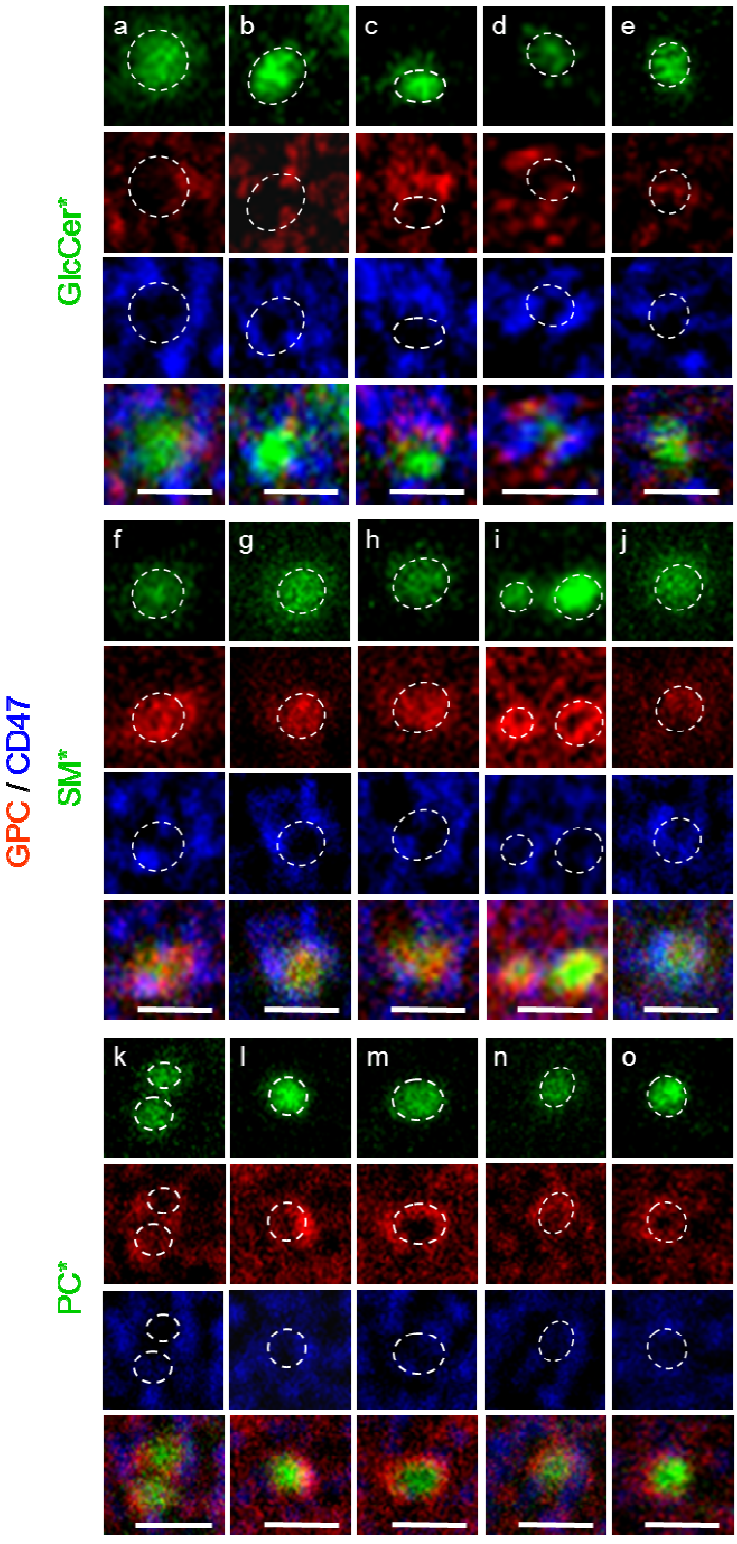

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