Enhanced receptor–clathrin interactions induced by *N*-glycan–mediated membrane micropatterning

Juan A. Torreno-Pina^{a,1}, Bruno M. Castro^{a,1}, Carlo Manzo^a, Sonja I. Buschow^b, Alessandra Cambi^{b,c}, and Maria F. Garcia-Parajo^{a,d,2}

^aICFO-Institut de Ciències Fotoniques, 08860 Castelldefels, Spain; ^bDepartment of Tumour Immunology, Radboud Institute for Molecular Life Sciences, Radboud University Medical Center, 6500, HB Nijmegen, The Netherlands; ^cNanobiophysics, MESA+ Institute for Nanotechnology and MIRA Institute for Biomedical Technology and Technical Medicine, University of Twente, 7500 AE, Enschede, The Netherlands; and ^dICREA-Institució Catalana de Recerca i Estudis Avançats, 08010 Barcelona, Spain

Edited by Jennifer Lippincott-Schwartz, National Institutes of Health, Bethesda, MD, and approved June 16, 2014 (received for review February 2, 2014)

Glycan–protein interactions are emerging as important modulators of membrane protein organization and dynamics, regulating multiple cellular functions. In particular, it has been postulated that glycan-mediated interactions regulate surface residence time of glycoproteins and endocytosis. How this precisely occurs is poorly understood. Here we applied single-molecule-based approaches to directly visualize the impact of glycan-based interactions on the spatiotemporal organization and interaction with clathrin of the glycosylated pathogen recognition receptor dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN). We find that cell surface glycan-mediated interactions do not influence the nanoscale lateral organization of DC-SIGN but restrict the mobility of the receptor to distinct micrometer-size membrane regions. Remarkably, these regions are enriched in clathrin, thereby increasing the probability of DC-SIGN–clathrin interactions beyond random encountering. *N*-glycan removal or neutralization leads to larger membrane exploration and reduced interaction with clathrin, compromising clathrin-dependent internalization of virus-like particles by DC-SIGN. Therefore, our data reveal that cell surface glycan-mediated interactions add another organization layer to the cell membrane at the microscale and establish a novel mechanism of extracellular membrane organization based on the compartments of the membrane that a receptor is able to explore. Our work underscores the important and complex role of surface glycans regulating cell membrane organization and interaction with downstream partners.

pathogen receptor DC-SIGN | clathrin-dependent endocytosis | STED nanoscopy | cell membrane compartmentalization | protein glycosylation

Glycans are fundamental cellular components ubiquitously present in the extracellular matrix and cell membrane as glycoproteins or glycolipids. Glycan-binding proteins such as galectins, siglecs, and selectins are mostly multivalent and thus thought to cross-link glycoproteins into higher-order aggregates, creating a cell surface glycan-based connectivity also called glycan lattice or network (1–3). By concentrating specific glycoproteins or glycolipids while excluding other cell surface molecules, surface glycan-based connectivity can organize the plasma membrane into specialized domains that perform unique functions (1, 3–6). Nevertheless, direct observation of glycan-mediated ligand cross-linking in living cells remains challenging (7). Notwithstanding, there is no doubt that surface glycan-based connectivity is essential in the control of multiple biological processes including immune cell activation and homeostasis, cell proliferation and differentiation, and receptor turnover and endocytosis (1, 5, 6, 8).

Clathrin-mediated endocytosis (CME) constitutes the primary pathway of cargo internalization in mammalian cells regulating the surface expression of receptors (9). Formation of clathrin-coated pits (CCPs) starts by nucleation of coat assembly at distributed positions in the inner surface of the plasma membrane, where it continues to grow or dissolve rapidly unless coat

stabilization occurs (10, 11). One event that clearly correlates with successful CCP stabilization is cargo loading (11). Recent studies show that cargo molecules diffuse randomly on the cell membrane until they meet growing CCPs, with the extent of cargo interactions regulating CCP maturation (12). As such, factors that affect cargo mobility within/at the cell surface will inevitably impact on CCP maturation and successful internalization. In the context of surface glycan–protein interactions, it has been shown that glycoproteins with an intact glycan-based connectivity exhibit reduced lateral mobility and this correlates with compromised endocytosis (3, 13–17). How this precisely occurs is poorly defined, although fluorescence recovery after photobleaching on the EGF receptor (EGFR) suggested that cell surface glycan-based interactions restrict EGFR dynamics and localization into membrane regions away from endocytic platforms (14, 17). Whether this is a general mechanism for glycosylated proteins or specific to EGFR is not known. Moreover, visualization of receptor interactions with the endocytic machinery under the influence of the glycan network has not yet been attained.

In this work we applied superresolution nanoscopy and developed a dedicated dual-color single-molecule spatio-dynamic exploration approach to visualize the impact of glycan-based interactions on the spatiotemporal organization and clathrin

Significance

Glycan-based interactions can organize the plasma membrane into specialized domains that perform unique functions. One of their major roles is to regulate the turnover of receptors on the cell membrane. However, there is no clear picture on how this occurs. In this work we visualize cell membrane micropatterning mediated by glycans using a combination of super-resolution imaging techniques and dual-color single-particle tracking. We find that this micropatterning corrals the receptor dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) into clathrin active regions, thereby increasing clathrin–receptor interactions, and potentially influencing clathrin-mediated endocytosis of DC-SIGN-bound ligands. We also establish that clathrin–receptor encounters do not occur in a random fashion and further substantiate the dynamic and transient behavior of clathrin interactions with their cargo before successful internalization.

Author contributions: J.A.T.-P., B.M.C., S.I.B., A.C., and M.F.G.-P. designed research; J.A.T.-P., B.M.C., and S.I.B. performed research; J.A.T.-P., B.M.C., C.M., A.C., and M.F.G.-P. contributed new reagents/analytic tools; J.A.T.-P., B.M.C., and C.M. analyzed data; A.C. and M.F.G.-P. supervised research; and M.F.G.-P. wrote the paper.

The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

¹J.A.T.-P. and B.M.C. contributed equally to this work.

²To whom correspondence should be addressed. Email: maria.garcia-parajo@icfo.es.

This article contains supporting information online at www.pnas.org/lookup/suppl/doi:10.1073/pnas.1402041111/-DCSupplemental.

interaction of a glycosylated membrane receptor involved in pathogen recognition and uptake. We focused on the transmembrane glycoprotein dendritic cell-specific intercellular adhesion molecule-3-grabbing nonintegrin (DC-SIGN) given its importance in supporting primary immune responses such as pathogen recognition and uptake on immature dendritic cells (imDCs), signaling, and cell adhesion (6, 18–20). Moreover, DC-SIGN contains a single *N*-glycosylation site, organizes in nanoclusters at the cell membrane (19, 21–23), and internalizes bound antigens via CPPs for subsequent processing and presentation to T cells (20, 24–26). Our work provides insights on how surface glycan-mediated interactions tune spatiotemporal micropatterning of receptors on the cell membrane, potentially regulating interactions with the endocytic machinery and underscoring the importance and complex role of surface glycans on cell membrane organization and function.

Results

Glycan-Based Interactions Do Not Affect DC-SIGN Nanoclustering.

Glycan-binding proteins cross-link surface glycoproteins into higher-order oligomers (1, 2, 4, 5). Because DC-SIGN forms nanoclusters on the cell surface and has a single *N*-glycosylation site (19, 21–23), we first investigated the role of *N*-glycosylation on the nanoscale organization of DC-SIGN using stimulated emission depletion (STED) nanoscopy. We used CHO cells stably expressing wild-type DC-SIGN (wtDC-SIGN) and a receptor variant presenting a point mutation within the DC-SIGN *N*-glycosylation motif (denoted as N80A), known to prevent receptor glycosylation (27). This cell system recapitulates DC-SIGN essential activities, such as antigen binding, internalization, and trafficking (23–25). STED images on fixed immuno-labeled cells expressing either wtDC-SIGN or N80A at comparable expression levels (Fig. S1) showed well-separated bright fluorescent spots (Fig. 1*A*, Upper). Spot size distributions were generated from multiple superresolution images (Fig. 1*B*). As a control for impaired nanoclustering, we include the spot size distributions obtained for a DC-SIGN mutant lacking the neck region (Δ Rep), known to abrogate nanoclustering (23, 27). Interestingly, the spot size distributions for wtDC-SIGN and N80A are similar, with average values around 160 nm, in agreement with previously reported wtDC-SIGN values (21, 23) and significantly larger than for Δ Rep. We also generated fluorescence intensity distributions of DC-SIGN spots (Fig. 1*C*) and compared them with the intensity arising from individual Abs on glass. The clear shift in the DC-SIGN intensities toward higher values compared with the Ab signal evidences the nanoclustering of DC-SIGN, with no DC-SIGN molecules being in a monomeric state, i.e., no overlap between DC-SIGN and Ab intensities. Furthermore, considering that antibodies typically bind to 1–2 antigens, an average of 5–10, 6–12, and 1–3 molecules per spot was obtained, respectively, for wtDC-SIGN, N80A, and Δ Rep. These results thus show that glycosylation does not significantly affect DC-SIGN nanoscale organization on CHO cells.

Similar experiments were performed on imDCs (Fig. 1*A*, Lower). Quantitative analysis confirms DC-SIGN nanoclustering on imDCs (Fig. 1*B* and *C*) with an average density of 3.5–7 molecules per nanocluster, in agreement with previous results (19, 21–23). To test the effect of cell surface glycan-mediated interactions on DC-SIGN nanoclustering, we treated imDCs with lactose. Lactose impairs cell surface glycan-based connectivity promoted by galectins, by competing with their major ligands, branched *N*-linked protein glycans, dissociating bound galectins from the cell surface (14, 17, 28). Although lactose treatment highly reduced the surface levels of galectin-9 and -3 on imDCs (Fig. S2), it had no effect on DC-SIGN nanoclustering (Fig. 1*B* and *C*). These results indicate that DC-SIGN nanoscale organization is independent of the glycosylation state of the receptor and/or on glycan-based interactions at the cell surface.

Fig. 1. DC-SIGN nanoclustering is independent of its glycosylation motif. (*A*, Upper) Representative STED images of wtDC-SIGN (Left) and N80A (Right) on CHO cells. (Lower) DC-SIGN on imDCs (Left) and on imDCs treated with 200 mM lactose (Right). (Insets) A magnified view of the receptor nanoclusters for each case is shown. Spot size (*B*) and normalized intensity distributions (*C*) of all fluorescent spots for each experimental condition, including data from the Δ Rep mutant. Three hundred spots obtained from multiple STED images at each experimental condition. Data from individual Abs nonspecifically attached to glass are also shown in *B* and *C* to illustrate the spatial resolution of STED (ca. 110 nm) and sensitivity for single Abs detection, respectively. Statistical significances were obtained with two-tailed Student's *t* test. **P* < 0.05; n.s., not significant.

DC-SIGN Glycosylation Does Not Influence Nanocluster Interactions in Living Cells.

To capture potential effects of glycan-based interactions on DC-SIGN nanoclustering in living cells we applied dual-color single-particle tracking (SPT). We labeled DC-SIGN using two different quantum dots (QDs) at concentrations to increase the probability of capturing nanocluster interaction events in case they would occur, while allowing for single trajectory recording (Fig. 2*A*). Two-dimensional trajectories of spatially close QDs (red and green) were generated (Fig. S3) and their separation distance was plotted vs. time (Fig. 2*B* and *SI Materials and Methods*). In the case of interacting nanoclusters, dual-color trajectories should exhibit correlated motion with separation distances between QDs close to or below 160 nm (nanocluster size) for periods longer than random coincidence events (29). We found that neither wtDC-SIGN nor N80A trajectories exhibits correlated motion (Fig. S3) or persistent nearness <160 nm compared with simulations of random coincident events (Fig. 2*C* and *Movie S1*), demonstrating the absence of dynamic interactions between nanoclusters regardless of DC-SIGN glycosylation state.

Fig. 3. Membrane exploration maps reveal glycan-mediated confinement of DC-SIGN at the mesoscale. (A) Scheme illustrating how membrane exploration maps of DC-SIGN are generated from dual-color SPT movies at high labeling densities, and example of such a map overlaid with the fluorescence image. (B) Representative membrane exploration maps for wtDC-SIGN and N80A on CHO cells (8,000 localizations per map, 30 frames per second), and DC-SIGN on untreated and lactose-treated imDCs (10,000 localizations per map, 30 frames per second). Each blue point corresponds to a localization event. Gray background corresponds to the cell surface area. Black contour lines delineate the edges of the membrane (scale bars, 400 nm). (C) Implementation of the fixed grid box-counting algorithm. Boxes of decreasing sizes are used and the number of boxes containing at least one localization event is recorded. (D) Normalized number of boxes containing at least one spatial localization event vs. box size. The solid lines are the fit to the data using a double-exponential decay function. (E) Degree of mesoscale compartmentalization (expressed in percent), as extracted from the weight coefficient of the second exponential decay (Table S1). Error bars represent the error on the fitting.

Mesoscale Compartmentalization Influences Nanoscale Transient Confinement of DC-SIGN. Next, we investigated the impact of DC-SIGN mesoscale compartmentalization on its nanoscale dynamic behavior. We performed SPT of DC-SIGN nanoclusters on CHO cells (wtDC-SIGN and N80A), and on untreated and

lactose-treated imDCs. In all cases DC-SIGN nanoclusters were mobile (Fig. 5A). We analyzed the trajectories to detect regions of transient arrest (i.e., transient confinement zones, TCZs) (30) (Fig. 5A) and used cumulative probability analysis of the squared displacements to characterize the sizes of TCZs and the mobility of DC-SIGN inside TCZs (Fig. S5 and *SI Materials and Methods*). In addition, we determined the characteristic duration of the TCZs and the average number of TCZs per area (Fig. S5 and *SI Materials and Methods*). Although the average duration of the TCZs was roughly similar in all samples (Fig. 5B), the nanometer-scale TCZs sensed by DC-SIGN nanoclusters in unperturbed cells were larger in size compared with those found on the glycan-perturbed counterparts (Fig. 5C). Moreover, DC-SIGN diffusion coefficients inside TCZs were significantly larger in unperturbed cells compared with those obtained on N80A and on lactose-treated imDCs (Fig. 5D). Finally, the TCZ surface density, i.e., number of TCZs per area, was lower on cells with an intact DC-SIGN glycan-based connectivity compared with those where these interactions have been disturbed (Fig. 5E), in nice correlation with the restricted surface exploration maps shown in Fig. 3. Altogether, these results indicate that the mesoscale organization brought about by glycan interactions influences the TCZs encountered by DC-SIGN and further supports the existence of different mechanisms responsible for the occurrence of TCZs.

Fig. 4. Glycan-based mesoscale compartments correlate with regions enriched in clathrin. (A) Still frame of a dual-color SPT movie taken in total internal reflection geometry showing individual DC-SIGN QDs (red) overlaid with the clathrin-YFP signal (green) on the surface of a CHO cell (Movie S2). Scale bar, 5 μm. (B) Membrane exploration maps of wtDC-SIGN and N80A overlaid with the normalized CLC-YFP image. Black points correspond to individual localizations of wtDC-SIGN or N80A (~12,500 localizations per map). Arrows highlight localizations close to clathrin-rich regions. Scale bars, 200 nm. (C) Distribution of the normalized CLC-YFP intensity associated with each localization of wtDC-SIGN (blue) or N80A (orange) on CHO cells extracted from the exploration maps. To avoid false colocalization of the receptors with clathrin due to background, only localizations associated with clathrin-YFP intensities ≥ 0.2 were considered. (D) Difference between the normalized frequency of localizations of wtDC-SIGN and N80A as a function of the normalized clathrin signal. (E) Total percentages of wtDC-SIGN and N80A localizations associated with the normalized clathrin-YFP fluorescence signal for values <0.6 and >0.6 as extracted from C. At least 30,000 localizations from 6 cells from different days at each experimental condition.

N-glycosylation Enhances DC-SIGN Clathrin Interactions. Based on our data, we further questioned whether: (i) the detected TCZs might correspond to dynamic interactions of DC-SIGN with clathrin and (ii) glycan-based connectivity would impact on DC-SIGN-CCP interactions. To address these questions we analyzed DC-SIGN trajectories on double-transfected cells (wtDC-SIGN or N80A and CLC-YFP) for the presence of TCZs and quantified their proximity to clathrin (Fig. 6A and Movie S3). Strikingly, ~80% of the wtDC-SIGN TCZs occurrences were located inside or in proximity to clathrin-rich areas, whereas this percentage decreased to ~40% for N80A (Fig. 6B).

We further analyzed the TCZs with respect to their proximity to clathrin, both for wtDC-SIGN and N80A. The majority of the N80A TCZs occurs outside clathrin with diffusion values (Fig. 6C) similar to those shown in Fig. 5C and D, indicating that these TCZs bear no relation to clathrin. In remarkable contrast, wtDC-SIGN TCZs inside clathrin showed similar characteristics

Fig. 5. Mesoscale compartmentalization influences nanoscale transient confinement of DC-SIGN. (A) Representative trajectories of wtDC-SIGN and N80A on CHO cells, and DC-SIGN on untreated and lactose-treated imDCs. Regions of TCZs are detected and shown in circles and highlighted by arrows. Scale bar, 400 nm. (B) Average duration of TCZs. (C) Average size of the TCZs. (D) Average diffusion inside TCZs. (E) Average number of TCZs per area. Error bars correspond to the errors on the fitting (Fig. S5). wt-DC-SIGN: 283 TCZs on 755 trajectories. N80A: 483 TCZs on 614 trajectories. ImDC: 293 TCZs on 171 trajectories. ImDC+lactose: 107 TCZs on 155 trajectories. Minimum of 50 cells from 10 to 20 separate experiments at each condition.

as those found for DC-SIGN with intact glycan-based connectivity (compare Fig. 5 C and D with Fig. 6 C and D), revealing that dynamic interaction with clathrin leads to the transient arrest of DC-SIGN. Regrettably, the poor transfection efficiency (<5%) and low cell viability of transfected imDCs prevented analogous studies on these cells. Importantly, these data imply that DC-SIGN–CCP interactions do not occur by random encounters between mobile receptors and CCPs. Instead, DC-SIGN micropatterning in regions enriched in clathrin, brought about by the glycan network, favors cargo–clathrin encounters. Interestingly, N80A TCZs events proximal to clathrin showed similar

diffusion to those of wtDC-SIGN TCZ (Fig. 6 C and D), indicating that glycosylation does not interfere on the dynamics of the interaction itself, but it affects the probability with which these interactions occur.

Consistent with the above data, we observed increased clathrin-dependent internalization of virus-like particles bound to DC-SIGN on both CHO and imDCs with an intact glycan-based connectivity compared with their glycan-perturbed counterparts (Fig. 6 E and F). Collectively, our data reveal that glycan-based interactions promote mesoscale compartmentalization of DC-SIGN, thereby fine-tuning the occurrence of dynamic interactions with clathrin and possibly enhancing CME of antigens bound to DC-SIGN.

Discussion

In this work we directly visualize cell membrane micropatterning mediated by glycans using a combination of superresolution imaging techniques and dual-color single-particle tracking. We find that this micropatterning corrals receptors into clathrin-enriched regions, thereby increasing clathrin–receptor interactions, and potentially influencing clathrin-mediated endocytosis of receptor-bound ligands. We also establish that clathrin–receptor encounters do not occur in a random fashion and further substantiate the dynamic and transient behavior of clathrin interactions with their cargo before successful internalization.

Cell surface glycan-mediated interactions have been thought to promote the formation of high-order aggregates of several membrane proteins (1–3, 5). Contrasting with these expectations, one of the key observations of our work is that glycan-mediated interactions do not contribute to receptor nanoclustering, nor do they promote dynamic interactions between nanoclusters. However, the overall lateral mobility was considerably affected, with glycosylated DC-SIGN nanoclusters dynamically exploring restricted areas of the cell surface. These results demonstrate that glycan-based interactions regulate the microscale organization of the receptor and, importantly, establish a previously unidentified extracellular mechanism of membrane organization based on the compartments of the membrane a molecule is able to (or unable to) explore. Moreover, our results indicate that these interactions are mediated (directly or indirectly) by cell surface galectins. Indeed, treatment of imDCs with lactose drastically affected the spatial exploration of the receptor and reduced galectin-9 surface levels (Fig. S2), an important glycan-binding protein found

Fig. 6. N-glycosylation enhances DC-SIGN clathrin interactions and virus-like particle internalization via CCPs. (A) Representative wtDC-SIGN and N80A trajectories (white lines) overlaid on the normalized CLC-YFP image. Black points (highlighted with arrows) show the occurrence of TCZs (Movie S3). Scale bars, 400 nm. (B) Percentage of TCZs near clathrin-rich areas for wtDC-SIGN and N80A. Because clathrin images are diffraction-limited, we considered clathrin-rich regions as those having intensity >0.7. Data from at least eight cells from different days. $**P < 0.01$. (C) D_{2-4} inside TCZs for wtDC-SIGN and N80A, in relation to clathrin proximity. Due to the low number of wtDC-SIGN TCZ events outside clathrin, we could not determine D_{2-4} values here. (D) Sizes of TCZ inside clathrin. Error bars in C and D correspond to the error on the fitting (Fig. S5). (E) Merged confocal microscopy images of internalized gp120-QD (red) and CCPs (green) on CHO cells expressing wtDC-SIGN or N80A, and on untreated and lactose-treated imDCs. Images represent one focal plane in the middle of the cell body. Representative cells from multiple experiments are shown. Scale bars, 5 μm . (F) Pearson correlation coefficient between gp120-QD and clathrin. Values $\pm$ SEM are average of multiple images from several cells from different days of experiments. $**P < 0.01$.

on mDCs phagosomes together with DC-SIGN and the transmembrane glycoprotein CD44 (31).

An interesting finding of our work is that glycan-based interactions seem fundamental in fine-tuning DC-SIGN interactions with clathrin, by confining the receptor in regions enriched with this endocytic protein. Remarkably, we observed that clathrin distribution is not homogeneous but highly localized in permissive regions of the membrane, supporting earlier findings that indicated CCPs nucleation at predefined sites (11, 32). It has been further shown that these regions are specialized cortical actin patches that might efficiently organize CCP nucleations (32). How exactly glycan-based interactions sense these clathrin-rich regions is currently unknown. It is conceivable that there might be areas of the cell membrane enriched in actin where several key players colocalize in space and time, including CD44 (33) and clathrin (32). DC-SIGN could then be maintained in these regions through its interactions with CD44, most likely involving galectin-9, which would cross-link both proteins through their glycosylated motifs. Initial studies in our group indeed support this hypothesis (Figs. S6–S8), which is currently under further investigation.

Recent intriguing single-molecule experiments have shown that CCPs do not permanently capture cargo molecules before internalization (12). Instead, cargos diffuse on the membrane until they randomly encounter CCPs and become transiently arrested in a “catch-and-release” fashion (12). Our results substantiate the dynamic and transient behavior of clathrin interactions with their cargo before successful internalization, and importantly extend these findings by showing that the spatial proximity of DC-SIGN to clathrin enhances the probability of successful cargo–clathrin interactions. Our data thus imply that CCP–cargo interactions do not proceed via random encountering but are greatly influenced

by the micropatterning spatial regulation of the glycan network. Interestingly, receptor glycosylation does not affect the dynamical aspects of this interaction, suggesting that the rates of clathrin-dependent endocytosis of DC-SIGN and its pathogenic ligands are not altered by glycosylation; merely the probability of encountering clathrin increases compared with a purely random process.

In summary, the quantitative methods described here identify a novel mechanism of membrane organization and provide a visual and quantitative picture on how glycan-mediated interactions contribute with an additional layer of complexity on the organization of the cell membrane. This mesoscale dynamic organization influences receptor interactions with clathrin, possibly impacting on CME but likely also influencing other cellular processes.

Materials and Methods

Description of the materials and experimental methods can be found in *SI Materials and Methods*. Single- and dual-color SPT measurements were performed as described in ref. 29 and are expanded in *SI Materials and Methods*. TCZs occurring within trajectories were detected by an algorithm described by Simson et al. (30). Generation of time-dependent membrane exploration maps and analytical tools used in this study can be found in *SI Materials and Methods*.

ACKNOWLEDGMENTS. We thank M. Rivas for technical assistance. STED images were obtained at the Super-resolution Light Nanoscopy (SLN) facility at Institut de Ciències Fotoniques. This work was supported by the Spanish Ministry of Science and Innovation (MAT2011-22887), Human Frontiers Science Program (Grant RGP0027/2012), and the European Commission (Grants 288263 and 284464). A.C. is the recipient of a Meervoud Grant (836.09.002) from The Netherlands Organization for Scientific Research (NWO). B.M.C. acknowledges the EC-Marie Curie COFUND action (ICFONEST). S.I.B. acknowledges European Molecular Biology Organization Foundation for short-term Fellowship (ASTF 367–2011) and NWO (Aard-en Levenswetenschappen 822.02.017).

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