Toxoplasma gondii Tic20 is essential for apicoplast protein import

Giel G. van Dooren*,†, Cveta Tomova‡, Swati Agrawal§, Bruno M. Humbel‡, and Boris Striepen*†§

*Center for Tropical and Emerging Global Diseases and †Department of Cellular Biology, University of Georgia, Athens, GA 30602; and ‡Electron Microscopy and Structural Analysis, Department of Biology, Faculty of Sciences, Utrecht University, 3584 CH Utrecht, The Netherlands

Edited by Thomas E. Wellems, National Institutes of Health, Bethesda, MD, and approved July 22, 2008 (received for review April 22, 2008)

Apicomplexan parasites harbor a secondary plastid that has lost the ability to photosynthesize yet is essential for the parasite to multiply and cause disease. Bioinformatic analyses predict that 5–10% of all proteins encoded in the parasite genome function within this organelle. However, the mechanisms and molecules that mediate import of such large numbers of cargo proteins across the four membranes surrounding the plastid remain elusive. In this work, we identify a highly diverged member of the Tic20 protein family in Apicomplexa. We demonstrate that Tic20 of Toxoplasma gondii is an integral protein of the innermost plastid membrane. We engineer a conditional null-mutant and show that TgTic20 is essential for parasite growth. To characterize this mutant functionally, we develop several independent biochemical import assays to reveal that loss of TgTic20 leads to severe impairment of apicoplast protein import followed by organelle loss and parasite death. TgTic20 is the first experimentally validated protein import factor identified in apicoplasts. Our studies provide experimental evidence for a common evolutionary origin of import mechanisms across the innermost membranes of primary and secondary plastids.

Apicomplexa | plastid | chloroplast

Organelle acquisitions through endosymbiotic events have been major drivers of eukaryotic evolution. The incorporation of a cyanobacterium into a heterotrophic eukaryote led to the formation of plastids (e.g., chloroplasts), enabling eukaryotes to become autotrophic. It is thought that a single, so-called “primary,” endosymbiotic event led to the acquisition of chloroplasts in a lineage that later evolved into eukaryotic phyla such as red algae, green algae, and plants (1). An alternative means by which eukaryotes have obtained plastids is through a process of “secondary” endosymbiosis. Here, a eukaryote containing a primary plastid is incorporated into a heterotrophic eukaryote. Secondaryly derived plastids are found in numerous lineages of ecologically, economically, and medically important organisms, including diatoms, dinoflagellates, and Apicomplexa. Apicomplexa are a phylum of obligate intracellular parasites that include Plasmodium species, the causative agents of malaria, and Toxoplasma gondii, which causes severe encephalitis upon congenital infection and in immunocompromised patients. The plastids of apicomplexans are known as apicoplasts and are thought to function in several essential metabolic pathways such as fatty acid, heme, and isoprenoid biosynthesis (2, 3). Because of the phylogenetic and biochemical divergence of these pathways from their host counterparts, they are heavily pursued as potential targets for antiparasitic drugs.

A key step in the conversion of an endosymbiont into a full-fledged plastid is the transfer of endosymbiont genes to the nucleus of the host. This action affords the host cell control over its endosymbiont but requires the evolution of molecular machinery to enable the import of proteins encoded in the nuclear genome back into the organelle to carry out their role. This targeting process has been well studied in plants, where multisubunit protein complexes mediate protein translocation across both membranes that bound the organelle (4). However, protein translocation into secondary plastids is a fundamentally different process. Secondary plastids are surrounded by three or four membranes and reside within the endomembrane system, with plastid-targeted proteins requiring an N-terminal hydrophobic signal peptide to mediate the first step of protein import (5). Although the protein motifs required to direct proteins into secondary plastids are reasonably well characterized (6), little is known about the molecular mechanisms that mediate import. A fascinating evolutionary question is whether protein import into secondary plastids required the evolution of novel machinery or whether organisms were able to retool existing mechanisms to accommodate novel means of protein targeting. Four membranes surround the apicoplast of Toxoplasma gondii, and in this work we demonstrate that protein import across the innermost membrane of this secondary plastid requires machinery derived from primary plastids.

Results and Discussion

Tic20 Homolog in Apicomplexa. We performed iterative BLAST searches to identify homologs of the plant inner chloroplast membrane translocase component Tic20 in apicomplexan parasites. We identified Tic20 homologs from all of the available genomes of apicomplexans, with the noted exception of the plastidless genus Cryptosporidium. Alignments of apicomplexan Tic20 homologs with plant, red algal, diatom, and cyanobacterial counterparts [supporting information (SI) Fig. S1] reveal the presence of an N-terminal extension with characteristics of a bipartite apicoplast targeting sequence. Similarity to plant and algal Tic20 homologs resides in the C-terminal portion of the protein, although very few residues are conserved among all homologs depicted (Fig. S1).

TgTic20 Is an Integral Protein of the Inner Apicoplast Membrane. Cloning of the full ORF of TgTic20 revealed the presence of three introns and a predicted protein size of 43.4 kDa. We generated a transgenic parasite line expressing TgTic20 fused to a C-terminal HA tag and monitored its localization by immunofluorescence assay (Fig. 1A). TgTic20-HA (green) localized to a small, apical organelle that overlapped with acyl carrier protein (ACP; red), a marker for the apicoplast stroma. A Western blot of cells expressing the TgTic20-HA transgene revealed a major protein species of ~25 kDa and a less abundant species of ~40 kDa (Fig. 1B). This finding suggests that the N-terminal portion of the protein is cleaved to yield the mature protein of ~20 kDa, consistent with the N terminus of the protein functioning as an apicoplast-targeting domain that is processed upon import into the apicoplast (7, 8). In silico modeling of the protein structure of TgTic20 suggests the presence of four transmembrane domains in TgTic20, found in close proximity to the putative C-terminal targeting sequence.


The authors declare no conflict of interest.

This article is a PNAS Direct Submission.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. EU427503).

1To whom correspondence may be addressed. E-mail: giel@uga.edu or striepen@cb.uga.edu.

This article contains supporting information online at www.pnas.org/cgi/content/full/0803862105/DCSupplemental.

© 2008 by The National Academy of Sciences of the USA.
TgTic20 is an apicoplast integral membrane protein. (A) Immunofluorescence assay depicting an eight-cell T. gondii vacuole. TgTic20-HA (green) colocalizes with the apicoplast marker ACP (red). (Scale bar: 5 μm.) (B) Western blot of protein extracts from the TgTic20-HA line with anti-HA antibodies. A mature TgTic20 protein species is labeled at ~23 kDa, whereas a weaker precursor band is labeled at ~40 kDa. (C) Proteins were extracted from the TgTic20-HA/APT1-YFP line and fractionated into soluble (S) and membrane pellet (P) fractions by sodium carbonate treatment, or into soluble (S) and detergent (D) phases by Triton X-114 phase partitioning. Total protein extracts (T) are shown in the first lane. (D) Transmission electron micrograph of the TgTic20-HA cell line, where TgTic20-HA is labeled with 10-nm gold beads and ACP with 15-nm gold beads. Arrowheads show TgTic20-HA labeling at the membranes of the organelle. (Scale bar: 100 nm.)

**Fig. 1.**

succession at the C terminus of the protein (Fig. S1). To determine whether TgTic20 is an integral membrane protein, we performed sodium carbonate extractions and Triton X-114 phase partitioning. Sodium carbonate extractions resulted in TgTic20 localizing to the membrane fraction, much like the characterized apicoplast phosphate transporter [APT1 (9)] and unlike the soluble ACP (Fig. 1C). Much, but not all, TgTic20 localized to the detergent (i.e., membrane) phase during Triton X-114 phase partitioning, again consistent with a membrane localization for TgTic20.

To characterize further the subcellular localization of TgTic20, we performed transmission electron microscopy on parasites expressing TgTic20-HA, labeling with anti-HA and anti-ACP antibodies. This action revealed the localization of TgTic20-HA to membrane-bound organelles that also contained ACP (Fig. 1D), consistent with the apicoplast localization of this protein. Localization of TgTic20-HA within the apicoplast was generally confined to the membranes of the organelle, consistent with the membrane localization of TgTic20, whereas ACP was distributed throughout the entire organelle.

Four membranes surround the apicoplast, and until now it has been difficult to determine to which membrane a given apicoplast membrane protein localizes. To determine the membrane to which TgTic20 localizes, we made use of an established self-assembling split pea green fluorescent protein (GFP). In this system, the C-terminal β-strand of GFP (GFP-11) was removed from the remaining 10 β-strands (GFP 1–10) of the molecule. This GFP-11 was engineered with the ability to self-assemble with GFP 1–10 if both molecules localize to the same compartment (10). As a proof of principle, we first targeted GFP 1–10 to the apicoplast stroma by adding the N-terminal apicoplast-targeting domain of ferrodoxin-NADP⁺ reductase (FRN). By itself, this protein was unable to fluoresce (data not shown). We next fused GFP 11 to the C terminus of ACP and transfected this into the FNR-GFP 1–10 cell line. The resultant line revealed fluorescence that colocalized with an apicoplast red fluorescent protein (RFP) marker (Fig. 2B), consistent with the C terminus of TgTic20 residing in the apicoplast stroma. Flow cytometric analyses of the various cell lines described above supported the results obtained by microscopic analysis (Fig. S2A). One concern was that the observed apicoplast fluorescence might result from retention of FRN-GFP 1–10 in an outer membrane. To control for this possibility, we monitored cleavage of the apicoplast-targeting leader of FRN-GFP 1–10, a measure for whether proteins are accessible to the stromal processing peptidase enzyme that likely resides in the apicoplast stroma (8). We found no difference in processing of FRN-GFP 1–10 whether expressed by itself or with interacting components (Fig. S2B), indicating that most FRN-GFP 1–10 protein resides in the stroma and consequently that TgTic20-GFP 1–10 does not prevent targeting of FRN-GFP 1–10 to the stroma.

We conclude that TgTic20 is an integral protein of the inner apicoplast membrane, with its C terminus residing in the apicoplast stroma. Assuming that the predictions of four transmembrane domains are correct, the N terminus would also be in the stroma, resulting in the model for TgTic20 topology presented in Fig. 2D. Recently, candidate proteins that likely localize to outer membranes of the apicoplast have been identified (9, 12), and the split GFP assay may help to pinpoint the residence of these and other proteins to a specific membrane or apicoplast compartment.
Parental cell lines (Fig. 3) reduced parasite growth in the knockout cell line but not in the parental cell line. We predict that Tic20 localizes to the inner membrane of the apicoplast, with the C terminus in the stroma. At similar levels in both the absence and presence of ATc (Fig. 3), green fluorescence colocalizes with the apicoplast stromal marker FNR-RFP. This model for localization of Tic20 was confirmed by fluorescence assays that revealed two differently sized molecular species: a slow migrating mature protein where the N-terminal targeting leader has been cleaved (7, 8). We asked whether leader processing was affected in the conditional Tic20 mutant. To facilitate these studies, we generated a cell line in the conditional Tic20 mutant background that expressed a ‘synthetic’ apicoplast-targeted protein, consisting of the apicoplast-targeting leader of TgFNR fused to mouse dihydrofolate reductase (DFHR; a reporter protein typically used for organellar import assays in other systems [15]) and a C-terminal HA tag for detection. To gain a dynamic measure of the timing of defects on protein import in the apicoplast, we established several assays for successful protein import into apicoplasts (Fig. 4). First, we examined processing of the N-terminal targeting domain of Tic20 (Fig. 4A, Top). As a more sensitive measure for Tic20 abundance, we immunoprecipitated Tic20 protein from \( \times 10^7 \) parasites. We measured immunopurified protein levels by Western blotting and found that after 2 days growth on ATc, we could no longer detect Tic20 protein from a constitutive promoter (iTic20/Etic20, Fig. 4B). We asked whether leader processing was affected in the conditional Tic20 mutant. To facilitate these studies, we generated a cell line in the conditional Tic20 mutant background that expressed a ‘synthetic’ apicoplast-targeted protein, consisting of the apicoplast-targeting leader of TgFNR fused to mouse dihydrofolate reductase (DFHR; a reporter protein typically used for organellar import assays in other systems [15]) and a C-terminal HA tag for detection. To gain a dynamic measure of the timing of defects on protein import in the Tic20 mutant, we conducted pulse–chase labeling experiments. We incubated mutant parasites growing in host cells for 0, 2, 3, 4, and 5 days on ATc in the absence (green diamonds) or presence (blue squares) of ATc. We could no longer detect Tic20 protein from a constitutive promoter (iTic20/Etic20, Fig. 4B). We asked whether leader processing was affected in the conditional Tic20 mutant. To facilitate these studies, we generated a cell line in the conditional Tic20 mutant background that expressed a ‘synthetic’ apicoplast-targeted protein, consisting of the apicoplast-targeting leader of TgFNR fused to mouse dihydrofolate reductase (DFHR; a reporter protein typically used for organellar import assays in other systems [15]) and a C-terminal HA tag for detection. To gain a dynamic measure of the timing of defects on protein import in the Tic20 mutant, we conducted pulse–chase labeling experiments. We incubated mutant parasites growing in host cells for 0, 2, 3, 4, and 5 days on ATc.
and radiolabeled proteins with $^{35}$S-amino acids for 1 h (pulse). We then washed the radiolabel and incubated in medium containing an excess of unlabeled amino acids for an additional 2 h (chase). We purified proteins of interest by immuno- or affinity purification, separated them by SDS/PAGE, and detected them by autoradiography. After 5 days growth on ATc, precursor FNR-DHFR-HA protein was made at levels similar to that formed in cells grown in the absence of ATc, indicating that knockdown of TgTic20 does not affect synthesis of apicoplast-targeted proteins (Fig. 4C). However, after 2 days growth in ATc there is a 35% reduction in formation of mature, processed FNR-DHFR-HA, decreasing to undetectable levels at day 4 (Fig. 4C; Fig. 4D, green diamonds).

We also monitored processing of the native apicoplast protein ACP in the TgTic20 mutant. Mature ACP contains only one sulfur-containing amino acid, making detection difficult. The experiment shown in Fig. 4C suggests that ACP is processed at day 2 on ATc and not beyond, but detection levels are too low to draw a definitive conclusion. As a control, we monitored processing of microneme protein MICS, which occurs in a post-Golgi compartment of the secretory pathway (16). Even after 5 days of incubation on ATc, MICS is processed (Fig. 4C), suggesting that TgTic20 knockdown does not affect other parts of the secretory pathway.

Although we suspect that precursor protein cleavage is a solid marker for whether proteins are able to traffic into the apicoplast stroma, it has not been formally shown that the processing event occurs here. Therefore, we sought to establish independent measures for successful protein targeting to the apicoplast. Several apicoplast enzymes are modified posttranslationally by cofactors after import into the stroma. One such modification is the biotinylation of acetyl-CoA carboxylase [ACC (17) Fig. 4B], a protein involved in biosynthesis of fatty acids. We purified biotinylated proteins by using an immobilized streptavidin column. Radiolaabeled biotinylated ACC is not yet detectable after the 1-h pulse. In the absence of ATc, we observe robust biotinylation of ACC during the 2-h chase (Fig. 4C). Biotinylated ACC is reduced after 2 days of incubation in ATc and severely reduced after 3 days (Fig. 4C; Fig. 4D, red triangles), consistent with the results of the leader-processing assay. T. gondii contains a second major biotinylated protein, the mitochondrial pyruvate carboxylase (PC) enzyme (17). Levels of biotinylated PC remain unchanged after incubation in ATc.

A second postimport modification is lipoylation of the E2 subunit of pyruvate dehydrogenase complex (PDH-E2). Lipoylation of PDH-E2 is solely mediated by apicoplast-targeted LipA and LipB and requires a substrate synthesized de novo within the apicoplast stroma [octanoyl-ACP (2, 18) Fig. 4B]. In addition to apicoplast PDH-E2, T. gondii contains several lipoylated E2 subunit proteins in the mitochondrion (mito-E2 (2, 18)). The mitochondrion contains a specific protein (LpLA) that functions in the addition of the lipoyl moiety to the E2 enzymes (18), suggesting that, much like the apicoplast, lipoylation can only occur after successful import into the organelle. We purified lipoylated proteins by using an antibody against lipoic acid. After the 1-h pulse, mito-E2 enzymes are labeled, consistent with rapid import into mitochondria (Fig. 4C). As with biotinylated ACC, lipoylated PDH-E2 is not observed until the 2-h chase. Lipoylation of the apicoplast PDH-E2 is reduced after 2 days of growth on ATc and severely reduced after 3 days, whereas modification of mitochondrial enzymes was not affected, even after 5 days of incubation on ATc (Fig. 4C; Fig. 4D, blue squares).

Together, these data indicate that knockdown of TgTic20 impairs import of apicoplast-targeted proteins into the stroma of the organelle, but does not impair targeting of proteins to other destinations of the secretory pathway or to the mitochondrion. To rule out the possibility that defects in apicoplast protein import

**Fig. 4.** TgTic20 is essential for apicoplast protein import. (A) Regulation of the inducible TgTic20-c-myc protein. TgTic20 parasites were grown for 0–4 days on ATc. Proteins were extracted and subjected to Western blotting with either anti-c-myc or anti-GRAB antibodies (as a loading control) or subjected to immunoprecipitation of the inducible TgTic20-c-myc protein followed by Western blotting with anti-c-myc antibodies (Bottom). (B) Schematic depiction of the three protein import assays used in this work. We measured cleavage of preprotein leader sequences by the stromal processing peptidase (SSP), biotinylation of ACC by a holocarboxylase synthase (HCS), and lipoylation of PDH-E2 by Lip8 and LipA. All three processes are thought to occur in the apicoplast stroma. (C) Pulse–chase analysis of proteins from the TgTic20 knockout line grown for 0, 2, 3, 4, or 5 days on ATc. Infected host cells were incubated in medium containing $^{35}$S-amino acids for 1 h and either harvested (P) or further incubated in nonradioactive medium for 2 h (C). After detergent solubilization, proteins were purified by immunoprecipitation or affinity purification and separated by SDSPAGE before detection by autoradiography. Protein bands marked by an asterisk in lanes containing biotinylated and lipoylated proteins represent contaminating host cell proteins. The band marked by an asterisk in HA pulldown lanes likely results from the use of an alternative internal start codon representing a shorter cytosolic version of FNR-DHFR-HA. (D) Quantification of bands in C. FNR-DHFR-HA values (green diamonds) were quantified as the percentage of the intensity of PC in the same lane. PDH-E2-LA values (blue squares) were quantified as a percentage of the intensity of the lowermost mito-E2 band in the same lane. Values for each day are expressed as a percentage of the no-ATc value. (E) Pulse–chase analysis of proteins from the TgTic20 parental (Tic20/c-tic20) line, performed in an identical manner to C.
Our results indicate that \( Tg^{\text{Tic20}} \) is required for apicoplast biogenesis. \( \text{(A)} \) \( Tg^{\text{Tic20}} \)-knockout parasites containing apicoplast-targeted RFP (FNR-RFP) were grown for 0, 4, or 5 days on ATc and subjected to live-cell imaging. (Scale bars: 2 \( \mu \)m.) \( \text{(B)} \) \( Tg^{\text{Tic20}} \) parental (green diamonds) or knockout (red squares) parasites containing apicoplast-targeted RFP were grown for 0–6 days on ATc. One hundred four-cell vacuoles were imaged at each time point. We graphed the percentage of vacuoles at each time point where every parasite in that vacuole contained an apicoplast.

Based on its integral membrane localization, it has been postulated that plant Tic20 forms part of the protein import channel of the inner chloroplast membrane (20), although no direct experimental evidence supports this. Our results suggest that knockdown of \( Tg^{\text{Tic20}} \) protein expression does not immediately ablate import. Two days after the addition of ATc, the amount of \( Tg^{\text{Tic20}} \) is below our limits of detection. At this time point, apicoplast protein import is clearly affected (as measured by three independent assays), yet still occurs at between 65 and 77% of the level of wild-type cells (Fig. 4D). This argues against \( Tg^{\text{Tic20}} \) functioning directly in an inner membrane import channel because the lack of an import channel would likely result in immediate ablation of import into the apicoplast. We considered the possibility that \( T. gondii \) harbors proteins that can partly complement the function of \( Tg^{\text{Tic20}} \). Plants contain multiple Tic20 paralogs and may also have additional nonrelated proteins with functions similar to Tic20 (21, 22). However, we did not identify \( Tg^{\text{Tic20}} \) paralogs in the \( T. gondii \) genome. Another possibility is that \( Tg^{\text{Tic20}} \) is an accessory or regulatory component of a putative import complex in the inner membrane.

In such a scenario, \( Tg^{\text{Tic20}} \) may influence the efficiency of protein import through this complex, assembly of the complex, or be involved in a separate process that is essential for functioning of the inner membrane import complex. Identifying and characterizing additional inner membrane import components should allow us to address these questions.

**Concluding Remarks.** During their intracellular development, apicomplexan parasites such as \( T. gondii \) must target large numbers of proteins to their apicoplast. Protein targeting occurs via the secretory pathway and requires proteins to cross four membranes before reaching the organelle stroma (5). There has been considerable speculation about how protein targeting across these four membranes is mediated (e.g., 5, 23), but there has been a distinct lack of functional evidence for the various models.

Emerging evidence suggests that \( T. gondii \) and other Apicomplexa belong to a eukaryotic “supergroup” known as the Chromalveolata (24, 25). Chromalveolates include other major eukaryotic groups such as dinoflagellates and heterokonts (including diatoms and brown algae). A distinguishing feature of chromalveolates is the presence of a plastid that was derived by secondary endosymbiosis from a red alga. Chromalveolate plastids, then, represent a cellular \( \text{ménage a trois} \) of three “founder” organisms: a cyanobacterium, a red alga, and a heterotrophic eukaryote. An early requirement in the acquisition of plastids is the evolution of protein import machinery. An intriguing evolutionary question is which of these founders “donated” the import machinery and whether the origin of individual translocons is tied to the origin of the membrane they cross. Three types of translocons of have been speculated to potentially act in apicoplast protein import: primary plastid-derived Tic and Toc complexes and, more recently, Der1-containing complexes retooled from their original role in protein retrotranslocation across the ER membrane (12). In this work, we show that the innermost apicoplast membrane is crossed using machinery derived (at least in part) from the inner membrane Tic translocation complex of the red algal plastid, and we note that Tic homologs are present in other chromalveolates such as diatoms (Fig. S1 (23)). Rather than evolving a fundamentally different means of protein import into secondary plastids, Apicomplexa and their chromalveolate cousins made use of the machinery already available from their primary plastid progenitors. It remains to be determined whether components of the Tic and Der1 complexes mediate import across other apicoplast membranes. The approaches for characterizing and localizing candidate apicoplast import proteins that we describe here provide an experimental framework to test these hypotheses conclusively.
Materials and Methods

Parasite Culture and Manipulation. Parasites were passaged in human foreskin fibroblasts and genetically manipulated as described in ref. 26. GenBank accession number for TgTic20 is EU427503. Plasmid construction and flow cytometry techniques are described in detail in SI Materials and Methods. All parasite strains described in this work were cloned by either limiting dilution or flow cytometry. Where indicated, parasites were grown in anhydrotetracycline (IBA) at a final concentration of 0.5 μg/ml.

Immunoprecipitation, SDS/PAGE, and Immunoblotting. For pulse–chase analyses, infected host cells were radiolabeled with 100 μCi/ml [35S]methionine/ cysteine (GE Healthcare) for 1 h. Cells were either harvested (pulse) or washed twice and incubated in parasite growth medium lacking radioactive amino acids for 2 h (chase) before harvesting. Proteins of interest were purified by immunoprecipitation or affinity purification and separated by SDS/PAGE using standard procedures (8) and detected by autoradiography or Phosphoimunoprecipitation or affinity purification and separated by SDS/PAGE for 2 h (chase) before harvesting. Proteins of interest were purified by twice and incubated in parasite growth medium lacking radioactive amino cysteine (GE Healthcare) for 1 h. Cells were either harvested (pulse) or washed

Microscopy. Immunofluorescence assays and light microscope imaging were performed essentially as described in ref. 26. Detailed procedures for electron microscopy are included in SI Materials and Methods.

Acknowledgments. We thank Geoff Waldo (Los Alamos National Laboratory), David Sibley (Washington University), Manami Nishi (University of Pennsylvania), Marc-Jan Gubbels (Boston College), and Markus Meissner (Heidelberg University) for plasmids; Jörn Lakowski (University of Georgia) for mouse cDNA; and Vern Carruthers (University of Michigan), Gary Ward (University of Vermont), and Geoff McFadden (University of Melbourne) for antibodies. We are grateful to Carrie Brooks, Lisa Sharling, Kylie Mullin, and Geoff Waldo for technical advice and discussions and especially to Julie Nelson of the Center for Tropical and Emerging Global Diseases Flow Cytometry Facility for performing cell sorting. This work was supported by C. J. Martin Overseas Research Fellowship (to G. G. V. D.), the European Network of Excellence “Three-Dimensional Electron Microscopy,” FP6, and the Dutch Cytron Consortium (to C. T. and B. M. H.), and National Institutes of Health Grant AI 64671 (to B. S.).

References