Jena Lab contributions in the past 20 years

Summary: The contribution made by our laboratory is the discovery of a new cup-shaped cellular structure the ***'porosome'*** at the cell plasma membrane, and how this structure is involved in the regulated fractional release of intravesicular contents from cells with great precision during cell secretion. The discovery of the '*porosome*' the universal secretory machinery in cells, has giving birth to a new paradigm in our understanding of a specific fundamental cellular process, revolutionizing our general understanding of the chemistry of life. In the course of our discovery of the *porosome*, and a molecular understanding of the associated structures and processes involved in cell secretion, our laboratory has additionally contributed to overcoming the 1 nm resolution barrier in the study of live cell structure-function using the atomic force microscope (AFM).

A time line of the *porosome* discovery, the elucidation of its structure, its chemical composition, and functional reconstitution into artificial lipid membrane and live cells (4-14, 43-45); the molecular assembly of membrane-associated t-SNARE proteins at the *porosome* base and v-SNARE proteins in the secretory vesicle membrane in a ring or rosette arrangement resulting in the establishing continuity between the opposing bilayers or ***'fusion pore'*** formation in presence of calcium (15-23,38); and the molecular mechanism of secretory vesicle volume increase required for intravesicular content expulsion during cell secretion (24-30,42), provide understanding of the fractional release of intravesicular contents from cells during the so called "kiss-and-run" mechanism of cell secretion. Consequently, the scores of publication by other laboratories on the *porosome* complex and the involvement of various *porosome* proteins in secretion and their altered states resulting in secretory defects (37,47-62), attest to the critical role played by the *porosome* in health and disease. It took nearly 20 years for the *porosome* to be included in textbooks.

Introduction: Secretion is a fundamental cellular process in living organisms, from yeast to cells in humans. Secretion is required for a plethora of physiological activities including neurotransmission, immune response, digestion, and hormone release, all required for maintaining homeostasis within a living organism. Correspondingly, secretory defects in cells are responsible for a myriad of debilitating diseases. Since the 50's, it was believed that secretory vesicles completely merge with the cell plasma membrane during secretion, resulting in release of the entire vesicular contents, an all or none mechanism. While this provides one mechanism for cell secretion, the observation of partially empty vesicles in cells following secretion [Fig. 1] is incompatible with complete vesicle merger, suggesting the presence of an additional mechanism that allows partial discharge of intra-vesicular contents during secretion. In a 1993 *Nature* article *[1993 Nature 363:497–498]* therefore, *Erwin Neher* wrote: *"It seems terribly wasteful that, during the release of hormones and neurotransmitters from a cell, the membrane of a vesicle should merge with the plasma membrane to be retrieved for recycling only seconds or minutes later."* We hypothesized that the proposed mechanism would involve a plasma membrane structure, which would serve to prevent the collapse of secretory vesicles at the cell plasma membrane, enabling the vesicle to instead transiently establish continuity with the cell plasma membrane, expel a portion of its contents, and disengage while remaining partially filled [Fig. 1a (√), 1b]. This mechanism would enable the cell to retain the integrity of both the vesicle membrane and the cell plasma membrane.

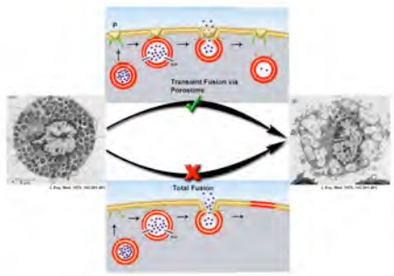

Fig. 1a. Electron micrographs of rat peritoneal mast cells, in resting (A, extreme left) and following secretion (B, extreme right). Note the fractional release of intravesicular contents following secretion (B) (*J. Exp. Med.* 1975, 142:391-401). This fractional release of intravesicular contents could only be achieved via the *porosome* (P)-mediated transient fusion mechanism shown (√).

The late *Bruno Ceccarelli* was a pioneer in the field of 'transient' or 'kiss-and-run' mechanism of secretory vesicle fusion at the cell plasma membrane enabling fractional release of intravesicular contents. Ceccarelli proposed in 1973 the presence of such a process in cells (1). Then in 1990 *Wolfhard Almers* hypothesized that the *fusion pore* (continuity established between the vesicle membrane and the cell plasma membrane), results from a "preassembled ion channel-like structure that could open and close" (2). A later 1992 review (3) by *Julio Fernandez*, opined that the difficulty in observing such channel-like structures, was the lack of ultrahigh resolution imaging tools to directly monitor their presence and study their dynamics in live cells.

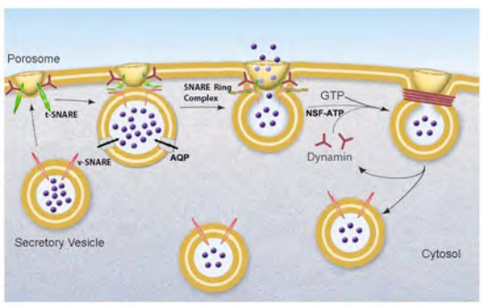

Fig. 1b: Schematic drawing of porosome-mediated fractional release of intravesicular contents during cell secretion. Secretory vesicles dock at the porosome base via t-SNAREs present at the pososome base and v-SNAREs present at the secretory vesicle membrane to form a ***t-/v-SNARE ring complex***, establishing continuity between the opposing membranes ***[fusion pore]*** through which pressurized intra-vesicular contents [intravesicular pressure established via active transport of water through aquaporin or water channels (AQP) at the secretory vesicle membrane] are expelled to the outside during cell secretion. Following secretion, the t-/v-SNARE rosette complex is disassembled by NSF-ATP and the fused lipid membrane is cleaved by dynamin-GTP. The resultant partially empty vesicle then dissociates from the porosome at the cell plasma membrane.

Discovery of the *'porosome'*: In the mid 1990's, motivated by the goal of identifying cellular structures at the plasma membrane involved in the regulated fractional release of intravesicular contents from cells, we employed the then newly developed imaging modality of AFM to study the morphology and dynamics of live pancreatic acinar cell surface at the nanometer scale during secretion. The major breakthrough came in 1995-1996, when circular pit-like structures containing 100-180 nm depressions or pores [Fig 2A,B] were observed at the apical plasma membrane of pancreatic acinar cells, where secretion is known to occur. During secretion, the depressions or pores grew larger, returning to their resting size following completion of secretion. We reported these results on January 1, 1997 in the Proceedings of the National Academy of Sciences (4). Five years later, new results from our laboratory established that the observed depressions are secretory portals at the cell plasma membrane (5,6). In January 2002 and February 2003, we reported in two seminal studies, one in Cell Biology International (5), and the other in the Biophysical Journal (6), that following stimulation of cell secretion, gold-conjugated amylase antibodies (the starch-digesting enzyme amylase being a major intravesicular product secreted by the exocrine pancreas) accumulate at depressions, establishing depressions to be secretory portals in the cell (5,6) and the name *'porosome'* was therefore assigned to the structure. The study reported in the Biophysical Journal (6), further demonstrated the presence of t-SNAREs at the *porosome* base facing the cytosol, firmly establishing *porosome* structures to be secretory portals (6). Electron micrographs of *porosome* at the apical plasma membrane (PM) of pancreatic acinar cells with docked secretory vesicle called zymogen granule (ZG) is presented in Fig 2 (Fig 2 top left and right). In Fig 2A, the *porosome* membrane (POM, yellow arrowhead) associated with the ZG membrane (ZGM) is shown. In March 2002, our laboratory in collaboration with *Lloyd Anderson* reported in the journal Endocrinology (ref. 7, Fig 2 cover illustration lower right), the structure of *porosome* and their dynamics at the cell plasma membrane in growth hormone (GH) secreting cells of the pig pituitary, and the accumulation of GH-immuno-gold at *porosome* openings following cell secretion (7). These results further demonstrate *porosome* to be plasma membrane-associated secretory portals in cells. In the same year in a separate study, we reported *porosome* structure-dynamics in chromaffin

cells (8), and in September of 2003 (9) following isolation of *porosome* from acinar cells of the exocrine pancreas, and determination of their composition and reconstitution into lipid membrane, the *porosome* was recognized as the universal secretory portal in cells (9). In the same study (9), morphological details of the *porosome* complex associated with docked secretory vesicles was revealed using EM (9) [Fig. 2A]. Finally in 2015-2016, isolated *porosomes* were functionally reconstituted in live cells (10) by our laboratory, establishing its critical function as the universal secretory machinery in cells and promise in therapy.

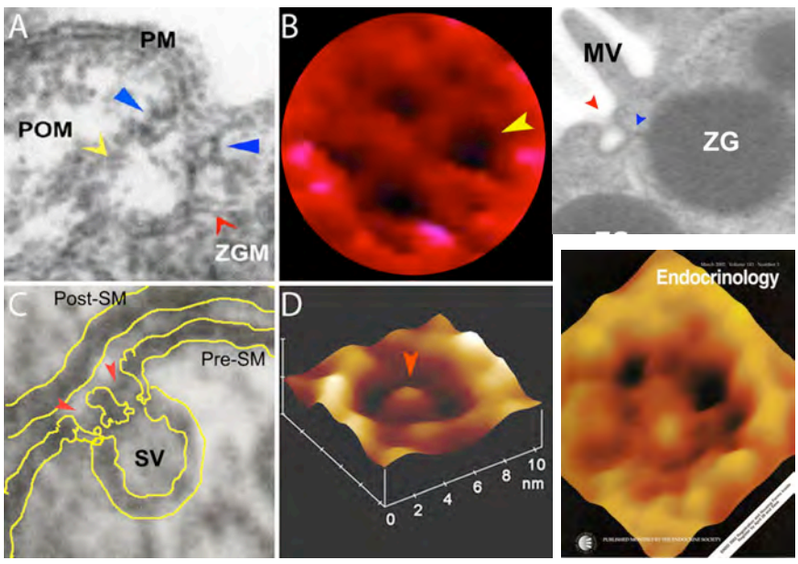

Fig. 2. Porosomes in the exocrine pancreas (A, B), neurons (C, D), growth hormone cell (Journal cover). (**A**) Electron micrograph of a single *porosome* at the apical plasma membrane (PM) of a pancreatic acinar cell showing the *porosome* membrane (POM, yellow arrowhead) associated with the membrane of a secretory vesicle called zymogen granule (ZGM). A circular ring structure (blue arrowhead) forms the neck of the *porosome* complex. (**B**) AFM micrograph of the apical surface topology of a live pancreatic acinar cell, demonstrating the presence of four openings or *porosomes* (one indicated by the yellow arrowhead). *Porosomes* in the exocrine pancreas range in size from 100-180 nm in diameter. (**C**) Electron micrograph of a neuronal *porosome* (red arrowheads) with a docked synaptic vesicle (SV) at its base, in the presynaptic membrane (Pre-SM) of the nerve terminal. Note the central plug in the *porosome* complex. (**D**) AFM micrograph of a neuronal *porosome* at the presynaptic membrane in an isolated synaptosome. Note the central plug (red arrowhead). The neuronal *porosome* is an order of magnitude smaller (10-17 nm in diameter) in comparison to the *porosome* in the exocrine pancreas. (**Extreme Top Right**) Electron micrograph of *porosome* (Fig 2 top left and right) next to a microvilli (MV) at the apical plasma membrane (PM) of a pancreatic acinar cell with docked secretory vesicle or ZG. (**Extreme Lower Right**) AFM micrograph of the apical surface topology of a live GH cell from pig pituitary demonstrating the presence of 100-180 nm in diameter *porosomes* (black circular openings). [Images obtained from: *Proc Natl Acad Sci* 94:316-321 (1997); *Biophys J* 85:2035-2043 (2003); *Cell Biol Int* 28:699-708 (2004); *J Microscopy* 232:106-111 (2008); *Endocrinology* 143:1144-1148 (2003).

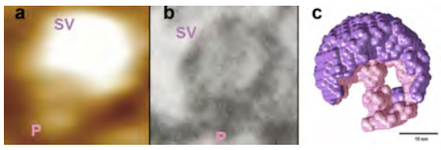

Fig. 3. **Docked synaptic vesicles at the neuronal *porosome*** in the presynaptic membrane of the nerve terminal, observed using AFM, EM, and small angle X-ray solution scattering (SAXS). (**a**) AFM micrograph obtained in fluid of a synaptic vesicle (SV) docked at the cup-shaped *porosome* complex (P) at the cytosolic compartment of the presynaptic membrane. Note the 35 nm SV docked to a 15 nm *porosome* complex. (**b**) An EM micrograph of a 35 nm SV docked to a 15 nm P at the presynaptic membrane [*Cell Biol. Int.* 28:699-708 (2004)]. Note the central plug of the *porosome* complex in the EM micrograph. (**c**) The averaged SAXS 3-D structure of synaptic vesicle (purple) docked at the cup-shaped neuronal *porosome* complex (pink) at the presynaptic membrane in isolated synaptosome is presented *(Bar = 10 nm)* [2014 *Micron* 56:37-43]. Note that AFM, EM, and SAXS, all demonstrate similarity in the docking and interaction of synaptic vesicles with the neuronal *porosome* complex.

In the past 20 years following the initial discovery of the *porosome*, employing a combination of approaches such as AFM, biochemistry, electrophysiology, conventional EM, mass spectrometry, and SAXS, we have reported the presence of *porosomes* in all secretory cells examined, including perhaps most significantly, neurons [Fig. 2C,D; Fig. 3] (11-14). Studies by our laboratory and those of other colleagues, establish this new cup-shaped supramolecular lipoprotein structure at the cell plasma membrane to be secretory portals that performs the specialized task of fractional release of intravesicular contents from cells during cell secretion (12-14). The discovery of the *porosome* provides new understanding on how cells secrete, resulting in a paradigm-shift in our understanding of the secretory process in cells.

The significance of the *porosome* discovery is reflected by the many publications on *porosomes* and associated transient fusion mechanism accompanied by fractional release of intravesicular contents from cells during secretion. In 2011 the *porosome* in hair cells was discovered by *Dennis Drescher* [*Cell Biol. Int. Rep.* (2011) 18:31-34.]; in 2010 *porosome* in RBL-2H3 and BMMC cells were reported by *Gang-yu Liu* [*J. Phys. Chem. B.* (2010) 114:5971-5982.]; *porosomes* in the exocrine pancreas have been further elaborated by the groups of *Elshennawy* in 2011[*J. Am. Sci.* (2011) 7:835-843.] and *Constantin Craciun* in 2013 [*Micron* (2013) 44:137-142.]

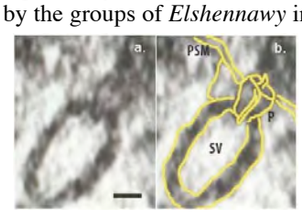

Fig. 4. Docked synaptic vesicles (SV) at the base of a cup-shaped neuronal porosome complex (P) in the presynaptic membrane (PSM) of the nerve terminal, observed using electron microscopy. *Micron* (2012) 43:948-953.

Porosomes have also been discovered by the group of *Márcia Attias* in the unicellular pathogen *Toxoplasma Gondii* in 2011[*J. Stru. Biol.* (2011) 177:420-430.]; by *Mzia Zhvania* in normal and diseased cat and dog brain neurons in 2012 [*Brain. Res. Bull* (2012) 87(2-3):187; *Cell Tissue Biol.* (2012) 6:69-72.; *Micron* (2012) 43:948-953 [Fig. 4]; and by the group of *Ilan Hammel* and *Isaac Meilijson* in 2012 [J. R. Soc. Interface 2012, 9:2516-2526]. It has also been demonstrated in the exocrine, endocrine, and neuronal cells that "secretory granules are recaptured largely intact following stimulated exocytosis in cultured endocrine cells" [*Proc. Natl. Acd. Sci.* 2003, 100:2070-2075]; "single synaptic vesicles fuse transiently and successively without loss of identity" [*Nature* 2003, 423:643-647]; and "zymogen granule exocytosis is characterized by long fusion pore openings and preservation of vesicle lipid identity" [*Proc. Natl. Acd. Sci.* 2004, 101:6774-6779]. Similarly, in recent years, a great number of *porosome*-associated proteins such as chloride, calcium, and potassium channels, rabs, SNAREs, dystrophin, dynamine, NSF, heterotrimeric GTP-binding proteins, GAPs, and myosin, among others have been implicated in secretion and diseases resulting from secretory defects (37,47-62).

Membrane fusion and secretory vesicle volume regulation for fractional release of intravesicular contents from cells: In the past two decades, our laboratory has also contributed to our understanding of the fundamental molecular process involved in Ca^{+2} and SNARE-mediated membrane fusion (15-23), and on secretory vesicle volume regulation required for the regulated fractional release of intravesicular contents (24-30) via the *porosome* during cell secretion. This process enables cells to precisely regulate the discharge of a portion of their intravesicular contents during a secretory episode, while retaining full integrity of both the vesicle membrane and the cell plasma membrane.

Role of SNAREs in membrane fusion: In 1988 *Richard Scheller* discovered a secretory vesicle associated membrane protein (VAMP-1 or v-SNARE) (31), and then in 1992 he and his team discovered another important protein present in the plasma membrane called syntaxin. Syntaxin is one of the two target SNARE or t-SNARE proteins (32). Then in 1989 Professor Michael Wilson discovered SNAP-25, the other t-SNARE protein (33). Understanding the properties of the three SNARE proteins in membrane fusion

requires a molecular understanding of their interactions, with the different SNARE proteins being present in opposing membranes (VAMP-1 in secretory vesicle membrane, and syntaxin and SNAP-25 in the cell plasma membrane). Since SNAREs are membrane-associated proteins, crystals of membrane-associated SNARE complex are required for X-ray crystallography. This has not been possible due to solubility problem of such membrane proteins. To circumvent issues associated with the solubility of membrane associated SNAREs, *Axel Brunger* and *Reinhard Jahn* in 1998 truncated the hydrophobic membrane anchoring domains of syntaxin and VAMP, to obtained crystals of a non-membrane associated t-/v-SNARE complex. Utilizing X-ray crystallography, Professors Brunger and Jahn determined the atomic structure of the soluble SNARE complex at 2.4Å, which they reported in Nature (34). It was unclear however, whether the structure of the resolved soluble SNARE complex was identical to the native membrane-associated SNARE complex.

To address this issue, we carried out high-resolution AFM studies combined with electrophysiological measurements. In a study reported in the Biophysical Journal in 2002 (15), we demonstrated that in the absence of membrane association, SNAREs fail to appropriately interact with each other or establish continuity between the opposing bilayers in presence of calcium. We further demonstrated for the first time that VAMP-1 proteins present in one membrane interact with syntaxin and SNAP-25 proteins present in an opposing membrane, and assemble in a rosette or ring configuration, establishing continuity between the opposing bilayers in the presence of calcium. While it had been hypothesized that the interaction between t-SNAREs and v-SNARE present in opposing bilayers form rosette or ring structures (35), the experiments confirming this was first reported by us in the 2002 in the Biophysical Journal (15) [Fig. 5], and further established using high resolution EM (18-23).

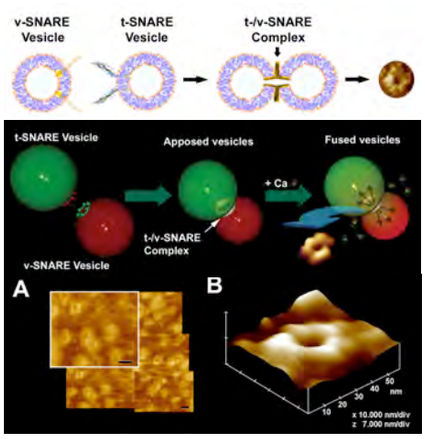

Fig. 5. Actual t-/v-SNARE ring complexes or rosettes formed following the interaction between membrane-associated t-SNAREs and v-SNAREs. Top panel is a schematic drawing depicting the interaction between t-SNAREs and v-SNAREs in opposing vesicles. AFM micrographs of the actual SNARE complex rings or rosettes are presented in the top right panel, in the middle panel, and in the right lower panel (15,16).

This SNARE rosette arrangement between opposing bilayers during membrane fusion is now widely accepted as the fundamental structure of the t-/v-SNARE complex associated with membrane fusion and cell secretion, and is widely published (36,37).

Role of Ca^{2+} in membrane fusion: In the 1970's, the late *Demetrious Papahadjopoulos* had proposed the involvement of inter-membrane Ca^{2+}-phospholipid complex in the fusion of opposing lipid membranes (38). To determine the involvement of Ca^{2+} in membrane fusion at the atomic level, Professor Jena performed X-ray diffraction studies involving t- and v-SNARE reconstituted liposomes (16). Results from this study demonstrated that SNAREs overcome the repulsive forces between the opposing negatively charged lipid membranes to bring them within a distance of 2.8Å (16). We therefore concluded that if calcium was involved in the bridging of opposing bilayers via oxygen of the phospholipid head groups, calcium must be present at the site where the t-SNARE vesicles and v-SNARE vesicles make contact. t-SNARE vesicles and v-SNARE vesicles associated in the absence of calcium, would therefore fail to establish continuity between the opposing bilayers since hydrated calcium (with 6 water molecules surrounding it) measuring nearly 7Å would be unable to fit within the 2.8Å spacing separating the two opposing membranes. In 2004, this hypothesis was tested and confirmed experimentally by our laboratory (17). From these results we further hypothesized that following bridging of the opposing phospholipids by hydrated Ca^{2+}, the loss of coordinated water associated with the calcium ion as well as those associated with the oxygen of the phospholipid head groups must result in local dehydration, lipid mixing, and membrane fusion. In collaboration with *Jeff Potoff*, we tested this hypothesis using blind molecular dynamic simulations involving dimethyl phosphate (DMP), calcium, and water molecules (14). Confirming this hypothesis, results from the study demonstrated that hydrated Ca^{2+} is capable of bridging phospholipid head groups, and that this process results in the expulsion of water from both phospholipid head groups and the calcium ion (39). The simulation further demonstrated that the distance between the anionic oxygen in DMP bridged by calcium is 2.92Å, which is in close agreement with the 2.8Å reported from X-ray diffraction measurements (16). These findings provide new insights into our understanding of the chemistry of membrane fusion in cells.

Secretory vesicle volume increase and regulated content expulsion during cell secretion: In the early 1990's it was reported that secretory vesicles undergo an increase in volume during cell secretion (40,41). However, the molecular mechanism underlying volume regulation of secretory vesicles, and the role of this volume increase on secretory vesicle function during cell secretion, was poorly understood. Studies by our laboratory showed that water channels or aquaporin in conjunction with several ion channels present at the secretory vesicle membrane regulate vesicle volume through GTP-binding G-proteins (24-30). The role of various ion channels at the secretory vesicle membrane was also demonstrated using single vesicle patch studies (42). In 2004 we reported that secretory vesicle volume increase is a requirement for the regulated release of intravesicular contents from cells (26). The relative increase in vesicle volume during cell secretion is proportional to the fraction of the intravesicular contents expelled.

In summary, studies by our laboratory in the past over 20 years demonstrate the presence of a new cup-shaped lipoprotein structure at the cell plasma membrane called *porosome*, -the universal secretory machinery in cells, and elucidated how the *porosome* is involved in the regulate fractional release of intravesicular contents from cells with exquisite precision involving membrane fusion and secretory vesicle volume regulation. We have isolated the *porosome* from a number of secretory cells including neurons, determined its composition, functionally reconstituted it in lipid membrane and live cells, and determined its dynamics and high-resolution structure using a variety of approaches including AFM, EM, and SAXS. Complementing the regulation of the *porosome* function, our studies have further contributed to our understanding of membrane fusion and secretory vesicle volume regulation, both required for the regulated fractional release of intravesicular contents during cell secretion. These results provide for the first time a molecular understanding of the regulated fractional release of intravesicular contents from cells during secretion. Recent studies by our laboratory using mass spectrometry, demonstrate interaction between the cystic fibrosis trans-membrane conductance regulator (CFTR) and the *porosome* complex in human airways epithelia, shedding light on the possible regulatory role of CFTR on the quality of mucus secretion via the *porosome* complex (43). Results from this study provide critical insights into the etiology of CF disease and for potential therapies. Similarly, our recent studies using mass spectrometry provide understanding of the lipidome of the neuronal *porosome* complex (44), and the role of Hsp90 in *porosome* assembly and function (45), has enabeled further understanding of the *porosome* structure-function (46). To determine the distribution of proteins within the *porosome* complex, our laboratory is currently engaged in single particle electron crystallography, cryoelectron microscopy, X-ray solution and neutron scattering, and chemical cross linking followed by mass spectrometry. In a recent publication (10), we report for the first time the functional reconstitution of the insulin-secretion *porosome* complex into live insulin secreting cells, resulting in an increase in the potency and efficacy of glucose-stimulated insulin release, and a promise for therapeutic applications. In the past decade, there have been scores of publication by other laboratories on the *porosome* complex and the involvement of various *porosome* proteins in secretion and in secretory defects, some referenced here (37,47-62), attesting to the critical role played by the *porosome* in health and disease.

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