UNIVERSIDAD NACIONAL AUTÓNOMA DE MÉXICO
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FACULTAD DE MEDICINA

EL NUCLÉOLO ANCESTRAL Y EL ORIGEN DEL ORGANIZADOR NUCLEOLAR

TESIS
QUE PARA OPTAR POR EL GRADO DE
DOCTOR EN CIENCIAS

PRESENTA
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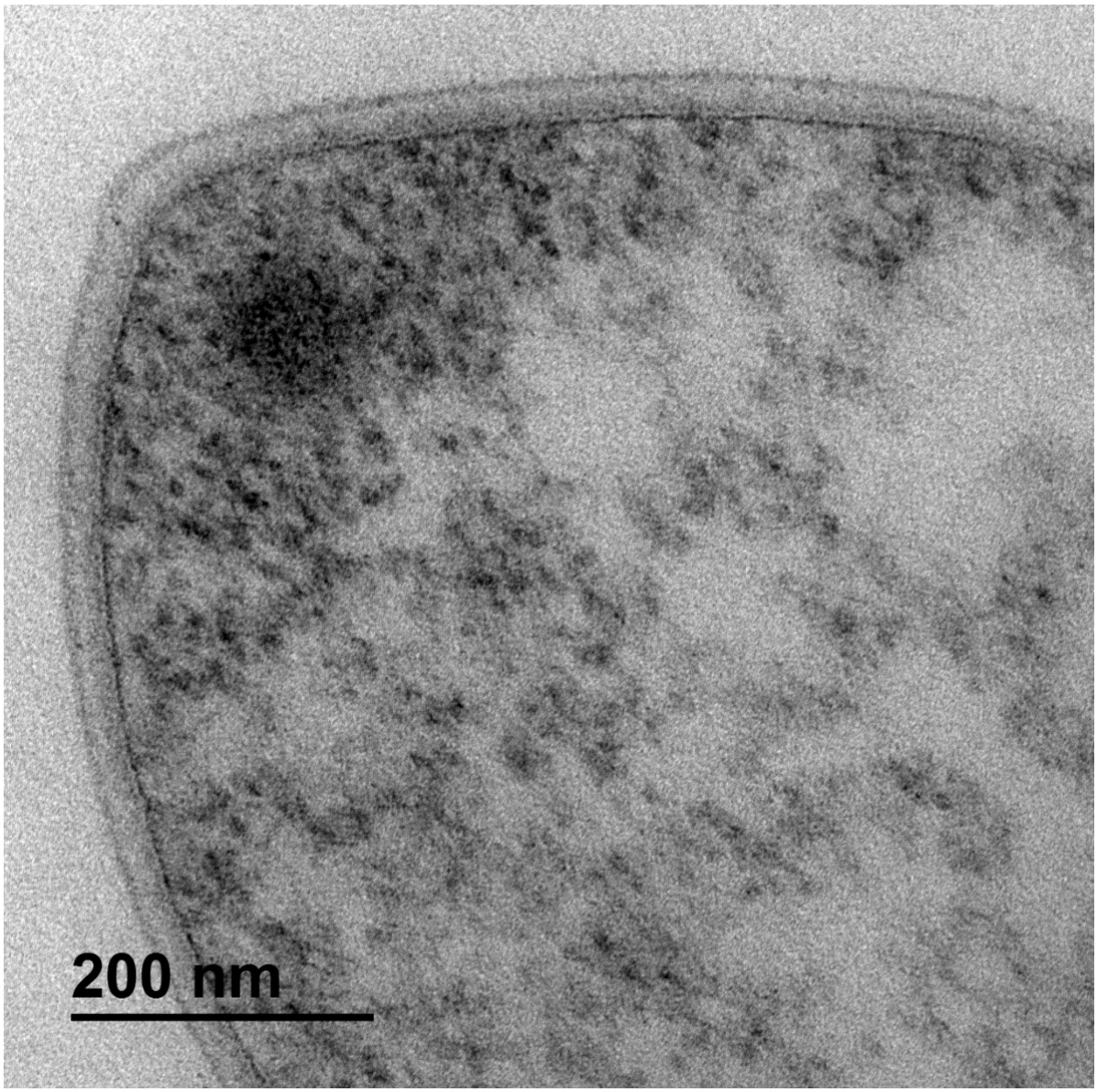

Portada: El nucléolo putativo de las arqueas: regiones fibrogranulares en S. solfataricus

A mis maestros:

desde mi madre hasta mis alumnos

Si pude ver más lejos,

fue sobre hombros de gigantes.

Newton

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CONTENIDO

Prefacio

Planteamiento del problema - Antecedentes generales - Justificación - Objetivos específicos

¿Es el nucléolo una condición *sine qua non* de las células eucariontes? Es decir, ¿son solamente los seres vivos que cuentan con un núcleo celular, que en árbol de la vida pertenecen al gran dominio Eucaria -como amebas, humanos, plantas, hongos, entre otros- los únicos con un organelo tan fascinante y enigmático como el nucléolo? ¿Por qué los procariontes - bacterias y arqueas- no habían sido descritos con este carácter celular, hasta este trabajo? ¿Por qué es fascinante el nucléolo?

Preguntas aparentemente simples, pero fundamentales para la ciencia, porque tratan sobre el origen de los eucariontes y la evolución de su arquitectura celular; acotadas al estudio del nucléolo, conforman el objeto principal para esta investigación. ¿Cual es el origen del nucléolo en el árbol de la vida?

Para comenzar, es preciso conocer al nucléolo; un objeto microscópico descrito ya hace más de 300 años, y cuya fascinación no cesa de perturbar a la ciencia. Observados por generaciones de microscopistas (Fig, 1), los nucléolos han contribuido a los grandes corolarios de la biología; desde la teoría celular, el concepto citogenético del gen, y la génesis de ribosomas; hasta las fronteras actuales de la microscopía, la superresolución, desde donde las más finas interacciones moleculares de la célula, pueden ser vistas *en vivo*. O cuando menos es nuestra aspiración.

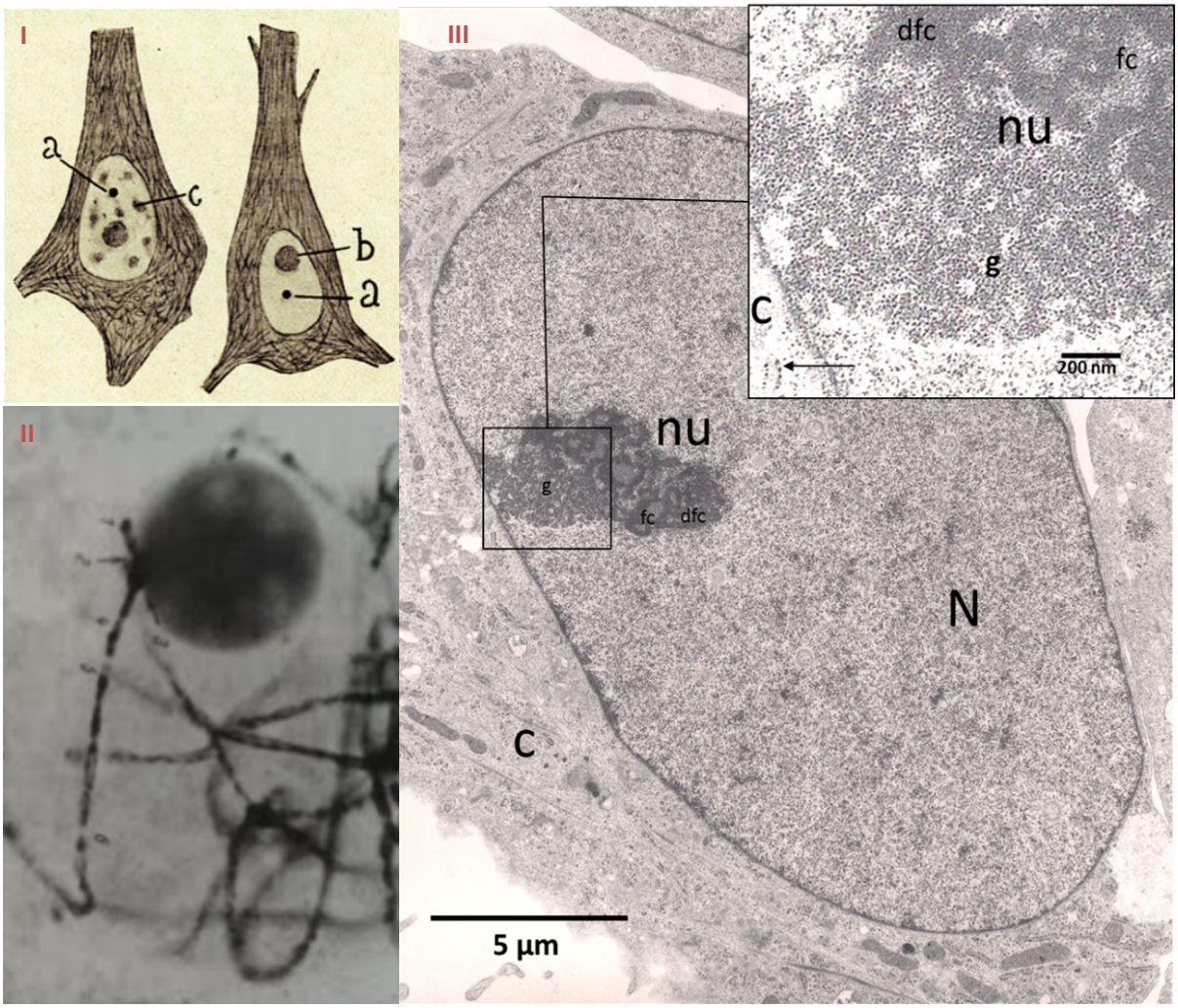


Fig. 1. Vistas del nucléolo a través de los siglos. (I) muestra el nucléolo y el cuerpo accesorio impregnados con nitrato de plata en una célula nerviosa, según una técnica de don Santiago Ramón y Cajal y don Pío del Rió Ortega. (II) muestra un organizador nucleolar en Zea Mays, unidad citogenética del nucléolo y la transcripción ribosómica, según la eminente nobel Barbara McClintock. (III) muestra un magnífico nucléolo del hígado de la rata canguro con sus componentes granulares y fibrosos, develado por el haz de la microscopía electrónica de transmisión.

A pesar de todo, los nucléolos son poco estudiados, desde otra gran corriente del pensamiento biológico: la evolución. Por la curiosidad y por la oportunidad que representan, esta investigación se aventuró a: observar, conocer, entender al nucléolo más allá de los modelos clásicos eucariontes; y proponer desde la diversidad nuevos descubrimientos nucleolares, relacionados con una inquietud fundamental para la humanidad: el origen y evolución de la vida.

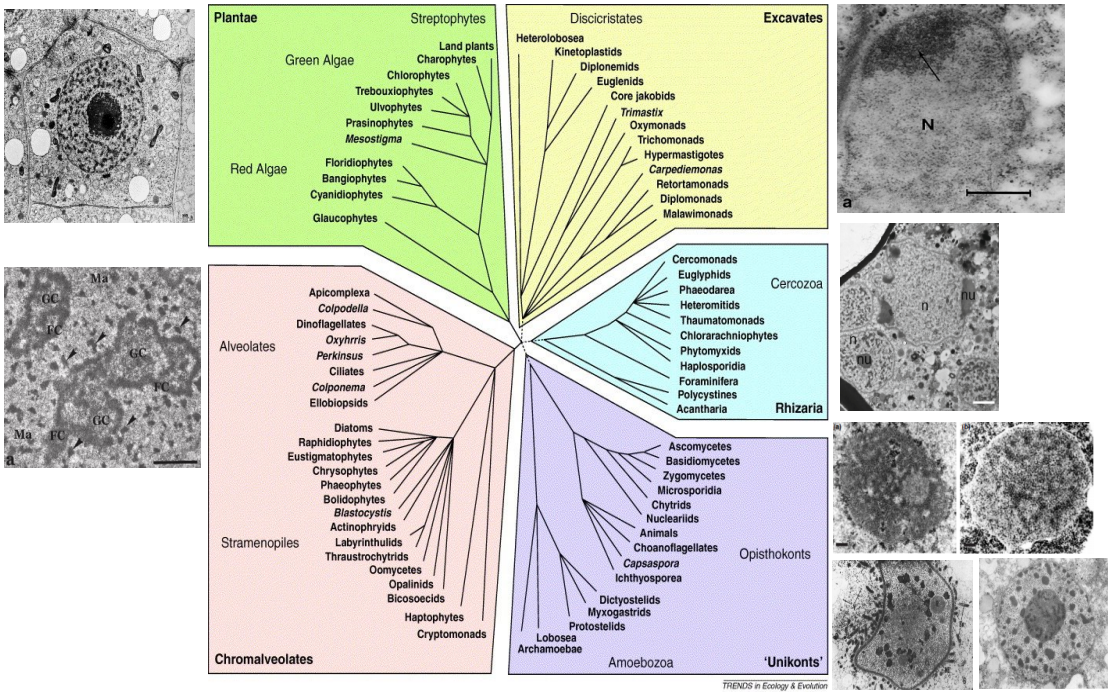


Fig. 2. La diversidad morfológica de los nucléolos en los grupos corona del dominio Eucaria. Como se muestra la filogenia de los eucariontes no ha sido resuelta aún y con ello la dificultad de establecer el primer nucléolo eucarionte. (Fuente: elaboración propia, 2020)

Para entrar en materia, pensemos primero: que el nucléolo es más que un organelo inquilino del núcleo celular. El nucléolo es un mundo nanoscópico, de estructuras y funciones; y se compone de ribonucleoproteínas, es decir de la interacción entre genes, proteínas y RNAs. Visto a través de los microscopios, es la estructura nanométrica donde ocurre la mayor actividad transcripcional de las células eucariontes; verbigracia, en el nucléolo sucede la transcripción de los genes ribosómicos y su ulterior maduración en precursores del ribosoma. Estos últimos, son la maquinaria molecular que la célula dispone, para sintetizar todas las proteínas con que funciona la bioquímica de la vida. El nucléolo no termina en la biosíntesis de ribosomas como antaño se ha dicho. Su naturaleza plurifuncional se caracteriza porque es el encargado de orquestar finamente el mantenimiento de la homeostasis celular, regulando la biogénesis de ribosomas a merced del estímulo de otros fenómenos y vías de señalización interesantísimos, como: el envejecimiento, el estrés, la proliferación, la diferenciación, el desarrollo y la muerte. En términos metafóricos, el nucléolo es un guardián de la homeostasis celular. Sus múltiples funciones convergen en la regulación de la biosíntesis de ribosomas; función indispensable, ya que, sin ribosomas el flujo de información paradigmático entre DNA-RNA-Proteínas, no es posible.

Organelo central del universo celular; es meritorio también pensar al nucléolo desde otras perspectivas más curiosas. Por ejemplo, al observar al microscopio manchas electrodensas, fibrosas, granulares, al fin nucléolos; las imágenes pueden

trascender el concepto convencional de compartimento celular electrodenso. ¿Qué momento ha sido, realmente captado por la instantánea de una micrografía? Tal vez, las manchas de electrones esconden la visión de un *puff* genético al alcance de nuestros ojos; y entonces, ver la manifestación física de los genes ribosómicos expresándose se convierte en un acto fascinante; un detonador de la creatividad con que pensamos nuevas teorías y generalizaciones para nuestras, frecuentemente abstractas observaciones. Ya sea para conocer sobre su naturaleza ultraestructural y funcional, o para indagar un poco sobre su origen, el nucléolo ha sido observado extensamente en organismos modelo y también ha sido descrito en una vasta diversidad de especies a lo largo de tres siglos de investigaciones. Así, una revelación importante es cierto que el nucléolo, no representa sólo un evento citogenético, sino un carácter biológico, que es constante y está bien conservado en la evolución: Por tanto, no sólo es una maravilla microscópica que conecta la dimensión de los genes con la de los organelos, sin un objeto natural de la evolución donde convergen la estructura con el proceso; la forma con la función.

Poco sabemos aún sobre el origen del enigmático nucléolo, a pesar de la vasta evidencia de observaciones cuyo significado es a veces difícil de explicar. La figura 2, muestra, por ejemplo, la gran diversidad de morfologías nucleolares en los mayores grupos de los eucariontes. Aunque el nucléolo es evidente en todos, no es posible identificar un arquetipo específico, y en la mayoría de los casos el estudio no ha ido más allá de una aproximación morfológica a la ultraestructura del nucléolo humano.

Pero el principal reto para descifrar el origen del nucléolo es que la filogenia de los eucariontes no está resuelta en cuanto al parentesco de los grupos corona. Por eso la tarea de relacionar al primer nucléolo eucarionte con un linaje en particular, permanece fuera de nuestro alcance.

No obstante, han ocurrido avances interesantes. Entre ellos, los estudios nucleolares, que inspiraron este trabajo. El descubrimiento microscópico del nucléolo del parásito *Giardia lamblia* en 2006 en México, cambió un paradigma de décadas y estableció las bases que guían la disección de nucléolos putativos en organismos no modelo. Previamente, *G. lamblia* fue considerado por décadas una excepción de la naturaleza; un eucarionte sin nucléolo carente de explicación. Sin embargo, cuando a tesón de una intensa verificación microscópica, el nucleolo de giardia fue confirmado, la conclusión evolutiva fue clara. El nucléolo es una sinapomorfía en el árbol de los eucariontes y posiblemente una propiedad de su último ancestro común (LECA).

Por otro lado, las preguntas también evolucionaron: ¿cuál es el nucléolo mínimo, arquetípico o ancestral? ¿Acaso el de *G. lamblia* o quizá, el de otro protista?

El organizador nucleolar (NOR) puede ser un punto de partida para estas preguntas (véase capítulo II). El NOR permite entender el desarrollo del nucléolo o nucléologenesis durante el ciclo celular; es decir, como se forma un nucléolo *de novo*. En este concepto descrito por McClintock y Giménez-Martin en animales y plantas, respectivamente, el NOR es un dominio cromosómico; un locus, donde inicia la formación del nucléolo, a partir de la reiniciación de la trascripción del rDNA; y el

reclutamiento de cuerpos pre-nucleolares, precisamente durante la anafase mitótica. Consecuentemente y como se explica en el capítulo II, resulta lógico comenzar por buscar el NOR o sus elementos si se indaga la evolución del nucléolo en el árbol de la vida. Pero hay una pregunta infranqueable. ¿Dónde comenzar a buscar? ¿En qué grupo de organismos se originó?

Por eso, resulta preciso revisar los trabajos, comentados en esta tesis, sobre la homología de proteínas nucleolares en diversos linajes de todo el árbol de la vida, así como las nuevas y reveladoras relaciones filogenéticas entre eucariontes, arqueas y bacterias.

A la fecha, es bien conocido que muchos elementos del nucléolo, como la fibrilarina y los RNA´s pequeños nucleolares (snoRNA`s) cuentan con homólogos, no sólo en todos los eucariontes, sino particularmente en las arqueas. En esta tesis, se hizo una revisión exhaustiva de esta evidencia, para entender los alcances y limitaciones de nuestra investigación. Quizá tan amplia como un *Gedankenexperiment* sobre el nucléolo ancestral en la tierra primitiva, nuestra propuesta dista de sólo teorizar, sobre el surgimiento del nucléolo en estas arqueas (capítulo I), sino literalmente estriba en observar y fundamentar experimentalmente.

Para esto, fue necesario proponer una plataforma disciplinar (capítulo II), que permitiese integrar herramientas experimentales y evolutivas, para definir objetivos y preguntas específicas. Dicha plataforma es una disciplina reciente llamada Biología Celular Evolutiva (ECB). ¡Cuán importante ha sido entender y aprender a pensar desde

los corolarios de la ECB! De esta manera nuestro planteamiento adquirió lógica. Si después del descubrimiento en Giardia, el nucléolo está presente en el último ancestro común de los eucariontes (LECA), entonces con herramientas similares es posible buscar sus elementos en otros linajes emparentados con los eucariotas, como las arqueas.

Las arqueas del superphylum TACK, son por ejemplo un grupo hermano de los eucariontes, según las más recientes propuestas de filogenia y proveen una propuesta de filogenia resuelta para eucariontes y arqueas. Así, no sólo es claro dónde empezar a buscar sino también es posible indagar, si el nucléolo es atribuible a un ancestro común arqueano o si su origen es idiosincráticamente eucarionte. En cuyo caso se encontraría en algún punto de la evolución entre el primer ancestro común de los eucariontes (FECA) y el último (LECA). Como se muestra en la figura 3, encontrar el nucléolo en algún linaje de TACK-Archaea, brinda elementos experimentales y teóricos para concluir sobre su presencia el LETackA, último ancestro común de Eukarya y el susodicho phylum de arqueas. En este sentido, la metodología desarrollada en el capítulo II propone una experimentación *in situ,* guiada por filogenias para la construcción de una narrativa histórica sobre la evolución del nucléolo.

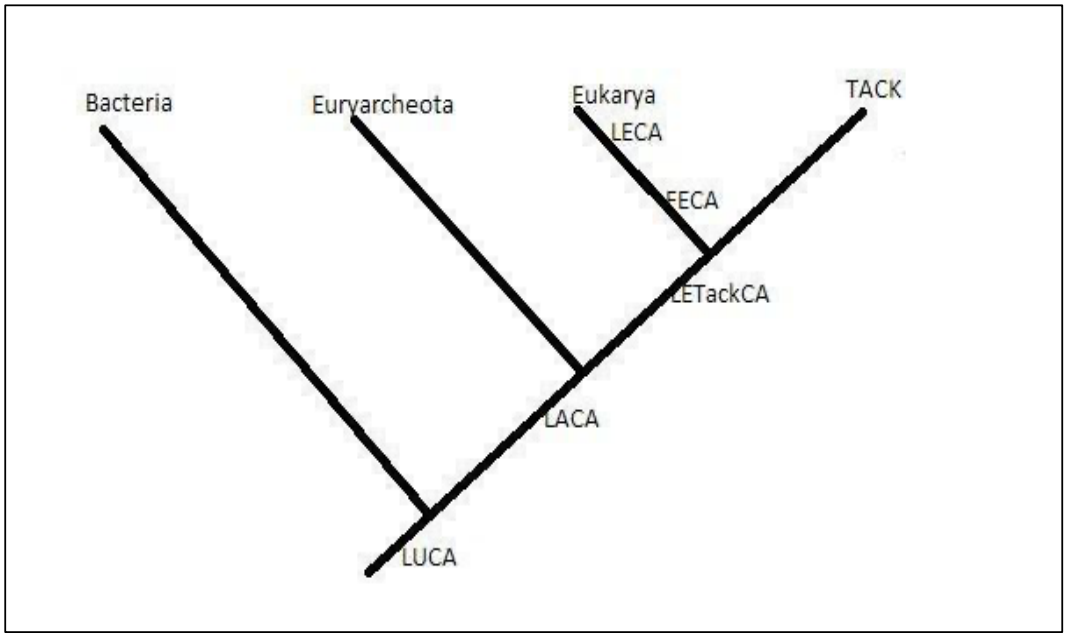


Fig. 3 Un árbol de la vida de dos dominios: Bacteria y Arquea incluyendo a Eucaria como grupo monofilético. El nucléolo ancestral podría encontrarse en cualquiera de los ancestros comunes: LECA, LETackCA, LACA o incluso LUCA. La consecuencia lógica es que, si al menos está presente en representantes de TACK, entonces ya estaba presente en LETackCA y por tanto en FECA. (Fuente: elaboración propia)

Siguiendo esta lógica, la pregunta central del proyecto de investigación versó:

¿Existen elementos nucleolares microscópicos y moleculares en algún representante de TACK-Archaea, que sugieren un origen del nucléolo anterior a FECA y por tanto su presencia en el último ancestro común de eucariontes y TACK-Arquea?

Ulteriormente, se derivaron tres objetivos específicos, que corresponden a los tres capítulos de esta tesis:

1. Conocer el estado del arte sobre el nucléolo y el origen de los eucariontes para contextualizar la importancia de esta investigación en el devenir científico.
2. Establecer una fundamentación teórica adecuada para estudiar el origen del nucléolo con perspectiva transdisciplinaria orientada tanto a la biología celular como a la biología evolutiva
3. Abordar experimentalmente si el nucléolo existe o no existe en arqueas.

Resultados y descripción de los capítulos

El proceso de investigación y los resultados se presentan a continuación en tres artículos compilados a manera de capítulos.

1) El primer capítulo es una revisión exhaustiva sobre el origen de los eucariontes como una de las mayores transiciones de la evolución. Aquí se resumen las diversas corrientes de pensamiento y teorías sobre el origen de los organelos eucariontes y se contextualiza al nucléolo como un objeto de estudio importante en los programas de investigación sobre el origen y evolución temprana de la vida. Este trabajo fue desarrollado como parte de una actividad académica del programa de doctorado, revisado por profesores acreditados del programa, y posteriormente fue publicado bajo el título **"On the ideas of the origin of eukaryotes: a critical review"** en el repositorio de libre acceso, "arXiv Quantitative Biology", de la Universidad de Cornell.

2) El segundo capítulo es un artículo original publicado en "Journal of Biosciences" intitulado, **"Evolutionary Cell Biology (ECB): lessons, challenges and opportunities for the integrative study of cell evolution".** En este trabajo los autores propusimos una manera original de estudiar el origen y la evolución del nucléolo de forma interdisciplinaria; partiendo de las premisas disciplinares del nuevo campo de la biología celular evolutiva. Así, planteamos la disección ultraestructural *in situ* de estructuras putativamente homólogas a organelos conocidos, en linajes inexplorados,

con el fin de esclarecer escenarios evolutivos relativos a la complejidad celular, basados en filogenias resueltas y premisas sobre ancestros comunes.

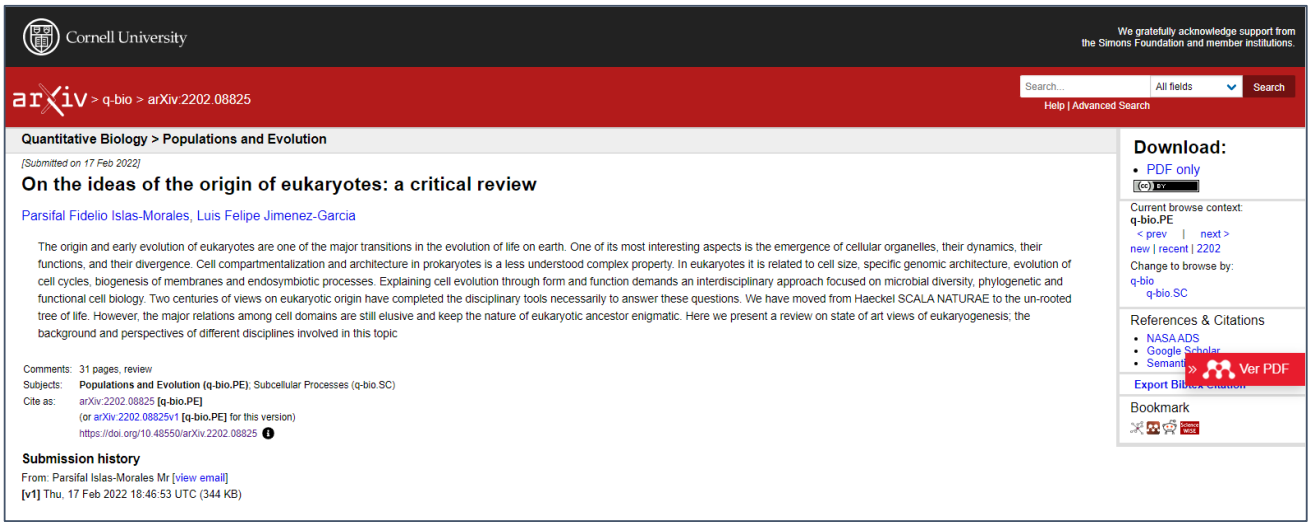
3) El tercer capítulo es un artículo de investigación original intitulado **"Ultrastructural and proteomic evidence for the presence of a putative nucleolus in an Archaeon**", publicado en la revista *Frontiers in Microbiology*. Este trabajo corresponde a la parte experimental de la investigación doctoral, poniendo en práctica las premisas y metodologías de los capítulos anteriores. Como trabajo integrador, presenta el resultado de que en *Saccharolobus solfactarius comb. nov. syn. Sulfolobus solfatatricus*, hemos constatado la presencia de estructuras nucleolares; elaborando sobre la relevancia de este descubrimiento para la biología, destacando por un lado: 1) la verificación de premisas sobre el origen del nucléolo en el ancestro común de eucariontes y TACK-Archaea; y 2) la relevancia de pensar en el nucléolo bajo un concepto de organizador nanoscópico entre el universo de los genes y el de la arquitectura celular. Un ensayo final resume las perspectivas generales y conclusiones más audaces de esta tesis doctoral.

Capítulo I

De las ideas sobre el origen de los eucariontes

Artículo de revisión

Islas-Morales, P.F. & Jiménez-García, L.F., *On the ideas of the origin of eukaryotes: a critical review*, 2022, arXiv:2202.08825 **[q-bio.PE]**



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[Submitted on 17 Feb 2022]

On the ideas of the origin of eukaryotes: a critical review

Parsifal Fidelio Islas-Morales, Luis Felipe Jimenez-Garcia

The origin and early evolution of eukaryotes are one of the major transitions in the evolution of life on earth. One of its most interesting aspects is the emergence of cellular organelles, their dynamics, their functions, and their divergence. Cell compartmentalization and architecture in prokaryotes is a less understood complex property. In eukaryotes it is related to cell size, specific genomic architecture, evolution of cell cycles, biogenesis of membranes and endosymbiotic processes. Explaining cell evolution through form and function demands an interdisciplinary approach focused on microbial diversity, phylogenetic and functional cell biology. Two centuries of views on eukaryotic origin have completed the disciplinary tools necessarily to answer these questions. We have moved from Haeckel SCALA NATURAE to the un-rooted tree of life. However, the major relations among cell domains are still elusive and keep the nature of eukaryotic ancestor enigmatic. Here we present a review on state of art views of eukaryogenesis; the background and perspectives of different disciplines involved in this topic

Comments: 31 pages, review

Subjects: **Populations and Evolution (q-bio.PE)**; Subcellular Processes (q-bio.SC)

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On the ideas of the origin of eukaryotes: a critical review

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Abstract

The origin and early evolution of eukaryotes is one of the major transitions in the evolution of life on earth (Smith and Szathamary, 1995). One of its most interesting aspects is the emergence of cellular organelles, their dynamics, their functions, and their divergence. Cell compartmentalization and architecture in prokaryotes is a less understood complex property. In eukaryotes it is related to cell size, specific genomic architecture, evolution of cell cycles, biogenesis of membranes and endosymbiotic processes. Explaining cell evolution through form and function demands an interdisciplinary approach focused on microbial diversity, phylogenetic and functional cell biology. Two centuries of views on eukaryotic origin have completed the disciplinary tools necessarily to answer these questions. We have moved from Haeckel's *SCALA NATURAE* to the un-rooted tree of life. However the major relations among cell domains are still elusive and keep the nature of eukaryotic ancestor enigmatic. Here I present a review on state of art views of eucaryogenesis; the background and perspectives of different disciplines involved in this topic

Contents

Ideas in Eukaryogenesis

Historically, there have been two reductionist perspectives: 1) one that reconstruct the history of organelles based on their biology and build up a hypothetical scenario to fit into *a priori* assumptions, and from which contradictions have arisen when trying to fit individual organelle origin into a wider model. Saltationst scenarios as those proposed by endosymbiosis contrast with gradualist scenarios as those proposed by archetypal hypothesis 2) the second perspective reconstructs the history of eukaryotic diversity based on phylogenetic hypothesis as a general picture. The disadvantages of phylogenetic algorithms are common to this kind of approach. Here we will try to contrast both perspectives into an integral view of the state of art of eukaryogensis and its disciplinary perspectives.

Palaeobiological perspectives

A usual tool in phylogenetic reconstruction is the use of fossil record to calibrate relaxed molecular clocks and topologies (Roger & Hug, 2006; Katz, 2012). This approach can led to a better picture of diversification times and coherence in diversification patterns. Among the resolved division between unikonts and bikonts, major lineages such as Excavata, Archeaplastida, Opistokonta, Amebozoa and SAR clade reveal strong basal divergence (Adl *et al.,* 2012). However phylogenetic relations and divergence sequence have not been resolved. The question about date and historic narrative of the crown group of eukaryotes can contrast with the many hypotheses about origin of eukarytoes (Knoll, 2014).

The fossil record can support radiation and diversification patterns, for example known eukaryotic diversification of certain lineages during the Phanerozoic. Crowngroup diversification should be traced back to the Neoproterozoic. The Proterozoic (542-2500 Mya) and the Archean (2500-4567 Mya) might have evidence on the nature of eukaryotic cells and the kind of environment; eukaryotic first diversification took place (Knoll, 2014). Cell like fossils have been found as vesicles in 3500 Mya old rocks in the Archean (Javeaux, 2011; Butterfield, 2004). Bangiomorpha, a Rodophyte like, has been dated

between 1100-1200Mya (Butterfied 2000). Laminar wall structure attributed to chlorophytes have been dated in mid-Proterozoic strata (Javeaux, 20004). Shells are also abundant in 1600 Mya sediments, however lack of ornamental diagnostic structures. Silica and calk tests of putative amoeba and simple foraminifera are found in the mid Neoproterozoic (Porter & Knoll, 2000). Nodules and vessels comparable to extant chrisophytes are dated 800Mya (Allison & Hilgert, 1986), before Ediacarian radiation. Most putative eukaryotes are difficult to characterize due to the lack of diagnostic characters. Javaux has established general identification criteria: size, complex ultrastructure and thickness of walls and shells (Jauveax, 2003).

Complementary to marginal morphologies, molecular fossils are a tool for paleobiologists. Steranes, derivate forms of sterols, are hallmarks of possible eukaryotic sterol biosynthesis (Pawlowska *et al.,* 2013). Methanogenic activities can be traced to 2700 Mya, suggesting that eocytes hypothesis can be drawn no later than 2700 Mya (Hayes, 1994; Knoll 2014).

Gradualistic and endosymbiotic scenarios are challenged since sterol synthesis in the Archean could just take place at nanomolar levels (Holland, 2006). Steranes register from the Archean are rare and can be due to contamination (Dutkiewicz *et al.,* 2006). Chronic increase of oxygen in ocean water is fond until de Proterozoic and supported by the divergence time of mitochondrial genes for aerobic respiration. Plastid acquisition can be traced before 1300 Mya. Chronic oxygenation can be due to cyanobacteria photosynthesis on microbial mats (Ambar *et al.,* 2007).

A comparison between early eukaryotic diversification and Ediacarian fauna and Cambiran carnivory has been proposed (Gingras *et al.,* 2011, Erwin *et al.,* 2011). Evolution of phagotrophy could had triggered the process of early diversification (Porter, 2011). Phagotrophy other than predation of little bacteria is found dispersive in several eukaryotic lineages suggesting that ancestral lineages evolved due to phagotrophic selective pressures. Fossils of ciliates, dinoflagelates and amoebas with protective armor support this scenario where size gain or aggregation could have been an adaptation against phagotrophy (Cohen & Knoll, 2012). Some authors even place eukaryogensis at the in the origin of multicellularity (Erwin *et al.,* 2011).

Theoretical perspectives

Endosymbiotic theory

In 1979 Lynn Margulis, based on the ideas of Mereschowski, proposed a theory on the endosymbiotic origin of mitochondria and undulipodia. A mutualistic relationship between two bacteria would lead to the total incorporation of both functions in a single cell. Coevolution and symbiosis became the basis to say that the origin of eukaryotes was chimeric and is in essence polyphyletic (Margulis & Bermudez,1984; Sapp, 2004)..

In 1990 Carl Woose proposed the so-called "standard model" where archaea are related to eukaryotes (Woese *et al*., 1990). The one gene (16S SSU) phylogeny by Woose provided a cue for the nature of the putative symbiotic partner of ancestral mitochondria: the archaea (Pace, 2006).

In same decade, the mitochondria´s origin was found within the lineage of the α-proteobacteria, as genomic alignments of the small ribosomal subunit revealed (Sapp, 2004). During coevolution, most metabolic and structural genes have disappeared from mitochondrial genome, so alotopic expression of their sequences in the nucleus became a condition that enabled form and function of these organelles (Gonzalez-Halpen *et al*., 2003. Mitochondrial ancestry was found to differ in various eukaryotic lineages suggesting that endosymbiosis could occur often in the course of evolution (Degli Esposti, 2014). Eukaryotic features were a byproduct of symbiotic co-evolution, however early endosymbiotic theory could not show evidence for this (Lang *et al*., 1999).

The Archeozoa hypothesis

In contrast to endosymbiotic theory, Thomas Cavalier-Smith proposed a gradualist view of the origin of eukaryotes in 1987. He emphasized on the structural and physiological constrains that would have been necessary for endosymbiosis: such as phagocytosis, cytoskeleton. He noted also on the lack of a logical, evidence based explanation for an

endosymbiotic origin of the cell nucleus. For Cavalier-Smith gradual evolution of ancestral forms is a need before endosymbiosis, which he does not, held as the main driver of eukaryogenesis.

The archeozoa hypothesis considers the engulfment of α-protobacteria by a protoeukaryotic ancestor, already capable of phagocytosis (Cavalier-Smith, 2009, Poole and Penny 1991). Eukaryotic features evolved not as consequence of mitochondrial pressures of selection, but as idiosyncratic characters. Eukaryotes evolved from a common ancestor within bacteria. The Neomuran ancestor proposed by Smith is related to extant endobacteria and was the ancestor of wall-less actinobacteria. This organism developed phagotrophy, endomembranes, mitosis, sex, nucleus, and cilium early before mitochondria were integrated by endosymbiosis (Cavalier Smith, 2001).

Cavalier Smith idea relies on cell biology, micropaleontology, and comparative protozoology. Extant organisms as *Pelomyxa palustris* and further amitochondriate Excavate were thought to be possible archeozoa living fossils (Cavalier Smith, 1991). *P. palustris* does indeed lack of mitochondria but replace them with enosymbiotic bacteria, furthermore endoplasmic reticula are absent. *G. lambia* also lack mitochondria and together with *Entamoeba hystolitica* were to be thought as basal eukaryotes. In the later years, hidrogenosomes, mitosomes were found in amitochondriates. This organelles harbor genetic hallmarks of secondary mitochondrial loss as demonstrated in *Gardia lambia* and *E. histolytica* (Dyall & Johnson 2000; Clark & Roger, 1995). Archeozoa hypothesis was partially dismissed as the idea of ancestral eukaryotes among extant representatives of amoeba and excavates could not be proved.

Endosymbiotic and archeozoa theory differed on the chronology of endosymbiotic events and thus the importance of endosymbiosis as a driver of organelle origin. They contrasted a co-evolutionary perspective against an idiosyncratic scenario.

The chimeric origin of the nucleus

The late work of Margulies proposed that the nucleus arose before the mitochondrial endosymbiosis as the result of symbiosis between an archaeal thermoplamsa (eocyte) and a eubacterial spirochete (Margullis *et al.,* 2010). Eocyte, spirochete and sulfur globules developed a syntrophic strategy as adaptive trait. The microbial community, *Thiodendron latens,* offers an extant example of such a consortium where sulfate reduction is provided as source of electron acceptors for spirochetes that provide for efficient carbohydrate metabolism or for endosymbiotic motility related benefits (Dubinina *et al.,* 2004). DNA exchange must have taken place. Vesicle formation due to membrane hypertrophies can take place as seen in *Gemmata obscuriglobis* (Lindsay *et al.,* 2001). This combination gave rise to the cytological structure known as karimastigont as direct ancestral state of the later eukaryotic nucleus. This structure converted into primitive microtubuli organizing center. Afterwards nuclei separated from MTOC and toke a central position in the cell, as seen in many early branching protest (parabasalids and hipermastiginds) (Margullis *et al.,* 2010). The extant ciliate *Mixotrichia paradoxa* harbor thousand of anchored spirochetes for motility purposes (Wenzel *et al.,* 2003).

Viral Eukaryogensis

The lack of transitional organisms between prokaryotes and eukaryotes has driven to speculative approaches additionally to the efforts of the genomic era. The viral eukaryogensis hypothesis was proposed by John Bell in 2001 based on a consortium theory where archaea, bacteria and complex DNA virus form a gradual symbiosis which results in the chimeric nature of eukaryotic cell (Bell, 2001). The main concerns of viral eukaryogensis are the gap between complexity in the eukaryotic cell cycle and genome architecture; and the prokaryotic features (Bell, 2009).

The hypothesis is based on a theoretical experiment on biological actualism. If the origin of eukaryotes took place in a prokaryotic world, then the features or a chimeric ancestor of

eukaryotes can be found in extant representatives of prokaryotic domains, including viri. Membrane and cytoplasm as known in eukaryotes are proposed to origin from archaeal wall-less methanogens as *M. eliziabethi* (Rose & Pirt, 1981). The syntrophic nature of methanogen in microbial consortia opens the possibility for them to establish symbiotic relation with other bacteria (Bell, 2009). This idea is consistent also with the argument in favor for an Archaean origin of the cytoskeleton and the membrane remodeling complex, despite these implications have not been discussed extended in the context of viral eukaryogensis (Koonin, *et al.,* 2006).

The most innovative element of viral eukaryogensis is the suggestion of the nucleus originated from a complex DNA virus. There is certainly molecular, cytological, and biochemical basis that argue for the genome a physiological complexity of DNA viri; especially giant viri form the NCLDV group (Villareal *et al.,* 2000; Bell, 2009). Mimiviri, pandoraviri and poxviri have been found capable of double strand DNA replication inside their capsid. Thus, their replication mechanism, in hyperpolyploid hosts, is the generation a viral factory. RNA capping is also a feature of NCLDV viri, suggesting that posttranscriptional modifications could have started in viral factories (Raoult *et al*., 2004; Koonin & Yutin, 2010).

In *Acantoamoeba polphaga:* mimiviri, rickettsias, nuclei and mitochondria coexist. Taking this as inspiration, a consortium of wall-less archaea, α-protobacteria and large DNA viri is plausible (Horn & Wagner, 2004). Giant viri normally set up to lysogenic phase as this ensure the protection their DNA within the host and coordinated propagation. Bacteriophage P1 like virus and recently described archaeal viri (Forterre. 2012) can be considered triggers of membrane evolution (Bell, 2013). Bacteriophages develop vesicles from bacterial membrane invagination, and viral factories need of vesicle formation to build new membrane bound capsids (Karhu *et al,* 2007). In this hypothesis viral factories also may have overtaken the protagonist role of protecting DNA. Giant viri acquire genome complexity through alotopic transfer of host genes. Fine gene regulation inside virus membrane could have offered an advantage in comparison to free swimming chromatin (Bell, 2013).

The conclusive remark of viral eukaryogensis is that it proposes a model of evolution of mitosis and sex in early eukaryotes. Mitosis can arise from mechanisms for chromosome *btw.* plasmid´s segregation. Conjugation could have arisen as a process triggered by predation via phagocytosis. Viral nuclei with certain genetic signatures can exchange information. Finally, meiosis and sex can be seen as consequences of errors in control mechanism for ploidy, and advantage for improving genetic diversity within the populations (Bell, 2006).

The communities and ecosystem approach

The ecosystem approach has been poorly integrated to the state of art of eukaryotic origins because of our ignorance on the nature of Proterozoic micro-ecologies. Paleobiology and astrobiology as scientific programs have given cues to ecosystem physical conditions and main ecological triggers in early evolution.

Recently some theoretical approaches to complex systems and communities' behavior have been argued towards an ecosystem first theory of the origin of life and eukaryogensis. Norris and Root-Bernstein propose that the eukaryotic cell originate from the integration of hyperstrucutres in prokaryotic cells based on the principle of molecular complementarity (Norris & Root-Bernstein, 2009). For them, eukaryotic features are the result of a process of progressive lost through selection of efficient ecologies, rather than individual traits. Systems theory establish that the elements of a complex system evolve in the context of a common ecology (Hunding *et al.,* 2006). Furthermore, ecologies give rise to new ecologies. Departing from the ontological question that the unrooted tree of life sets: ¿What was the nature of LUCA? The authors argue on a scenario were the evolution of molecular ecologies give rise to highly complex protocells *btw.* protecosystems. Later, a progressive loss of complexity gives rise to individual cells (Norris *et al.,* 2007). The origin of eukaryotes can be traced to their emancipation from complex molecular ecosystems (Norris & Root-Bernstein, 2009).

The Bioenergetics based scenarios

Metabolism based hypothesis suggest that Eukaryotes arose from the invasion of an archaeon by a protobacteria (Martin & Müller. 1998; Rivera & Lake, 2004; (López-García & Moreira, 1998). This could have been the start point for metabolic selection pressure that conformed either metabolic compartmentalization or DNA isolation through the nucleus. Following hypothesis argue in favor of this with consequences on the context of accepted phylogenetic inferences.

Quantitative approaches

Bioenergetics hypotheses argue that endosymbiotic events provided new orders of magnitude of energetic demand (Lane & Martin, 2010). In words of Nick Lane an important energetics divide is fond between prokaryote and eukaryotes. The last expanded their genome, expression and metabolic surface over 200,000-fold. The hypothesis is that endosymbiosis with facultative anaerobic α-proteobacteria resulted in genomic asymmetry by accumulation of bacterial genomes and metabolic activity. The demand of increased space and gene expression was solved by mitochondrial coordination that ensures form expanded metabolic surface (Lane, 2011). Examples for expanded metabolic surface can be seen in bacterial gigantism. Those organisms are hyperpolyploid and solve genetic asymmetry through metabolic membrane sustainability (Soppa, 2014).

The hydrogen hypothesis (Martin & Müller. 1998)

In the hydrogen hypothesis, eukaryotes are a result of spontaneous symbiosis between a euryarchaeal methanogen and facultative anaerobic eubacteria. It is also a syntrophic relation were first anaerobic respiration in eubacteria produces hydrogen, acetate and CO_2 as products which the archaeon uses for methanogenesis. In potentially anoxigenic media such as the reducing atmosphere of the Archean, dependence between the two types could lead to engulfment of the bacteria by the wall-less euryarchaeota. Endosymbionts could have kept genes for aerobic respiration, leading to different kinds of eukaryotes depending on adaptive radiation triggered by oxygen availability. Amitochondriate with mitoses result

in fermentations, amitochondriate with hydrogenosomes result from reversion to PDH metabolism (Martin & Müller. 1998). Main disadvantage of hydrogen hypothesis is that current phylogenies tend to dismiss the idea of euryarchaeal origin.

The syntrophic hypothesis

The syntrophic hypothesis proposes that euryarchaeal methanogens coexist with δ-proteobacteria (ancient myxobacteria) with whom they enter a primary endosymbiosis in the Archean eon (Moreira & López-García, 1998). Fermentative sulfate reducing δ-proteobacteria provided the methanogen the selection pressure for building up sulfate reduction metabolic compartments. As the result of this endomembrane, probably nucleus arose. α-proteobacteria endosymbiosis was secondary and led to evolution of mitochondria after oxygenation of oceans in the Proterozoic (López-García & Moreira, 2006).

The eocyte hypothesis

The eocyte hypothesis was proposed by Lake as phylogenetic explanation of eukaryogenesis in accordance with the standard model of Woose. If ribosomal sequences place an un-rooted tree domain tree of life, Eukaryotes should have evolved from extant Crenarchetoa (Eocytes) as they share most conserved genes (Rivera and Lake 2004). The nature of this evolution was elusive. Eukaryotes could be rooted within the Crenarchaea or in a discontinuous scenario, endosymbiotic events happened to members of Crenarchaea (Eocyte) which gradually converted into modern eukaryotes. Phylogenetic technical advances such as models that reduce long branching attractions and fast evolving artifacts were considered to this hypothesis (Cox *et al.,* 2008). However, the phylogenies resulted in elusive polytomies. Archaea groups were not enough to provide strong signal and solve the phylogeny, which remained enigmatic.

From Carl Woose´s standard model to the comparative genomic diversity of Archaea

Improvement of phylogenetic studies took place in the following years by concatenation of highly conserved proteins, comparison of phyletic patterns, and comparison of protein domain architectures. They showed that eukaryotes harbor bacterial and archaeal homologues in addition to idiosyncratic genes. The identification of these genes and domains helped to understand their story as products of lateral gene transfer or vertical inheritance and relate it to their function. Bacterial homologues in eukaryotes correspond to functional operational characters and bioenergetics processes, while archaeal homologs correspond to fundamental information flow and housekeeping tasks (Koonin, 2015; Cox *et al.,* 2008). Despite efforts in phylogenetic discrete topologies between Eukarya and Archaea were elusive until the 2010. Vellai argued that, according to the standard model, the ancestral eukaryotes are placed outside the extant archaeal diversity (Vellai *et al*., 1998). This panorama was close to change with novel scientific endeavors. Namely the discovery of 5 new phyla within the archaea in less than 10 years

Archaea diversity: new groups, new topologies.

Already in 2007, extensive studies suggested sisterhood relation between thermoplasmatales and eukaryotes (Pissani *et al.,* 2007), supporting cytological and biochemical considerations previously noted by Margulis and Stolz regarding ultrastructural features (Margulis & Stolz 1984).

Before 2008, the differential distribution of eukaryotic homologues between crenarchaeal and euryarchaeal was considered evidence for chimeric scenarios where two archaea coevolved and then endosymbiosis occurred (see hydrogen hypothesis). In 2008, Yutin proposed that the crenarchaeal gene input to euryarchaea is significant, and that horizontal gen transfer can explain the chimeric distribution of protoeukaryotic genes within the Archaea (Yutin *et al.,* 2008).

The 2002 discovered Korarcheota phylum was genome-sequenced in 2008 (Huber *et al*., 2002; Lapidus *et al.,* 2008). The same year, marine mesophilic Thaumarcheota (Brochier-

Armanet *et al.*, 2008) were identified. The phylogeny started to fill the gaps within the evolutionary relationship of crenarcheaota and eukaryotes. The new archaeal phyla were found in different habitats. Aigarchaeota were discovered in 2011 (Nunoura *et al.,* 2011). The new diversity allowed establishing sisterhood between Taumarcheaota, Aigarcheota, Crenarchetoanad and Korarcheota. This resulted in a new superphylum: the TACK archaea (Guy & Ettema, 2011).

Resolving the tree of life in the postgenomic era

Major advantage had been made since the serendipitous proposal of Woose (Woose *et al.*, 1990). Contemporary trees of life use 28 to 49 universal markers (Cox *et al.,* 2008; Foster *et al.,* 2009; Guy & Ettema, 2011). The new view is a two domain of life. Eukaryotes are part of a polyphyletic branch in archaeal phylogeny (Williams *et al.* 2013; Nesselquist & Gogarten 2013; Williams & Embley 2014; Guy & Ettema 2014). They are sisters of the TACK superphylum supporting previous suggestion about a chrenarchaeal origin (Williams & Embley 2014).

Massive gene gain was described at the basis of the TACK superphylum (suggesting that archaeal branches suffered reduction of genomes from a last common ancestor that had a complex genome (Wolf *et al* 2012). Reduction can be attributed large effective populations under strong selection pressures, as expected in extremophiles (Forterre, 1995). Complexity ca arose form explosive genome expansion in early eukaryotes, either close to LACA or TACK ancestor. (Wolf and Koonin, 2013).

In 2012 a phylogeny using 32 universal markers succeed to root the whole TACK with eukaryotes as they sister branch but failed to explain their fine relation within the TACK (Williams *et al.,* 2012; 2013). However, the picture of eukaryotic origin close to the TACK is consistent with the presence of informational homologs such as RNAse polymerase subunits RPB8 (Koonin *et al.,* 2007); RPC34 (Blombach *et al.,* 2009) and transcription factors (Daniels et al 2009). In contrast euryarchaeal homologs are mostly operational genes (Yutin *et al.,* 2012).

Two-domain tree of life

Super matrix approach in phylogenomics allowed avoiding long-branching attractions and politomies artifacts in 2010. Eukaryotes were placed next to poorly understood Korarchaeota (Gribaldo *et al.* 2010). Simonetta Gribaldo, on a conference on major transition of evolution held in Mexico City in 2015 argued about the importance to define topologies, not only in the TACK, but to improved super matrices at the root of bacteria and Archaea (Gribaldo, 2015). The consequences of rooting the tree of life are as we have seen fundamental for eukaryogensis (Poole & Penny, 2007). For example, the Archeozoa hypostasis could be plausible again if the phylogenetic tree of life can be single rooted. If only one domain exists, then gradualistic change from the bacteria could provide evidence for Cavalier-Smith's *Neomuran* revolution.

In 2015 new genomes assemblages form the Arctic Ocean were named Lockiarcheaota (Spang *et al*., 2015). They revealed to be the sister group of eukaryotes and solved the topology towards the other TACK members. Lockiarcheaota is only a metagenomics assemblage but has revealed to contain genes specific for membrane remodeling vesiculation and other typical eukaryotic features (Spang *et al*., 2015; Embley & Williams, 2015; Nasir *et al.,* 2015). The two-domain tree of life became relevant in the light of a rooted tree of the TACK superphylum: a scenario of gradualist speciation where eukaryotes are included.

The dispersal prokaryotic Eukaryome

Eugene Koonin has pointed out the concept of dispersal Eukaryome, regarding the extended distribution or eukaryotic homologs in Prokarya and the specific distribution in the different lineages of Archaea (Koonin, 2015). The classical perspective of searching cytological homology between eukaryotes and prokaryotes, which defined archeozoa and endosymbiotic theories, has moved to functional and comparative genomics of specific cell systems. Here I will summarize some of Koonin arguments about specific cell systems.

Ubiquitin system

Ubiquitin system was believed to be a eukaryotic feature evolved from prokaryotic enzyme synthesis. In 2011 ubiquitin putative homologs of archaea E1 and E2 were found to harbor operon structure in *Candidatus Caldiarchaeum subterraneum*, the only representative of Aigarchaeota (Nunoura *et al*, 2011). Further analysis found sequence similarity to eukaryotic E1 and E2 genes (Koonin, 2015). Also, the sulfur carrier homolog URM was found in Sulfolobales suggesting that the ubiquitin system is part of an early degradation pathway in the TACK (Marakova and Koonin, 2010).

Cytoskeleton

Cytoskeletal features were thought to be sinapomorphies of eukaryotes and a requirement of protoeukaryotic cells. Distant prokaryotic homologs of tubulin are found in septation proteins FtsZ and MreB. Comparative genomics revealed actin homologs in crenarchaeal Thermococcalees, Korarchaeota and Aigarchaeota. These proteins can coil in the same way as eukaryotic actins (Bernarder *et al.,* 2011). Recent tubulins homologs were found in Thaumarchaeota completing the picture of actin tubulin system as a probable feature of the TACK ancestor (Yutin and Koonin 2012).

Cell division

Mostly all prokaryotes share division mechanisms based on Z-ring formation by FtsZ. Some variants of cell division such as endospore formation and offspring viviparity

(Enterobacteriales, Firmicutes and Myxococcales) have been described in endosymbionts but poorly characterized at the molecular levels. (Angert, 2015). Additionaly to FtsZ; a homolog on the ESCRT-III membrane remodeling complex has been found in crenarchaeal orders (Lindas *et al.,* 2008) and in some euryarcheota (Makarova *et al.*, 2010). The Thaumarchaeota *Nitrospumilis marins* uses ESCRT-III as primary cell division machinery (Pelve *et al.*, 2011). Comparative genomic analysis also provides evidence of a third system in thermoprotiales, which in the context of a dispersive distribution of ESCRT-III and FtsZ suggest that the three systems might have coexisted at the root of the TACK (Koonin, 2015). Furthermore, it has been appointed that cell cycle variation in prokaryotes led to genome expansion and polyploidy, as suggested for the giant bacteria *Epulopisicum fishelsoni* and the whole euryarchaeota clade (Katz and Oliverio, 2014). Thus, the picture of a progressive TACK archaea genome reduction and explosive genome expansion in eukaryotes and has gain much more discursive support.

Endocytic system

Studies on ESCRT in eukaryotic phylogeny have revealed that endocytic systems are diverse among eukaryotic groups. Endocytosis is mediated by novel proteins AP5 and TOM-1, which act on three different ESCRT systems (I; II; III) in different configurations depending on the eukaryotic major clade. This suggest that all variants of configuration of endocytic Pathway already existed in the LECA (Wideman *et al*., 2014). This could be consistent with a scenario where phagotrophy was an important trait that leads to adaptive radiation in early eukaryotic evolution.

Nuclear envelope

From the perspective of comparative biochemistry, it has been shown that the nuclear pore complex (NPC) and the endomembrane system could have coevolved. They are present in the form of a proto-coatomer in all major eukaryotic lineages suggesting that the LECA already had a NPC (DeGrasse *et al.*, 2009). Furthermore, multiple DNA binding domains have been described in major eukaryotic groups suggesting a diversity of membrane protein-nucleic acid interaction in the LECA (Devos *et al.,* 2006). Finally, the existence of multiple systems responsible for chromosome and plasmid segregation in bacteria suggests

that chromosome segregation could have triggered nuclear membrane evolution and mitosis (Dawson &Wilson, 2015).

Nucleolus

Staub *et al*. searched for homologues of nucleolar protein domains based in the nucleolar proteome of S. *cerevisae.* Many protein domains were found in archaea (Staub *et al*., 2004). There are at least 25 nucleolar domains shared between arches and eukarya; 13 between prokarya and eukarya , exclusive of eukarya 25 and 29 present in the three domains . For example, enzyme domains and remodeling factors RNA bases are homologous to archaea. Among these, were discovered: Sm protein, present in the H subunit of RNA polymerase I (Hermann *et al.*, 1995) ; splicing factors (U1 , U2 , U4) ; CBF transcription factor (Burley *et al.*, 1997) ; proteins (S3A , S4 , L15 , L31) ; and ribosomal factors (eIF - 5th , eIF-5^{a}_N , eIF6 , eRF1_1 , eRF1_2 , eRF1_3) used in the translation process (Koonin, 1995). There are 29 exclusive domains of eukaryotes that have to do with fundamental functions of nucleolus (Staub et al, 2004) . These include the HMG genes, ribosomal domains (Ribosomal_L6e, Ribosomal_L14e , Ribosomal_ L22e , Ribosomal_L27e) (Ghallagher *et al.* , 1994), the recognition domain of the SRP (Birse *et al*. , 1997) , the PARP domains polymerases (Smith, 2001) and chromatin remodeling complex CHROMO (Koonin *et al*. , 2005) SSU complexome proteins were founded as homologues in archaea (Wen et al., 2013) and that the length of the intergenic spacer may affect nucleolar size and structure (Thiry et al., 2005).

Interference RNA´s

Regulation of gene expression through enhancers, transcription factors, and interference RNA might seem as an idiosyncratic invent of eukaryotes towards evolution of genome complexity. *Argonauts* families are found be originated in Euryarchaeota (Marakova *et al.,* 2009). *Dicers* don't have direct homologs, but some protein domains resembled homology between RNAse III and bacterial protein domains. The archaeal counterpart of Dicer helicase domain is a homolog in euryarchaeal helicase (Shabalina & Koonin, 2008) Thus

interference RNA might be an idiosyncrasy of eukaryotes on the basis of protein domain adaptation to new genomic architecture.

According to Koonin the picture of a dispersal Eukaryome among the prokaryotes but with a clearly functional as phyletic pattern on the TACK superphylum, suggest that eukaryogensis took place in the root of the TACK. Genome expansion occurred at the root of the archaea and genome reduction at their branches. Thus, complexity might be related to complex genomes either contemporary to LACA or to the ancestor of the TACK (Wolf & Koonin, 2013; Koonin, 2015).

The disciplinary debate

The archeozoa hypothesis by Cavalier Smith, which originally suggested a bacterial origin of the archeozoa, can be dismissed by the fact that eukaryotic features as we know them today, were unsustainable at the complex forms of the eukaryotic ancestor, due to their unsustainability in the context of prokaryotic cells. A complex archaeon is a plausible model for gradual evolution of genome complexity and at some point, assimilation of endosymbiotic protobacteria as energy resources (Lane, 2011). Definitive topologies in phylogenetic hypothesis might reveal a better approach to the discrete relation of eukaryotes to the TACK however the so called phylogenomics impasse can lead to misunderstanding in teleological basis of the questions around eukaryogensis. On one hand the question about the abstract nature of the protoeukaryotic cell and on the other hand the question on an historic narrative of the gain of eukaryotic features from FECA to LECA. The last has been the source of controversy between the endosymbiotic theories and the idiosyncratic scenarios but also a sort of speculation discourse and tautology until recent years.

Margullis later theory on the origin of nucleus departs form the necessity of microbial evolution to include cytological data set in the molecular era. For her, Woose classification is partial, as the separation between archaea and bacteria from cytological point of view is as plausible as separation between archaea and eukaryotes. The last could have only emerged from several chimeric episodes due to their complexity level, but not form gradualistic process. Cytological characters have become more attention today in the context of better-known archaeal diversity. Also, symbiotic relations in microbial ecosystem became relevant for defining evolutionary traits in gradualistic as saltationst scenarios.

Although viral eukaryogensis is an attractive scenario it should regarded as the archeozoa hypothesis jut as an actualistic view to the problem of eukaryotic origin. Phylogenetic basis is required to prove homology between viral genomes and the other three domains. If a

putative virome can resemble de dispersal nature of the Eukaryome, especially in those genes that are thought to be idiosyncratic, practical advantages can be made.

The ecosystem first approach is clearly much more theoretical than biological. Despite its tendency to autorganization theory, some aspects are important. First, that an ecosystem first approach can be implemented for the rise of major transitions of evolution in which the evolution of ecosystem is a fundamental part toward explaining evolution of species. And secondly that the debate between xenogenesis and idiosyncrasy is still open so far, the evolutionary traits at the root of the tree of life remain an open question. In my opinion so far, our methods cannot provide data to construct a sharp ecological causality, the complex system approach must remain as a metaphysical domain but not as a scientific theory.

In the paleobiolgical approach, just relative datation of LECA is possible yet. However, the paleobiological reconstruction of eukaryotes originated over chronical oxygenation in microbial mats ecosystem, where phagotrophy triggered cell size evolution and radiation is to be consider in exosystemic and metabolic perspectives.

A certainly important contribution has been de discovery and classification of archaeal diversity. From the modern phylogenomics methods a skeleton for historic narrative has been proposed. The TACK superphylum is now an open sea for scientific endeavor. Scientists are starting to view to the biology of those organism rather than to their genomes. We have moved from the reductionism of ribosomal phylogenies to the multiplicity of genomics and now to integrative biology. Therefore, a new disciplinary basis is needed.

Evolutionary cell biology

The *-omic's era* come with the challenge of relating sequence information with biological meaning of structure and function. The cell might be still a valid conceptual framework to address this interaction. As Lynch said, "*All aspects of biological diversification ultimately trace to evolutionary modifications at the cellular level" (Lynch, 2014).* The research program proposed by Lynch and collaborators in 2014 departs from the premise that a transdisciplinary interaction between cell biology and evolutionary science is possible to understand complex aspects of the major transitions in evolution. But it also challenges to encompass epistemic differences of both disciplines. Can we use the mechanistic nature of cell and molecular biology to explain the phenotypic diversity of cells? How can we integrate both disciplines if cell biology is reductionist in comparison to the integrative nature of evolutionary biology addressing diversity through historic narrative? One should recognise that evolutionary biology has become more reductionist and less speculative in terms of cell evolution due to the realm of well-resolved phylogenies. In this sense the cell biological approach does not conflict with the reductionistic nature of functional disciplines such as cell biology, but with the fact that cell studies are limited to few model organisms. In summary, if we want cell biology to be complementary to evolutionary inquiry, the approach must address diversity and retrieve a comparative approach. Therefore, Lynch *et al* proposal focuses on four questions (Lynch *et al.,* 2014):

- Why are cells the way they are and why aren´t they perfect?
- How do cellular innovations arise?
- Where do cellular innovations map onto the tree of life?
- How can evolutionary cell biology be effectively implemented?

The inquiry process in evolutionary cell biology departs from genomic and proteomic analysis, being based on functional constrains known from model organisms. Protein domain analysis has proven a powerful tool to address homology of functional entities. Mapping these characters on comprehensive phylogenies may reveal important insights on the biology of common ancestors. Then, functional studies can be conducted to prove specific putative functions in non-model organisms. Morphology plays two major roles: to

establish better morphological characters according to new phylogenies, and to assess experimental studies on structure, topology, and localization of cell functions, related to phylogenies. The reconstruction of ancestral states of an organelle's architecture is an example (Lynch, 2014; Richardson *et al.,* 2015). As a result, evolutionary narratives will be able to rely on more statistical and functional evidence.

Eukaryogenesis from the perspective of evolutionary cell biology

As we have seen efforts on eukaryotic origins have taken places form different disciplines and modes according to the historical curse of biology (Lynch & Field, 2014). We need to integrate these different approaches but not only in the discursive argumentation of biological essays, but in the design of experiments on functional genomics, cytological description and phylogenetic reconstruction. Thirty years earlier Doolittle argued towards a comprehensive understating of comparative cell physiology form evolutionary perspective (Doolittle, 1980; Hartwell *et al.,* 1999). Evolutionary cell biology should recognize the value of cytological and morphofunctional diversity of all eukaryotes, and therefore proposes research outside the canonical model organisms and turns to the tradition of compared cytology in an exercise that reconciles morphology, biological actualism and phylogenomics. Thus, new theories try to reconcile paradigmatic events as endosymbiosis, karyogenesis, the origin of cilia and endocytosis, in the light of the current evolutionary constrains of functional systems.

Cell topology across the FECA-LECA evolution

A recent paper by Gabaldón & Pittis is a remarkable example that calculates stemlenghts for eukaryotic protein families along the FECA-LECA branch, gives striking evidence for the potential sequentiality in which organelles could arose: -*The very teleological question that has driven many of previous efforts*-. According to Gabaldon, LECA already harbored all eukaryotic features and mitochondria were the last to be incorporated. Endomembrane system, nucleus and non-proteinaceous features were acquired long before (Gabaldón & Pittis, 2016). Does this mean gradualist evolution is a more plausible scenario than

chimeric origin? Comparative genome architecture and proteomics revealed eukaryotes as chimeric assemblages of archaeal and eubacterial genes and protein domains. Horizontal gene transfer is an explanation for chimerism but syntenial ancestry of genes from bacteria throughout archaea and eukarya must be taken in account. This is especially important to discern between functional genes acquired through HGT and linear evolving characters. One remarkable account is that eukaryotic proteins are related to cellular compartments and compared to their prokaryotic homologues; concluding that some early emerging organelles have chimeric prokaryotic ancestry. In this sense, the next approach could be considered to explore the cell biology of prokaryotic groups harboring eukaryotic protein families. Bacterial and Archaeal evolutionary cell biology is a promising field, expanding the comparative approach to prokaryotic cell architecture.

Nuclear architecture and evolution

Nuclear architecture has always been related to evolution by the question on the origin of the cell nucleus. Despite its complexity, previous attempts addressing evolutionary hypotheses stayed at the speculative level. The discovery that *Giardia lambia* (a flagellated protozoan and intestinal parasite with pediatric relevance) harbors a nucleolus by Jiménez-García et al in 2006 was for us the motivation to address the evolution of the nucleolus from a functional and evolutionary approach. The nucleolus is a multiproteic complex of close to 300 different proteins that are involved with processes as important as programmed cell death, metabolic regulation, cell differentiation, stress and aging (Boulon, *et al.,* 2010). All these processes converge to the core process occurring in the nucleolus, namely, the transcription and maturation and synthesis of ribosomal RNAs and components. The nucleolus should be considered a cell structure and a genomic location, therefore a nuclear domain that controls fundamental eukaryotic features relating them to the scale of ribosomal gene expression. Thus, the discovery of Giardia´s nucleoli implicated a major advance, namely that nucleoli represent a character shared among all eukaryotes (a sinapomorphy) and thus, LECA should also harbor a nucleolus.

Nucleolus evolution

The nucleolus has crenarchaeal and actinobacteria shared ancestry (Gabaldón & Pittis, 2016). I would like to conclude with the thought that non canonical organelles have been shown to be the early emerging features in eukaryogensis. Ribonucleoproteins and nuclear bodies are important to be consider as proteinaceous organelles or systems, harboring a large diversity of interactions and nanoscale morphologies (see Table 1).

The diversity of nucleolar morphology among eukaryotes and especially protists was already known since the 19th century based on strong cytological evidence (Montgomery, 1900, Heath, 1980). In fact, our knowledge on the mammalian and yeast nucleolus does not guarantee that the polymorphic structures of protists are homologous to these model nucleoli. Additionally, our definitions of nucleolus and nuclear bodies are undergoing reconsideration as the nanometric nature of protein-nucleic acid interaction and its relation to gene expression is being studied as a dynamic process (Mistelli, 2005). We face the challenge of understanding the evolution of processes of cell entities that are highly dynamic. We propose that the functional and structural knowledge on morphology, function, and development of Giardia´s nucleoli (Jiménez-García *et al*., 2006, Lara *et* a., 2016) and other early branching eukaryotes as *Entamoeba histolytica* (Vázquez-Echavarría *et al., 2009*), *Chaos chaos*, and *Pelomyxa palustris* (Islas-Morales et al, unpublished), increases our understanding of a 'primitive' nucleolus. Therefore, consistent evolutionary characters will necessary. Properties like, the behavior of nucleolus during mitosis, its distribution, and the presence of transcriptionally active polymerases have attracted our interest towards discrete elements of nuclear architecture that can justify characters at the evolutionary level. Thus, our current work focuses on applying an evolutionary cell biology approach with a focus on the emerging concepts of nuclear architecture.

Conclusive remarks

One important lesson is to be learned from this review is that the question on the origin of eukaryotic cells demands trans-disciplinary work. Evolutionary cell biology is the product of a profound understanding of the epistemic aspects of evolutionary theory and its difference with functional approaches such as experimental genomics and cell biology. However evolutionary theory can become a practical unifying concept and theoretical background in the questions that evolutionary cell biology aims to address. For this understanding of evolution beyond adaptation must be communicated, but also experimental and theoretical methods for the early evolution of life must improve. Only then research projects can find a methodology of evolutionary narratives that can be supported by experimental evidence form cell biology. For eukaryogenesis immediate efforts must be driven to improve model organisms for lower eukaryotes and Archeaea. This will improve research on the origin of genome architecture, expression patterns and the compared cell biology of diverse cell systems.

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Capítulo II

Biología Celular Evolutiva: lecciones, retos y oportunidades para el estudio integrativo de la evolución de las células

Artículo

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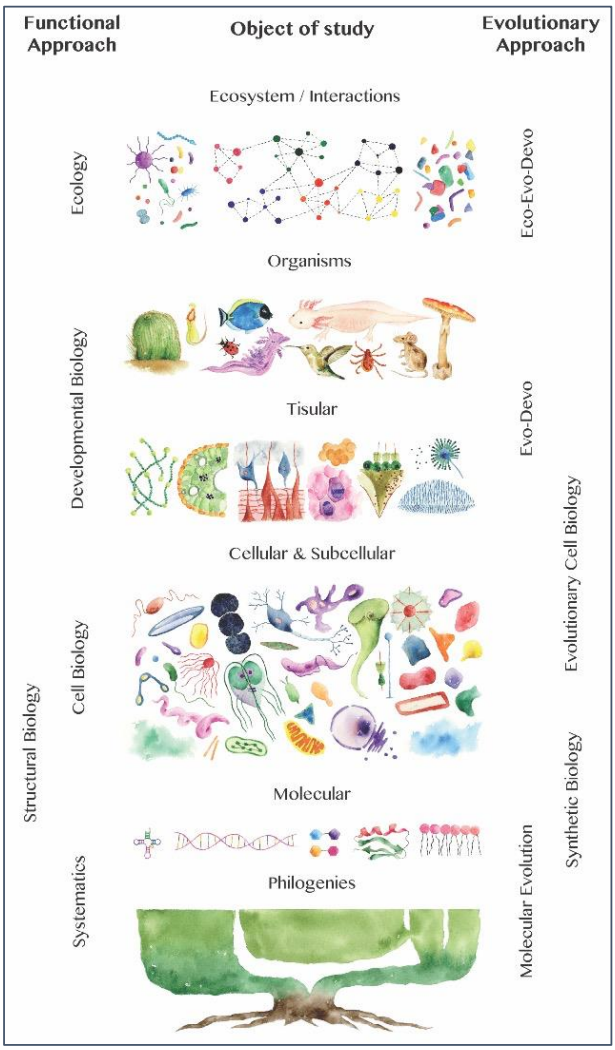

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Review

Evolutionary Cell Biology (ECB): Lessons, challenges, and opportunities for the integrative study of cell evolution

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Evolutionary Cell Biology (ECB) has gained increasing attention in the last decades. Here we explore whether ECB is truly inter-disciplinary through the combination of cellular and evolutionary biology to offer evidence-based insights regarding the major questions of cell evolution. Since 2012, ECB asserts to utilize the increasing potential of high-throughput omics data (*in silico*) with morpho-functional (*in situ*) information, although challenges remain for a complete integration. For instance, the limited number of model organisms and cultivation techniques available excludes the majority of the extant diversity of cells from the scope of experimental inquiry. At the conceptual level, the simplification of evolutionary processes influenced by cultural views of evolution, such as adaptationism or Scala Naturae, challenges effective interdisciplinary work. Without a profound understanding of evolutionary theory and an integrative view of cell biology, the formulation of questions and experiments properly addressing evolution and diversification of cell complexities can become misleading. In 2009, we advanced the discovery of a nucleolus in the flagellated unicellular eukaryote *Giardia lamblia*, and studied nucleolus diversity in other lineages via electron microscopy. Since then, studying evolutionary questions at the cellular level became central to our research. We think that new methodological advances are re-shaping and strengthening the ECB research program and opening its door to experimental scientists. For example, the discovery of new archaea and protozoa and subsequent investigations that coupled *in situ* approaches with *in silico* approaches has proven that comprehensive morpho-functional information can be obtained that can only be understood through the merging of the cell biological and evolutionary discipline. Motivated by this, we here explore the history, the challenges, and the opportunities of ECB to motivate researchers to join this emergent field of research. We outline elements that contrast the current ECB discipline from previous integrative attempts. We conclude by elucidating the current disciplinary constraints of ECB and propose considerations towards successfully employing ECB to answer questions pertaining to the evolution of cellular complexity.

Keywords. Archaea; bacteria; eukaryotes; evolutionary cell biology; nuclear architecture; nucleolus

1. Introduction

> With the theory of evolution, the cell theory is the most important generalization in biology. There is, however, a missing link between these theories that prevents an even more general and unifying concept of life
>
> –Mazzarello 1999

What is evolutionary cell biology (ECB)? A general view is that ECB is an attempt to integrate evolutionary and cell sciences into a major discipline that can address the origins and diversity of cellular complexity, which spans the last centuries. A more recent view posits the name ECB entails a defined and consensual research program of 10 questions, based on the interdisciplinarity between omics, functional experiments, and phylogenetics (Lynch *et al.* 2012; Ford Doolittle 1980). In fact, ECB defines itself as: 'the study of patterns of variation in cellular features within and between species and of the mechanisms (molecular building blocks and population-genetic underpinnings) responsible for their establishment and maintenance' (Lynch *et al.* 2012). As with previous attempts, the 2012 research program of ECB cannot escape the fact that integration between two historical and contrasting epistemic entities is sought. Cell theory and evolutionary theory are probably the most important corollaries in biology, but they depart from different conceptual frameworks: empiricism and historicism, respectively (Mazzarello 1999). Thus, any ECB definition seeks implicitly the integration of two major biological disciplines and is, therefore, a motivation to develop a shared methodology taking into account authors, ideas, theories, techniques, and philosophical views in biology. Its purpose: the ability to study cells, their form, function, and changes.

Parallel to the consolidation of the ECB research program, our research group started working on the evolution of the nucleolus employing comparative Electron Microscopy among protozoa. In 2008, we could confirm that *Giardia lamblia* has a nucleolus (Jiménez-García *et al.* 2008) with the evolutionary implications to be studied using comparative functional and evolutionary approaches in line with the ECB proposed methodology. Evolutionary patterns in cellular diversity are addressed with hypotheses and experiments that transversally derive and use state-of-the-art techniques: 'big data' omics, experimental cell biology, and evolutionary phylogenetics (Lynch *et al.* 2014; Brodsky *et al.* 2012; Lynch *et al.* 2012). Here, omics is understood as the high-throughput acquisition and inventory of biomolecular metainformation of cells, organisms, or environments. These metadata should relate to a cellular context to become meaningful. Cellular context is often represented by morphological information and experiments on cells (*in situ*), but recently also by single-cell genomics. The availability of 'big data' omics technologies, with high-throughput evolutionary bioinformatics and functional *in situ* techniques (e.g., CRISPR-Cas, single-cell genomics) is the first argument that supports the technical capacity of ECB (Lynch *et al.* 2014). Lynch and collaborators have mentioned important technical and conceptual challenges for ECB: first, the lack of model organisms and cultivation techniques from most unicellular lineages; second, the over-simplification of evolution in experimental biosciences; and third, the difficulty to build research programs raising a mixed community of evolutionary and cell biologists (Lynch *et al.* 2014).

Thus, 8 years after the inception of ECB, we think it is time for an examination: how close are we to addressing evolution at the level of cellular organization? The question is intriguing and motivating for biologists that are addressing evolution at the cellular level.

'The Cell' is what we recognize as the structural and physiological unit of all living things since the 19th century (Gonzalez Recio 1990). With ECB, we want to explain the extant diversity of cells; how the forms and functions of their organelles evolved and contributed to organismal complexity, by taking advantage of state-of-the-art techniques in omics, microscopy, and cell biology. The challenges faced are not just technical, but also historical and discipline-inherent. While other reviews on ECB focus on recent perspectives or just mention ECB within a major debate between the integration of functional and evolutionary disciplines in biology, this review explores four centuries of ECB from a disciplinary and historical point of view to understand how the inquiry processes have changed. We are especially motivated to raise consciousness about the philosophical constraints, but also methodological and cultural challenges (figure 1).

2. Understanding of cell evolution through history

2.1 *Pre-disciplinary attempts*

The aspiration to integrate evolution – the notion of change in species — in the understanding of cells was

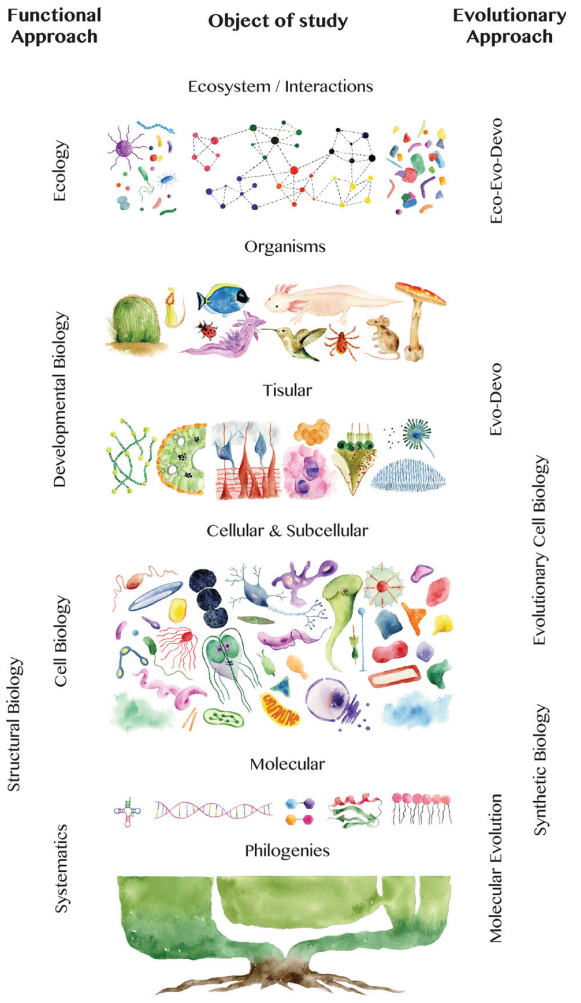

Figure 1. Theoretical position of Evolutionary Cell Biology (ECB). The figure shows an artistic representation of the living diversity emphasizing different levels of organization, which are study objects of biological disciplines. Considering the historical functional/evolutionary divide of biosciences, we show that novel disciplines such as ECB, Evo-devo, and Eco-evo-devo, have the potential to fulfill the gap of evolutionary approaches and research programs that are needed to address evolution at the cellular, tissular, developmental, and ecological level. We recognize the importance of already consolidated evolutionary disciplines (not shown) such as population genetics and comparative biology. Classical approaches are crucial as sources of transdisciplinary planning of new research strategies. Coincidences between disciplines are to be found in the first instance by sharing the same objectives and objects of study.

already present at the beginning of the 17th century. Robert Hooke's Micrographia (Hooke *et al.* 1667) and Anton van Leeuwenhoek's letters on 'Animalcules' opened a new world of (single) cells, followed by a philosophical revolution about life, its diversity, and its origin (Caspers 1964). These early works were driven by the empirical and realistic nature of Francis Bacon's research program, which aimed at the integration of empirical and theoretical knowledge. Therefore, Leeuwenhoek's proclivity to develop work based on experimental and observational evidence was revolutionary. Before Bacon, scholastics had been the principal philosophical program, based mostly on dissertation, hermetics, and logics, but without experimentation and truthful representations of what was seen. Scientific illustration became a technical advantage the new world unveiled by lenses (Alpers 1983). Microanatomical observations did not find a proper theory in the 17th century (Recio 2016). However, Leeuwenhoek's legacy provides interesting ideas that link the fact of microbial diversity to the idea of change and continuity in microscopic forms. He outlined important consiliences and discordances. For example, Leeuwenhoek proposed a divide between so-called gigantic monsters (protozoa) and little animalcules (bacteria) based on morphology, differential cultivation conditions, and their observed abundance. He pointed out similarities between the morphology and movement of bacteria and those of organelles in the protozoan *Colpidium colpoda*. The comparison may be considered the first, albeit unconscious, observations of mitochondria (Lane 2015). He also speculated on the ecology of microscopic organisms based on his observations, e.g. that rotifers disperse by means of transport on the feathers of aquatic birds from one pond to another, explaining the spread in geographical distribution and their relation to each other (Keilin 1959). Leuwenhoek's experiments on the anaerobic cultivation of *infusoria* (amoeba) led him to be convinced that the continuity of life in microbes is based on cell division. Based on this, he supported Reddi's ideas against spontaneous generation (Fildes 1951; Lane 2015) that dominated since Aristotle. Thus, even before disciplines such as cell biology and evolutionary biology were established, early microanatomists started an ontological survey detailing questions and ideas about single-cell organisms, their functions, diversity, and origins.

2.2 *Early interdisciplinary attempts: cell theory encounters Darwinian methodology*

Two centuries after Leeuwenhoek, cell theory was converting progressively into the major axiom

experimental life sciences, hosted under the term physiology. The great advantage was defining cells as the anatomical, functional, and ontogenetic unit of plants and animals. Paraphrasing Nurse, with the cell theory *'The living found its atom'*, cells became a model to explain or grasp the basic units of organisms (Nurse 2003a). In parallel, the Darwinian Theory of Evolution revolutionized biology. In fact, evolution as changes in biological species through time had been the subject of study for centuries, similar to the cells described by Hooke, but it was not until these two theories were accepted that biology became a scientific discipline. The search for a major generalization of both theories became immediately a challenge. It meant the chance to unify biology, a single theory linking form, function, and change, and solving the issue of the origin of cells in the light of evolution (Albarracín 1983). However, interactions between evolutionists and cell physiologists were unilateral rather than interdisciplinary. Cell physiology and cytochemistry built an understanding that the basic unit of reproduction and life continuity was a cellular process, such as mitosis, leaving questions on variation at the cellular level still pending and relying on ideas such as *gemmules* and protoplasm. This remained until 1930 when the detailed mechanisms of inheritance by chromatin and chromosomes were discovered and contextualized as the cellular explanation of Darwinian variation.

Another challenge was that cell evolution and microbial diversity were hard to assess from a Darwinian perspective at first. Ernst Haeckel attempted a comparative approach to link elements of the cell theory with those of Darwin's theory systematizing uniformities, sequentialities, consiliences, and discordances in his observations of microbial diversity (Dayrat 2003). His classification of protozoa and monera at the base of the tree of life represented a major change to the prevalent Linnaean classification, where microbes were still allocated according to Aristotle under the names of Vermes (Worms) and Chaos (formless) (Lane 2015). With Haeckel, single cells became the origin of life and the cells' uniform character relating all life forms. It became evident that cells are diverse and that subcellular characters relate to the classification of unicellular organisms, but also functions of different cell types and tissues in animals, plants, and fungi. To address this evolution of cellular types and functions, Haeckel tried to extrapolate cell generation and evolution with embryonic development.

Haeckel´s biogenetic law is an interpretation of Virchow´s corollary '*Omni cellula e cellula*' suggesting that animal development is the result of cell proliferation. Accordingly, the simplest state of development is a single cell, in this case, the fertilized egg (Gonzalez Recio 1990). This was unfortunately misleading because cell proliferation and differentiation were just starting to be understood, and certainly not all single cells are the same. However, Haeckel thought of the fertilized egg as the homolog of the ancestral unicellular state, and therefore it should resemble the most ancient state of the phylogeny. Logical at first, he was unable to find evidence for his biogenetic law among protozoa due to their diverse and complex cellular organization, which made it impossible to associate their cell morphology with any of the developmental steps of animals. Boveri´s work on the organization of chromatin contributed to the understanding of the complexity in the nuclear structure during development. But even at the level of the cell nucleus, common to all eukaryotes, Haeckel noticed that the wide diversity of morphologies in the nuclei of protozoa was incompatible with his biogenetic law and could not be related to any ontogeny steps in animals.

To the discredit of the biogenetic law, its notion of less and more evolved organisms by means of complexity (from bacteria to protozoa to invertebrates to vertebrates to humans as the most evolved organisms), became one of the most common misunderstandings in biology (Maehle 2011). This representation, also called *Scala Naturae*, is strongly misleading because it depicts evolution as stepwise progress mirroring animal development, rather than the sum of changes over time following a directionless diversifying process.

2.3 *Modern attempts: the concept of bacteria cell in comparative biochemistry*

For long, cell theory and evolutionary theory concentrated mostly on eukaryotes, despite bacteria being the most abundant and diverse group of unicellular organisms, and leaving out their economic and medical relevance. In the early 20th century, bacterial cells started to be understood and classified by comparative biochemistry. Alfred Kluyver's work on fermentations advanced the knowledge on metabolic diversity and prompted the use of biochemical characters in a new bacterial taxonomy (Lane 2015). In 1962, van Niels and Stanier published '*The concept of bacterium*' (Stanier and Van Niel 1962) incorporating the advances of biochemistry and electron microscopy to begin to provide a definition of bacteria. The relevance of biochemical characteristics in phylogenies was stated by Kluvyer as follows: 'What is true for the butyric acid

bacteria is true for elephants'. In reference to this, years later Jacques Monod stated: 'What is true for *E.coli* is true for elephants'. Bacterial model organisms became the experimental platform for cellular metabolism (Lane 2015). Evolutionary implications are present in these statements. Namely, that metabolism is conserved across lineages as distant as bacteria, elephants, and plants. Metabolic characters might be useful for a functional taxonomy of bacteria, but are not necessarily evidence of natural groups, since they are present in different lineages. In contrast, the fine *organization of the cellular machineries is more diverse* across the tree of life and evidences specific characteristics of some groups of organisms. Thus, Steiner and Niel concluded that, despite sharing basic principles of metabolism with other organisms, bacteria are a monophyletic group, because they have substantial differences in cell architecture compared to eukaryotes, and these differences are uniform among bacteria, and thus, make them a natural group (Stanier and Van Niel 1962; Lane 2015). Bacteria were no longer called *monera* and thought of as an artificial group.

Recognizing the value of characters as homologies and homoplasies at the cellular level is important in not misunderstanding evolution. While many molecular functions are conserved (homolog), cell structures can evolve independently linked to different functions (homoplastic). Thus, a certain metabolic pathway can be found in different cell compartments across species.

A common misunderstanding in early biochemistry was simplifying the evolution of a certain pathway to the 'adaptation towards thermodynamic perfection'. For example, Hans Krebs suggested that the origin of his homonymous pathway in the prebiotic world took place because multiple steps of degradation and oxidation are energetically 'perfectly adapted' in contrast to direct acetate oxidation (Baldwin and Krebs 1981). Similarly, Jacques Monod was convinced that allosteric regulation was the result of linear adaptation towards perfect enzyme and substrate interaction (Morange 2010). (This would mean that a metabolic pathway that involves several steps and degrees of molecular and cellular complexity can only evolve in one direction and should be the same in all livings, rather than being part of a larger process of metabolic diversification. Following this notion, how would one explain the exceptions and alternative pathways to the Krebs cycle that are found in the extant microbial diversity? Furthermore, the above posits remain to explain how 'imperfect' parts of the Krebs cycle remained over evolutionary time or were recruited into the pathway in the first place.

The problem is to dismiss variation and diversification (there is only perfection in a given context), which are found in cellular diversity that evidences cases of co-evolution and adaptive radiation. Thinking of evolution just as a process of selective pressure shaping a perfect adaptation is a common mistake in biochemistry (Morange 2009, 2010). Metabolism evolves, but it is not disentangled from a changing cellular context. To address these issues and to be consistent with evolutionary theory, one has to understand the importance of randomness, imperfection, which becomes readily visible if one moves away from model organisms and focuses on diversity across organisms. In fact, evolution has no direction and not every feature is the product of adaptation to the environment. A conclusion of these early attempts integrating evolution and biochemistry is that biologists, despite recognizing evolutionary theory, still struggle to apply it to their understanding of the molecular biology of the cell, which is a complex and diverse collection of features, not just an adaptive trait. Effective ECB should therefore emphasize that teleological and panadaptationist views are to be avoided.

2.4 *Direct antecedents of ECB*

Systematic and evolutionary biology were revolutionized when biochemical and morphological taxonomies were extended through the use of molecular characteristics. Shortly after the discovery of the DNA double helix structure in 1953, Francis Crick wrote: 'Biologists should realize that before long we shall have a subject which might be called 'protein taxonomy'—the study of amino acid sequences of proteins of an organism and the comparison of them between species (Crick 1958; Cobb 2017). In 1962, the Zuckerkandl and Pauling paper on Molecules as documents of evolutionary history outlined how changes in amino acid and nucleotide sequences can be used to build phylogenies and construct evolutionary relationships between species (Zuckerkandl and Pauling 1965).

In the next decade, advances in DNA and protein sequencing were developed and gave rise to the discipline of molecular evolution (Stent 1968). The informative value of molecular characters relies on the uniformity of the central dogma (DNA-RNA-Protein) across species, which also explains the source of variation at the molecular level. Was the cell substituted by DNA as the basic unit of life? In molecular evolution, characters are condensed into 4 letters (A, C, G,

T) and phylogenies are inferred using statistical approaches (Grahame and Avise 1995).

However, linking molecular phylogenies with complex cellular features became not meaningful until after Woese's 1990 paper on the tree of life (Woese, Kandler, and Wheelis 1990). With a vision of microbial diversity, Woese suggested a revolution to Steiner & Niel's concept of bacteria (Stanier and Van Niel 1962). Life is divided in three domains Eukarya, Bacteria, and Archaea based on the supremacy of one molecular character –the ribosomal gene - over morphology and metabolism. But what happens with the history of all complex cell features: Do they rely only on gene sequences? Luckily, the ribosomal gene phylogeny of Woese was consistent with later investigations on archaeal cell structure; structural differences sustained the vision of a three domains tree of life (Zillig 1991). However, the tree remained un-rooted. A one-gene phylogeny is reductionist and unable to explain gradual changes in cell complexity. One has to ask the impossible question of how archaea derived from bacteria. If the tree was rooted, the question would be how archaea had originated within bacteria, and the same applies to how the eukaryotes originated within the archaea. When Lynn Margullis proposed endosymbiosis, she took the distance of Woese's molecular reductionism and developed her theory based on comparative morphology (Sagan 1967). Later her theory got validated by molecular data, evidencing the chimeric nature of genomes and organelles. She concluded that genes are not enough to explain evolution, it is necessary to incorporate morphology and cell biology to phylogenomics in order to address the evolution of different degrees of cell complexity (Margulis and Fester 1991; Dyall and Johnson 2000). In 1980, Doolittle already anticipated the need for an integrative view of cell evolution: 'Developments in micropaleontology, RNA and protein sequencing, and the analysis of genome organization suggest a view of early cellular evolution radically different..' (Ford Doolittle 1980). Thirty years later, the tree of life is an always-changing conception due to the integration of more data, in particular big data coming from *omics* approaches. However, our view of early cell evolution is still debating between Woese´s un-rooted tree domain and the never-ending question: how did eukaryotic features arise? (Nick 2009). Gene evolution alone does not explain complex features, and comparative cell biology may not be simplified enough to the level at which gene evolution operates.

The use of metagenomics and high throughput phylogenomics has contributed to the solving of this problem. Only in the last ten years, five new phyla of archaea have been discovered due to metagenomics and were classified into a new superphylum embracing Thaumarchaeota, Aigarchaeota, Crenarchaeota, Korarchaeota, and recently Lokiarchaeota (TACK) (Nunoura *et al.* 2011; Guy and Ettema 2011; Spang *et al.* 2015). This new diversity, analyzed with novel supermatrix approaches that include molecular and morphological characters, led to propose the hypothesis of a two-domain tree of life. This means that eukaryotes are rooted within Archaea because TACK is its sister lineage. (Williams *et al.* 2013; Gribaldo *et al.* 2010; Guy and Ettema 2011). This rooting enables us to explore the cellular nature of LECA, which probably evolved gradually within the archaeal diversification. Such types of approaches are direct antecedents of modern ECB because they evidence that new tools in evolutionary biology enable the integration of molecular, functional, and morphological data that can become biologically meaningful to address questions from a cell biology perspective advancing the understanding of cell evolution.

3. The core of evolutionary cell biology

Modern ECB comes with the challenge of relating high throughput molecular approaches with a spatial context of cell structures and functions. This is essential to give a biological meaning to omics data at the cellular level of evolution. Here, the concept of the cell as the functional and structural unit becomes useful as its methodological framework expands and profits from new interdisciplines. Summarizing the biological meaning of cell evolution, according to Michael Lynch '*All aspects of biological diversification ultimately trace to evolutionary modifications at the cellular level'* (Lynch *et al.* 2014). This is a central corollary at the core of contemporary ECB. Cellular level means not just diversity of organelles and functions, but considering interactions and *in situ* phenomena that happen at the levels of the molecular and the organismic scale. Certainly, the exploration of cell dynamics *in vivo* and *in situ* with regards to diversity and evolution is an almost pristine field of study previously limited by the availability of tools that we have today such as time-lapse microscopy, *in situ* omics, and integrative phylogenomics. Thus, the modern ECB as proposed by Lynch and collaborators in 2012 claims that transdisciplinary interactions between cell biology and evolutionary biology are now possible, and it is useful to study intriguing jumps in cell evolution, such

as in eukaryogenesis and the origin of multicellularity (Lynch *et al.* 2014). Both are also considered major transitions in evolution (Smith and Szathmary 1997). As in previous attempts, these new interdisciplinary challenges need to encompass epistemic differences between functional and evolutionary biology.

Within Lynch's corollary, there is an implied assumption: we can use the mechanistic nature of the cell and molecular biology to explain the phenotypic diversity of cells. This is consistent with the fact that cell biology is a reductionist advancing explanation through empirical evidence and analysis of parts and finally synthesis. In contrast, evolutionary biology builds upon historical evidence and narrative as a methodology to confirm its theory. Can this evolutionary approach be integrated into the process of investigation of cell biology? First, one should recognize that evolutionary biology has become more reductionist due to the realm and acceptance of statistically resolved phylogenies. For example, analytical approaches can be applied to gene or protein sequences to predict an ancestral protein function, concluding that some protein domains can be considered evolutionary building blocks for certain biological functions. Furthermore, the results of these types of analysis can be proven using tools from cell biology, e.g. synthesizing ancient proteins in the laboratory and confirming functional properties. This is in fact a reductionist approach that addresses a question in evolution using functional biology. This interdiscipline has been called 'the functional synthesis' (Dean and Thornton 2007; Morange 2011a, b). At this level, the evolutionary approach (e.g., phylogenomics) does not conflict with the reductionist nature of functional disciplines such as cell biology or structural biology, because both have developed tools that are reductionist for the study of evolution and mechanisms of proteins.

It becomes more complex, functionally and evolutionary, when we focus on the gap, still unexplored, between single molecules and cellular landscapes. Cells provide a rich universe of study objects in evolution at different complexity levels (Nurse 2003b). This is linked but cannot be reduced to the study of biomolecules in the spirit of the functional synthesis. To this end to complex cellular features are converted into supramolecular and morphological characters using evolutionary bioinformatics and then hypotheses can be tested with experiments using cell biology approaches

Technical and disciplinary challenges are to be faced in modern ECB. The technical challenge is that the diversity of model organisms should increase in order to fairly represent the known cellular diversity. The disciplinary challenge is that for cell biology to be complementary to evolutionary inquiry, it has to look after information in diversity at the cellular level. A comparative approach, the same way as Darwin, is necessary for both disciplines to converge methodologically into the same object of study: evolution at the cellular level of organization.

The core of the Lynch *et al.* proposal takes this into account and asks four carefully formulated questions: (1) Why are cells the way they are and why are they not perfect? (2) How do cellular innovations arise? (3) Where do cellular innovations map onto the tree of life? (4) How can ECB be effectively implemented? (Lynch *et al.* 2014).

4. Inquiry process in evolutionary cell biology

A common example of ECB in action is the reconstruction of ancestral states of an organelle or the architecture of a cellular pathway (Lynch *et al.* 2014; Richardson *et al.* 2015). According to the authors, the inquiry process in ECB departs from metagenomics and metaproteomics analysis. One should compare the functional cellular constraints known in model organisms with the clues from meta-analysis of the unknown diversity. As in functional synthesis, protein domain analysis can be used as a powerful tool to address the homology of functional entities in the different cell types or lineages of microbes. Once, proteins, protein domains, and related cell features are mapped as characters on comprehensive phylogenies, one can formulate new questions based on bioinformatics analysis, for example, about the biology of a common ancestor. Here is where functional studies from cell biology (*in situ* experiments, microscopy, etc.) can be conducted to answer specific questions in non-model organisms. Comparative morphology using modern high throughput imaging tools plays two major roles in this process: first, it helps to revise and correct morphological characters with natural meaning in the new phylogenies, and second, it helps to resolve experimental studies regarding structure, topology, and localization of cell functions at the nano- and microscale.

As a result, evolutionary narratives will be able to rely on more statistical and functional evidence. Using cell features as hierarchical characters in addition to molecular characters, evolutionary bioinformatics can encompass complex cellular features into its analysis and formulate questions that are addressable using

recent advances in cell imaging and environmental microbiology, especially *in situ* approaches, given the lack of model organisms. Little by little more detailed cellular processes can be incorporated into this methodology and unveil consiliences and discordances at different levels of cellular complexity, advancing the understanding of cells.

5. Perspective for ECB in coming years

> The uniqueness of biology relies on the epistemology of evolutionary theory, an aspect every functional biologist should understand to grasp the nature of life
>
> –Mayr 2007

Based on this review, we think that technical challenges in microscopy, in situ omics, and bioinformatics will experience continuous improvements. However, the most prominent challenges in the near future of ECB are connected to educational and cultural constraints among biologists. In particular, our concern is that evolutionary theory can effectively pervade scientists from entering ECB.

The scholar Michel Morange has made a philosophical analysis of how evolutionary biology has pervaded functional biology in the last decades. He claims that previous attempts in the 19th and 20th to unify bio-disciplines with evolutionary biology failed, because of an improper understanding of evolution by experimental biologists. Morange explains that: 'The first obstacle was clearly an ignorance of the complexity of evolutionary theory, and of the transformations it underwent throughout the twentieth century (…). The vision of Darwinian evolution held by most functional biologists was closer to that of Herbert Spencer—in which progress is the motor of evolution than to the vision of Darwin himself' (Morange 2010). This has been reflected in historical examples provided in this review. Often in the history of cell biology, evolutionary processes have been over-simplified in the cultural understanding of biological adaptationism.

We think that evolutionary theory should not just superficially touch, but critically improve and pervade, the mechanistic explanations of biological phenomena in experimental disciplines and *vice versa*. This is critical for ECB to succeed. In this regard, increased attention to philosophy and the history of science in biological education should be improved to avoid oversimplification of ideas in evolution. Moreover, with a stronger philosophical and historical foundation, scientists realize that new disciplines such as ECB are objects of philosophical discussion and that research programs should benefit from a community prompting epistemological and historical culture in order to identify and critically examine common values between disciplines and achieve real transdisciplinarity.

We are convinced that ECB represents a promising opportunity for methodological transdisciplinarity and theoretical consilience between functional and evolutionary biology. ECB may not provide a revolutionary paradigm shift in biology, but the discipline shapes the convergence of two major theoretical frameworks towards robust theories around the origin and evolution of the cells, one of the most cryptic and unifying dimensions of life.

6. Conclusions

1. Since 2012 many research projects and programs are becoming consolidated around the conceptual framework of ECB. In order to remain successful, ECB should improve a profound understanding of evolutionary theory and its epistemic and methodological differences to functional cell biology. Among its scientific community, evolutionary theory must become a non-dogmatic and dynamic theoretical background. Thus, experimental hypothesis can organically incorporate evolution into functional approaches and *vice versa*.
2. ECB can also contribute to addressing the role of cellular modifications in the evolution of higher levels of biological organization such as tissues, or even the development of organisms.
3. Experimental advances in omics, microscopy, and cell biology are becoming essential for the process of inquiry in evolutionary cell biology because it facilitates the detailed study of cells from different lineages, enabling comparative approaches, and advancing evolutionary hypotheses with regards to the extant microbial diversity.
4. ECBs main challenge is educational and cultural. For ECB to be successful, coming generations of researchers and scientists need to acquire a broad and robust background in evolutionary theory and methodology, regardless of their specialization. However, misleading views and simplifications of evolutionary theory (e.g., adaptationism) are still common among the scientific community worldwide. Thus, education in biosciences should improve the teaching of evolution with the same

effort it has been improving technical skills in cellular, molecular, and computational biology. This is critical for biologists from different disciplines to understand each other methodologically and in order to establish integrative approaches built on common epistemic values.

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Appendix

Examples of ECB

Multicellularity and development: How organisms became multicellular is a question within the scope of inquiry of ECB that can contribute to the transdisciplinary research field of evolutionary developmental biology (informally, evo-devo). Evo-devo compares developmental processes across organisms to infer how developmental processes evolved. In the work of Nicole King on the origin of the multicellular condition, the cellular basis of development is split into characteristics and mapped onto the most recent eukaryotic phylogenies. Her work highlights that multicellularity has appeared many times in different lineages (e.g., across plants, fungi, and animals) and that many of the features of multicellularity were already present in unicellular ancestors. The different ways of how these ancestors evolved cell complexity sheds light upon the changes at the cellular level that prompted the evolution of developmental systems across the tree of life (King 2004).

Gradualism in eukaryogenesis – A focus for ECB: ECB has the potential of redefining fundamental questions in eukaryogenesis.

For example, gradualist scenarios are a more plausible way to explain the idiosyncratic cell complexity of eukaryotes, if we observe a rooted tree of life. Otherwise, the FECA-LECA transition is by itself a non-gradualist conception based on the assumption that ancient proto-eukaryotic lineages disappeared. In fact, if cell complexity was acquired gradually, we have limited understanding with regard to how simple or even how eukaryotic a hypothetical FECA should have been. In a gradualist scenario, the nature of the sister group of eukaryotes is critical to explore in order to define the attributes exclusively associated with eukaryotes and which attributes were already present in common ancestors. Based on this, we can ask: how are *in silico* evidenced homologs entities organized and functionally involved in the cells of the sister group of eukaryotes? Can we find them in any kind of ancestral compartment, or do they provide different functions related to a completely different cellular context?

In a two-domain tree of life, TACK-Archaea are the monophyletic sister group of eukaryotes. TACK-Archaea then represent a source of evolutionary characters and ancestral states through comparative approaches. As pointed out by Simonetta Gribaldo: in a two-domain tree of life late or early mitochondrial endosymbiosis does not affect a gradualist cellular diversification. FECA is no longer the un-rooted starting point of eukaryogenesis. And eukaryogenesis converts into a result of cell evolution within the TACK Archaea (Williams *et al.* 2013; Gribaldo *et al.* 2010).

The question for ECB would be: which eukaryotic cell features do TACK-Archaea have? Based on the extant and still unveiled diversity of TACK Archaea, how can we approach a real gradualist reconstruction for each common ancestor?

We should be aware of the possibility that many TACK-archaeal lineages, which represent intermediate states, might be already extinct or are highly modified. What is the proof of concept to establish homology between archaeal and eukaryotic compartments or structures? TACK archaeal genes and protein domains are heterogeneously conserved and derived among different eukaryotic families. The notion of a Dispersive Archaeal Eukaryome (Koonin and Yutin 2014) suggests that a comparative approach to prokaryotic and protist cell architecture is needed in order to relate molecular homologs to their cellular context, their putative function, and their evolutionary meaning. For instance, homology between structures can be established if the products of molecular homologs are localized in the same cellular feature(s) in two related lineages. Even if the compartment or structure is highly

derived, cell compartments of distant lineages can be homologous if molecular characters support a monophyletic relation and can be structurally related to a similar cellular context. Then the structure can be attributed to a common ancestor or be seen as a derived character.

Omics-driven ECB: In 2016 Pittis and Gabaldón proposed that the acquisition of mitochondria was a late event in eukaryogenesis. Using an omics approach these authors identified monophyletic groups of genes to be present in LECA with their prokaryotic orthologs. So-called Eukaryotic Protein Families (EPF) are monophyletic and relate to specific cell compartments being able to explore its history between prokaryotes and LECA, namely in the FECA-LECA transition. For instance, alpha-proteobacterial proteins represent mitochondria, while fibrillarin orthologs in Archaea represent nucleoli. By reconstructing phylogenies of each EPF, and evaluating branch lengths separating eukaryotic and prokaryotic sequences with a molecular clock methodology, they conclude that EPFs of archaeal origin tended to be closer to FECA, whereas genes of mitochondrial were closer to extant eukaryotes. The authors suggested that with this measure, as mitochondria have the shortest phylogenetic distance, they would have been the last organelles to be incorporated in an evolving FECA-LECA transition. In contrast, membrane trafficking compartments and ribonucleoproteins had been acquired long before the mitochondrial acquisition (Pittis and Gabaldón 2016). These studies have been criticized by Martin and collaborators, pointing out that the difference in stem lengths of EPFs are not statistically significant, due to the use of a strict molecular clock, meaning evolutionary rates can be considered the same across the complete phylogeny (Martin *et al.* 2017). According to Roger and collaborators: 'this objection fails to acknowledge the possibility that even if no single gene evolved in a clock-like manner, increases and decreases in rates across lineages and proteins could cancel so that the average of stem lengths of a large protein set may be roughly clocklike' (Roger *et al.* 2017).

Despite this methodological discussion, Pittis and Gabaldón conclusions are interesting in the light of ECB. Based on a two-domain tree of life, if EPF homologs existing in TACK-archaeal phyla are mainly orthologs, the search for the ancestral states of eukaryotic compartments in the order suggested by these authors should rely on experimental studies of extant TACK-Archaea. An approximation to the nature of early cell compartments could be addressed with the following question: How are these orthologs distributed and organized cells of Archaea?

Nuclear architecture: Nuclear architecture involves the form and function of nuclear bodies (Misteli 2005). These concepts are inherently related to the question of the origin of the cell nucleus. A special example is the nucleolus. The nucleolus is a multiproteic complex of close to 300 different proteins that are involved with processes as important as programmed cell death, metabolic regulation, cell differentiation, stress, and aging (Boulon *et al.* 2010). All these processes converge to the core process occurring in the nucleolus, namely the transcription and maturation of ribosomal RNA. The nucleolus can be considered a domain of ribosomal gene expression.

The discovery that *Giardia lamblia* (a flagellated protozoan and intestinal parasite with pediatric relevance) harbors a nucleolus (Jiménez-García *et al.* 2008) was for us the motivation to address the evolution of nuclear architecture from an ECB perspective. Phylogenies placed *Giardia* as part of 'primitive' or 'early branching lineage', known as diplomonads. These notions were misleading because eukaryotic phylogenies lack a root. We don´t know if *Giardia* were among the first eukaryotes to branch, and certainly, since *Giardia* are parasites with a long history of secondary losses and modifications of their organelles, there is no compelling argument to assume that they are primitive in comparison to other protozoa. However, this ambiguity of the evolutionary meaning of Giardia revealed the real value of our contribution. Before our confirmation, *G. lamblia* was considered the only anucleolated eukaryote. The discovery of *Giardia* nucleoli implicated a major advance, namely that, regardless of un-rooted phylogenies, nucleoli represent a character shared among all eukaryotes (a synapomorphy). The Last Eukaryotic Common Ancestor (LECA) should also harbor a nucleolus.

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Capítulo III

Evidencia ultraestructural y proteómica para la presencia de un nucléolo putativo en un arqueón

Artículo

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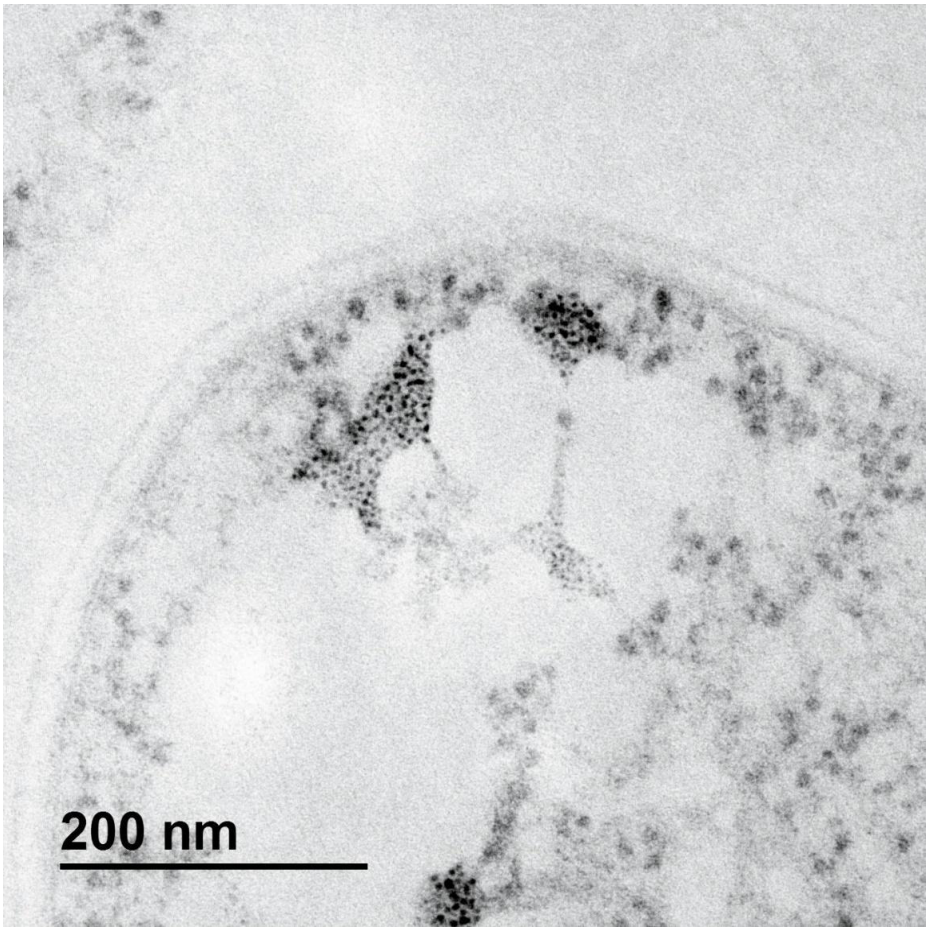

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Ultrastructural and proteomic evidence for the presence of a putative nucleolus in an Archaeon

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Nucleoli are subcellular compartments where transcription and maturation of pre-ribosomal RNAs occur. While the transcription of ribosomal RNAs is common to all living cells, the presence and ultrastructure of nucleoli has been only documented in eukaryotes. Asgard-Archaea, the closest prokaryotic relatives of eukaryotes, and their near relatives TACK-Archaea have homologs of nucleolar proteins and RNAs in their genome, but the cellular organization of both is largely unexplored. Here we provide ultrastructural and molecular evidence for the presence of putative nucleolus-like subcellular domains in the TACK crenarchaeon *Saccharolobus solfataricus* (formerly known as *Sulfolobus solfataricus*). Transmission electron microscopy (TEM) revealed consistent electron-dense fibro-granular compartments, also positive to the specific silver staining for nucleolar organizer regions (AgNOR). TEM also confirmed that ribosomal DNA (rDNA) is spatially distributed in non-random, clustered arrays underlying fine structures, as observed by ultrastructural *in situ* hybridization (UISH). To further explore these observations, proteomic sequencing of isolated bands from AgNOR-stained protein gels was conducted and compared against a compiled inventory of putative nucleolar homologs from the *S. solfataricus* P1 genome. Sequenced AgNOR-sensitive peptides encoded homologs of eukaryotic nucleoli proteins, enriched for nucleolus-related functions. Our results provide first evidence that subcellular domains of nucleolar-like nature are not exclusive to eukaryotes. Based on our data, we propose a model for a putative nucleolus in *S. solfataricus*. Whereas technical limitations and further aspects remain a matter for future functional studies, our data supports the origin of nucleoli within the common ancestor of Eukarya and TACK-Archaea, based on a two-domain tree of life.

KEYWORDS

AgNOR, Archaea, nucleolus, evolution, proteomics, *Saccharolobus*, TACK, microscopy

1. Introduction

Nucleoli are fibro-granular subcellular domains that are the site of ribosomal gene expression, maturation, and regulation. Ribosome biosynthesis comprises more than 50% of eukaryotic gene expression and dictates the formation of nucleoli (Hernandez-Verdun, 2006; Pederson, 2011). The disruption and formation of nucleoli (nucleologenesis) in eukaryotes occurs during mitosis,

particularly in telophase, when pre-nucleolar bodies (PNBs) are formed and then recruited as soon as rDNA transcription re-initiates at chromosomal domains called Nucleolar Organizer Regions (NORs). When the synthesis of pre-rRNA starts, a visible aggregation of ribonucleoproteins forms the nucleolus (Jiménez-García et al., 1989, 1994; Dundr et al., 2000; Hernandez-Verdun, 2006). Mature nucleoli are conspicuous, dynamic, and multi-functional compartments. They are made of rDNA, diverse types of RNAs, and over 300 proteins involved in the fine-tuning of ribosome biosynthesis that are coordinated with processes such as programmed cell death, metabolic regulation, cell differentiation, stress, and aging (Andersen et al., 2005; Grummt, 2013). Given this compositional complexity, the identification and study of nucleoli in most species have initially relied on ultrastructural features and cytochemistry, with the first choice of diagnostic being the presence of an electron-dense fibro-granular domain at the ultrastructural level that is positive to highly specific ammoniacal silver staining of the nucleolar organizer regions (AgNOR) (Smetana, 2011). Although a high degree of morphological heterogeneity is present across nucleoli of different lineages, those diagnostics have remained useful and have become nucleolar characters across phylogeny (Heath, 1980; Thiry and Lafontaine, 2005). Thus, our main understanding of nucleolus molecular physiology comes from functional studies developed in a small group of model organisms where specific antibodies and cell cycle markers are available (mammalian, amphibian, yeast, and plants), leaving a vast field of new research to be unexplored across many lineages and species (Islas-Morales et al., 2021).

In line with this, although ribosomal gene expression and maturation are common to all living cells, the presence of nucleoli has been attributed exclusively to eukaryotic cells, so far. Despite this paradigmatic view, some eukaryotic nucleoli remained elusive over decades. For instance, the ultra-small nucleolus in the protozoan *Giardia lamblia* was not evidenced until 2008 following classical ultrastructural observation and complimentary molecular approaches (Jiménez-García et al., 2008). The lack of a nucleolus in *G. lambia* was at the heart of the debate over the origin of eukaryotes, i.e., the idea of diplomonads and amoebas as proto-eukaryotes, as posited in the archeozoa hypothesis (Cavalier-Smith, 2002). Based on the ultrastructural evidence of a nucleolus in early branching eukaryotes, it is now accepted that the nucleolus existed in the Last Common Ancestor of Eukaryotes (LECA) (Jiménez-García et al., 2008).

The Tree of Life (ToL) has changed exhaustively over time and determining where and when the nucleolus originated could become a new field of study in evolutionary cell biology (Islas-Morales et al., 2021). In a two-domain ToL, where Eukarya and Archaea form a single life domain (Gribaldo et al., 2010), the nucleolus may have evolved gradually among and within sister lineages, rather than exclusively within the Eukarya (from the LECA). For instance, the recently highlighted Asgard-Archaea are the closest relatives of eukaryotes, including many unculturable and newly discovered groups of archaea such as the so-called Lokiarchaea that may display eukaryotic cell features (Spang et al., 2015; Liu et al., 2021). Also, within the Asgard-Archaea closest culturable relatives, the TACK Archaea, a significant number of eukaryotic homologies have been found *in silico* (Guy and Ettema, 2011). In line with this, homologs of major nucleolar elements such as fibrillarin and small nucleolar RNAs have been reported in TACK Archaea (Omer et al., 2000). Nucleolar homologs, for example, have been studied *in silico* in *S. solfataricus*, a culturable TACK-archaeon. Further, when expressed in transfected frog cells, some small nucleolar RNAs from *S. solfataricus* were observed to accumulate in the (eukaryotic) nucleolus (Omer et al., 2000). With the availability of the nucleolar proteomes of yeast and human (Andersen et al., 2005), studies now support that most nucleolar homologs across prokaryotic lineages are present mainly in Archaea (Staub et al., 2004). Furthermore, clusters of homologous genes for ribonucleoprotein complexes such as the SSU processome are also present in Archaea, but not in Bacteria (Feng et al., 2013). However, previous studies have proposed that a nucleolus-like compartmentalization could also occur for the transcription in the bacterium *Escherichia coli*, based on the co-localization of DNA polymerase and the bacterial nucleolar homolog NusB (Jin et al., 2017; Mata Martin et al., 2018). Yet, the search for a putative nucleolus ultrastructure in Archaea and Bacteria using experimental and ultrastructural approaches remains unexplored in the extant literature. We posit that much can be discovered regarding the understanding of archaeal cell structure and evolution when employing omics-oriented microscopy at the nano-level (Islas-Morales et al., 2021). Motivated by the above, we searched for initial evidence of a putative nucleolus ultrastructure in the crenarchaeon *S. solfataricus*. We chose *S. solfataricus* because it is a culturable organism from the TACK Archaea superphylum. Our approach followed ultrastructural examination with subsequent exploration and integration of molecular data. Although our data support the presence of a nucleolus in an Archaeon, technical limitations and further aspects remain to be addressed in future studies.

2. Materials and methods

2.1. *Saccharolobus solfataricus* cultivation

Cultivation was done according to standard procedures (Robertson, 2007). Briefly, the type strain *S. solfataricus* P1 DMSZ 1616 from the German Collection of Microorganisms and Cell Cultures[1] was grown at 80°C in media DSMZ88[2] and DSMZ182.[3] From a two liters bioreactor, samples were taken for processing in Transmission Electron Microscopy (TEM). Cells were harvested at log phase *S. solfataricus* with a cell density of $> 10^9$ cells/mL measured by optical density in a spectrophotometer at 600 nm.

2.2. Microscopy

We examined *S. solfataricus* using Light and Transmission Electron Microscopy (TEM). In the absence of specific antibodies, we focused on nucleolar cytochemistry, morphology, and cytogenetics, such as AgNOR staining positivity, fibro-granular nanodomains, and rDNA clustering (Goodpasture and Bloom, 1975; Scheer et al., 1993; Hernandez-Verdun, 2006; Pederson, 2011).

2.2.1. Light microscopy

Observations of *S. solfataricus* cells in bright field-, phase contrast-, and dark field-microscopy were performed on an Olympus BX41 upright microscope to confirm culture viability using DAPI stain. For visual assessment of AgNOR staining using light microscopy, cells were fixed and processed in slides according to previously described protocols

1 https://www.dsmz.de/collection/catalogue/details/culture/DSM-1616
2 https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium88.pdf
3 https://www.dsmz.de/microorganisms/medium/pdf/DSMZ_Medium182.pdf

(Jiménez-García, 1998; Andersen et al., 2005). We repeated this technique 10 times independently, counting 3,000 cells for each replicate. Following this, the totality of cells was positive to AgNOR staining, which became evident depending on the focal plane.

2.2.2. Transmission Electron Microscopy (TEM)

Standard TEM sample preparation with modifications was carried out for *S. solfataricus* cells (Robertson, 2007; Segura-Valdez et al., 2013). For general morphology with standard heavy metals contrasting, fixation was performed as follows: 300–500 mL of filtered (Millipore 5 µm) cell culture was fixed in 2.5% glutaraldehyde and 4% paraformaldehyde for 1 hour. Fixatives were added directly to the culture. Intermediate washes were carried out in a buffer composed of the salts of medium DMSZ182 adjusted to pH 4 to prevent osmotic damage. After fixation, cells were gently centrifuged at 2800 g and re-suspended in 1 mL of filtered 1% agarose. The suspension was poured into a small Petri dish reaching 2 mm height. This cell mat solidified and was prevented from drying using DMSZ88 medium-buffer. The mat was cut into 2 mm^2 pieces, each was dehydrated with ethanol: 30%; 50%; 70%; 80%; 90%; 95%; 3 × 100% at 10-min intervals at low temperature. Pre-inclusion was carried out with three washes in 100% propylene oxide at 5-min intervals and a final mixture of propylene oxide and Epon 611 (1:1 v/v) for 48 h, and subsequent polymerization in pure resin at 60°C. General contrast staining was performed using UranyLess EMS stain (Electron Microscopy Sciences #22409) and lead citrate under CO_2 limited conditions (Robertson, 2007). Imaging was done using a JEOL 1010 Transmission Electron Microscope at 80 kW.

2.2.3. Ultrastructural AgNOR silver impregnation

This specific staining technique for Nucleolar Organizer Regions (NOR) was performed in *S. solfataricus* cells according to previously described protocols for single cells providing superior results (Jiménez-García, 1998; Jiménez-García et al., 2008). Briefly, cells were double-fixed in 2.5% glutaraldehyde for 1 hour and then washed in Carnoy's solution (glacial acetic acid and 75% ethanol at 1:3 v/v) for 5 min. Cells were rehydrated in a series of 70% and 50% ethanol and bi-distilled water at 10-min intervals. Cells were embedded in 1% agarose cubes as described in section 3 to prevent loss of sample. The AgNOR reaction was started by adding drops of 50% silver nitrate solution (prepared with 1 g $AgNO_3$ in 2 mL distilled water) and incubating for 10 min in hot water (70°C), then washing 5 times with ice-cold water, and then adding 4 drops of the NH_4 reagent solution (4 g $AgNO_3$; 5 mL double-distilled water; 5 mL of concentrated NH_4OH at pH 12–13) and four drops of catalyst. The catalyst used consisted of a 3% formaldehyde solution that has been neutralized with sodium acetate crystals and adjusted to pH 5–6 with formic acid. After the cubes displayed a yellowish coloration, they were washed five times in ice-cold water. Subsequently, we proceeded with standard EM dehydration and embedding in Epon 600, as described under the TEM section. We repeated this procedure three times independently, sampling 30 random cells in each replicate. We calculated an AgNOR average signal density of 80% from a mean value of $n = 24 \pm 2$ cells showing a positive signal. Of note, some positive cells may have escaped our observation due to the focal plane.

2.2.4. Ultrastructural *in situ* hybridization of 16S and 23S ribosomal regions

We performed ultrastructural *in situ* hybridization (UISH) of 16S and 23S rDNA genes, because nucleoli are ribonucleoprotein compartments. According to standard procedures (Segura-Valdez et al., 2013), fixation was performed as follows: 300–500 mL of filtered (Millipore 5 µm) cell culture was fixed in a glutaraldehyde 2.5% and paraformaldehyde 0.5% mixture for 1 hour. Intermediate washes and dehydration were performed as detailed under general morphology. Embedding was carried out after dehydration using an increasing proportion of Lowicryl K4M and Ethanol (1:2; 1:1; 2:1; v/v) and absolute resin for 24 h at −20°C. K4M polymerization was carried out at low temperature under a UV light source. Imaging was done using a JEOL 1010 Transmission Electron Microscope operating at 80 kW.

To prepare the probes, aliquots of 1.5 mL of archaeal cultures were centrifuged at 10,000 g for 10 min, cell pellets were washed twice with 200 µL of sterile water and resuspended in 100 µL of ATL lysis buffer (Qiagen) to proceed with the DNA extraction using the DNeasy Blood & Tissue Kit (Qiagen). UISH was performed according to standard protocols (Segura-Valdez et al., 2013). Briefly, primers 27F and 1492R (Lane, 1991) and 189F and 2490R (Hunt et al., 2006) were used to amplify 16S and 23S rRNA gene regions from a *S. solfataricus* cell culture, respectively. Amplification was conducted until reaching a rDNA amplicon concentration of 1 mg/mL. UISH probes were generated using a nick translation mix following standard protocols (Segura-Valdez et al., 2013). The labeling reaction with Biotin dUTP was performed on ice with 16 µL sterile double distilled water containing 1 µg rDNA with equimolar amounts of the generated 16S and 23S amplicons and 4 µL of Biotin-Nick Translation Mix (Sigma Aldrich). The nick translation mix contained: 5 × concentrated reaction buffer, 50% glycerol, DNA Polymerase 1, DNase 1, 0.25 mM each of dATP, dCTP, dGTP, 0.17 mM dTTP, and 0.08 mM Biotin-dUTP. After brief centrifugation, the mixture was incubated at 15°C for 90 min and chilled to 0°C. The reaction was stopped with 1 µL 0.5 M EDTA (pH 8.0) and heating at 65°C for 10 min. The length of the probes (200–500 nucleotides) was checked using an agarose gel with a DNA size marker. Probes were stored in TE buffer (10 mM Tris HCl, 1 mM EDTA, pH 8.0) at −20°C. The hybridization reaction was performed "post-embedding" on 200 Mesh gold grids carrying sections from *S. solfataricus* in Lowicryl K4M resin. In order to hybridize, the native DNA present in the TEM sections on the gold grids as well as the amplicon DNA present in the hybridization mix (composed of 12 µL of probe amounting to ~170 ng/µL, 2.5 µL of 20x SSC, and 12.5 µL of formamide), both were denatured separately at 100°C in bowling water for 5 min. Hybridization took place by placing one drop of hybridization mix on the grid with the TEM sections and incubating at 37°C overnight, preventing evaporation. Subsequently, an immunoreaction was performed using GOAT-ANTI-BIOTIN antibody coupled to 10 nm Nanogold (EMS). This was incubated in PBS (1:10) for 30 min. After washing repeatedly in PBS and water, grids were contrasted with UranyLess stain (Electron Microscopy Sciences #22409) and observed in a Transmission Electron Microscope FEI Tecnai twin at 80 kW. We repeated this procedure three times independently, sampling 30 random cells in each replicate. We calculated an average hybridization signal density of 82% from a mean value $n = 24.6 \pm 1.2$ cells exhibiting a positive signal. Of note, some positive cells may have escaped our observation due to the focal plane.

2.3. Proteomics

For our proteomics approach, we employed an *in vitro* variant of the AgNOR method by staining *S. solfataricus* protein extracts that were separated on an SDS-PAGE protein gel (Lischwe et al., 1979). Our strategy was to utilize the conditions and specificity of the AgNOR

method employed for TEM. This resulted in a discrete banding pattern with remarkable contrast in the SDS-PAGE protein gel (Supplementary Figure S4) in contrast to CBB staining (Supplementary Figure S5). We excised AgNOR-positive bands that contained AgNOR-labeled peptides and subjected them to ancillary mass spectrometry analysis coupled to MALDI-TOF sequencing (Chevallet et al., 2006). The detailed procedures were carried out as detailed below.

2.3.1. AgNOR staining in SDS-PAGE gels from *Saccharolobus solfataricus* protein extracts

AgNOR staining in SDS-PAGE gels was done according to established procedures (Lischwe et al., 1979; Buys and Osinga, 1984; Trerè, 2000). Briefly, 300–500 mL of filtered (Millipore 5 µm) cell culture was centrifuged and concentrated into a pellet. The resuspended pellet was denatured at 100°C for 3 min in Laemmli buffer adding 4x SB containing 400 mM DTT (31 mg/500 µL) to a final concentration of 1×. This solution was then incubated at 90°C for 2 min and loaded onto a 10% 1-D polyacrylamide gel and run at ~10–12 mA. The SDS-PAGE gel was fixed in Carnoy's solution for 1 hour, washed with deionized water, and incubated for 2 hours in borate buffer (0.1 M Na_2S0_4 and 0.005 M $Na_2B_40_7$, pH 9.2). Incubation with 50% aqueous silver nitrate took place overnight at 50°C. Bands were already apparent after this step. The gel was placed in 3% formalin to contrast it more clearly (Goodpasture and Bloom, 1975; Lischwe et al., 1979). This method resembles the condition for nucleolar staining in cytological preparations (Trerè, 2000).

2.3.2. Mass spectrometry preparation and analysis

In preparation for MALDI-TOF, we followed an ancillary silver distaining protocol for mass spectrometry and subsequent standard procedures for in gel digestion and peptide extraction (Gharahdaghi et al., 1999; Chevallet et al., 2006; Shevchenko et al., 2006). Briefly, gel bands were covered in 30 mM aqueous potassium ferricyanide $C_6N_6FeK_3$ and 100 mM sodium thiosulfate $Na_2S_2O_3$ in equal volumes until the stain was removed approximately 6 min after washing with water. The pieces were soaked in 200 mM ammonium hydrogen carbonate $(NH_4)HCO_3$ for 20 min (approximately 0.15 mL per gel slice), washed again, and stored dry at −20°C. In-gel protein digestion was performed as follows: to extract the protein part for MALDI-TOF, the distained gel sections were reduced in 10 mM of dithiothreitol (DTT) in 100 mM $(NH_4)HCO_3$ at 37°C for 30 min, and then alkylated in 50 mM of iodoacetamide (IOA) in 100 mM $(NH_4)HCO_3$ for 1 hour, and subsequently washed, dehydrated, and rehydrated. Finally, the gel slices were digested with Trypsin (12.5 ng/µL) at 37°C for 16 h. Proteins were then extracted with 5% acetic acid and 50% acetonitrile (ACN, CH_3CN). After extraction, peptides were washed twice in 0.1% trifluoracetic acid TFA (CF_3COOH), eluted in 75% ACN, and completely dried by SpeedVac. The lyophilized protein extract was re-dissolved with 0.1% FA in HPLC-grade H_20 and quantified by NanoDrop at A280. Concentrations were normalized across samples for DIA/SWATH-MS analysis. Indexed retention time (iRT) standards (Biognosys, Ki30021) were added to the ready to inject peptide mixture at a 3:10 ratio (v/w) to Ultraflex III MALDI-TOF/TOF MS (Bruker).

2.4. Genomics

For our genomic analysis, we compiled a list of candidate nucleolar protein homologs in the *S. solfataricus* P1 genome for subsequent comparison to protein complements of eukaryotic nucleoli.

Additionally, we conducted a Gene Ontology (GO) analysis to identify enriched biological processes and molecular functions prevalent among precipitated proteins derived from the MS analysis of the AgNOR stained peptides in SDS-PAGE gels.

2.4.1. Nucleolus related genes in *Saccharolobus solfataricus*

Amino acid translated genes of the genome of *S. solfataricus* strain P1 (GenBank accession number NZ_LT549890.1) were annotated using the KEGG Automatic Annotation Server (KAAS) (Moriya et al., 2007) with the BBH (bi-directional best hit) method against the representative eukaryotic set of genes. Only gene features with KEGG assignments affiliated to the pathway "Ribosome biogenesis in eukaryotes" (ko03008) were considered in the analysis. In addition, a hidden Markov model (HMM) profile search was performed using HMMER3 v.3.1b2 (Eddy, 2011) to identify protein domains of the nucleolus reported previously (Staub et al., 2004).

2.4.2. Gene Ontology (GO) enrichment of AgNOR-stained proteins extracted from SDS-PAGE

Peptide sequences of the proteins extracted and analyzed with MALDI-TOF were aligned to the translated gene features of the *S. solfataricus* strain P1 genome (GenBank accession number NZ_LT549890.1) using the BLASTp algorithm (Supplementary Data). Only hits with a 100% identical match were allowed (no mismatches). All protein features were annotated against the UniProtKB/SwissProt database (The UniProt Consortium, 2018) and only hits with an E-value $<10^{-3}$ were considered. GO annotations were obtained from UniProt IDs by parsing a GOA gene association file (available at: ftp.ebi.ac.uk/pub/databases/GO/goa/UNIPROT/). A Gene Ontology (GO) enrichment analysis was done on the precipitated proteins using the "weight01" method implemented in the R package TopGO v.2.42.0 (Alexa and Rahnenfuhrer, 2022). Resulting *p* values were adjusted for multiple testing using the false discovery rate (FDR) method (Benjamini and Hochberg, 1995), and GO categories with an adjusted value of *p* (*q*-value) below 0.05 were considered enriched.

3. Results

By light microscopy, we consistently noticed a strong AgNOR impregnation in the totality of examined cells (Figures 1A1,A2). By general TEM, we frequently observed electron-dense intracellular bodies with an outstanding fibro-granular morphology in *S. solfataricus* cells at low and high magnifications (Figures 1B1,B2, 2; Supplementary Figures S1, S2), highly similar to the ultra-small nucleoli from *G. lamblia* (Jiménez-García et al., 2008). Furthermore, we observed a conspicuous, specific, and strong AgNOR silver impregnation in *S. solfataricus* cells by electron microscopy (Figures 1C1,C2). This result is relevant because ammoniacal silver impregnation of the Nucleolar Organizer Regions (AgNOR) is a specific technique for argyrophilic proteins associated to the NOR and evidences the presence of NORs, pre-nucleolar bodies, and consequently nucleoli at the ultrastructural level (Goodpasture and Bloom, 1975; Jiménez-García et al., 1994; Pederson, 2011; Grummt, 2013). Thus, for the first time we provide evidence that fibro-granular structures and AgNOR signals are present as discrete domains in Archaea.

Ultrastructural *in situ* hybridization (UISH) of 16S and 23S rDNA under denaturing conditions showed expected localization of both

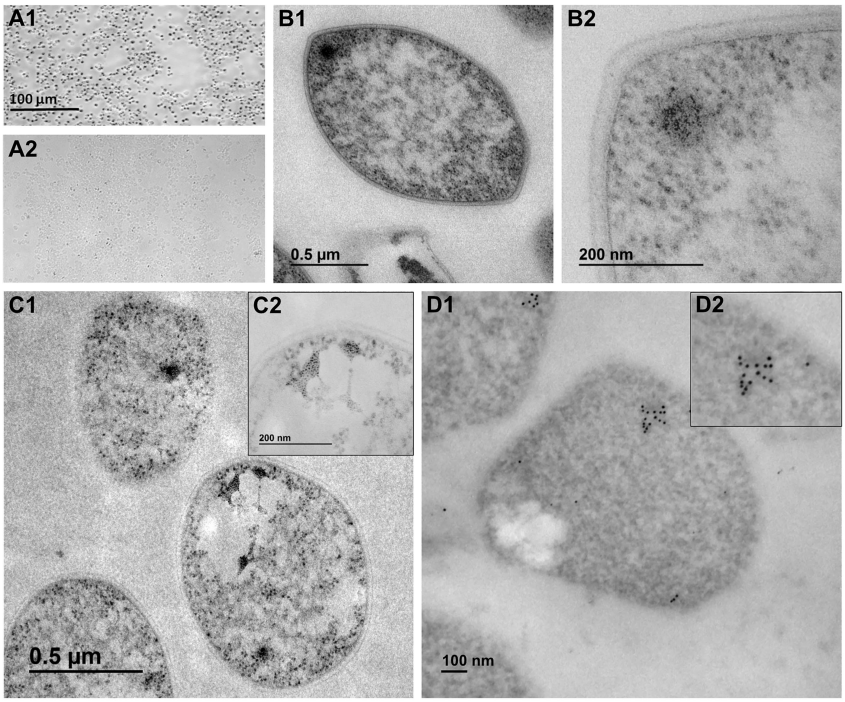

FIGURE 1
Light and electron microscopy observations support the presence of putative nucleolus-like domains in the crenarchaeon *Saccharolobus solfataricus* at the ultrastructural level. **(A1)** Light microscopy of the AgNOR reaction. **(A2)** Light microscopy of unstained *S. solfataricus* cells (negative control). **(B1)** Transmission Electron Microscopy (TEM) of *S. solfataricus* cells, with **(B2)** detail of discrete fibro-granular structures. **(C1)** Ultrastructural AgNOR impregnation of subcellular structures, with **(C2)** high magnification of AgNOR positive subcellular structures. **(D1)** Ultrastructural *In situ* hybridization (UISH) of 16S and 23S rDNA clusters, with **(D2)** magnified image showing an underlying electron-dense region.

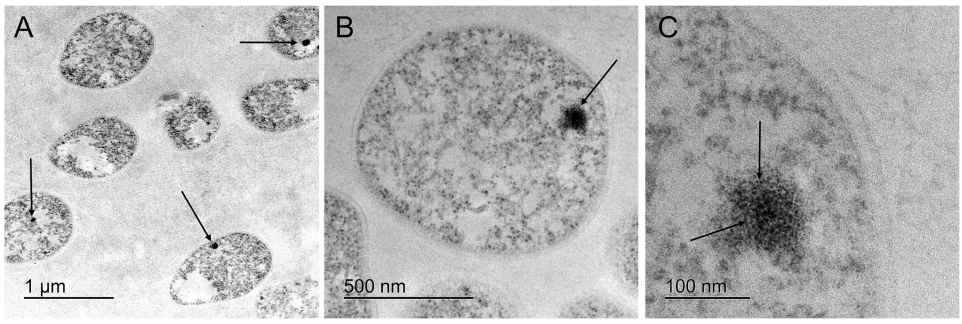

FIGURE 2
Transmission electron microscopy of fibro-granular, putative nucleolus-like domains in cells of the crenarchaeon *S. solfataricus*. **(A)** Arrows show electron dense regions, present in most cells at low magnification. **(B)** Single *S. solfataricus* cell with an electron dense domain, in which a fibro-granular morphology is evident. **(C)** Single *S. solfataricus* cell at high magnification. Arrows show the electron dense region where granules in the central part and fibers toward the outside can be observed. A differentiation of structural substrate is evident in comparison to the surrounding cytoplasm.

rDNA and rRNA. The distribution of ribosomal genes and their transcripts in *S. solfataricus* may correspond to single and discrete subcellular clusters with an underlying electron-dense region (Figures 1D1,D2). Notably, for UISH under non-denaturing conditions (Supplementary Figure S3), it is expected to only detect a signal from rRNA. Despite broader signal distribution, a concentration of discrete subcellular clusters was evident, corresponding to the observation under UISH under denaturing conditions. Although colocalization of rDNA and rRNA cannot be claimed, observations from UISHs under denaturing and non-denaturing conditions are complementary and suggest that ribosomal transcripts may be spatially related to a subcellular domain. According to nucleologenesis, while NORs are strictly associated with rDNA, pre-nucleolar bodies (PNBs) and other discrete elements of nucleolar origin do not necessarily contain

ribosomal genes (Jiménez-García et al., 1989, 1994). In this sense, our UISH observations are consistent with the notion of a single locus of rDNA in the *S. solfataricus* genome and complement the observation of multiple AgNOR silver impregnations associated to discrete subcellular domains with remarkable contrast. Following the above, we conclude that *S. solfataricus* AgNOR-sensitive proteins accumulate at specific locations and share a similar composition with eukaryotic NORs and nucleoli.

In addition to the AgNOR-based stainings, it is important to consider the consistency of ultrastructural observations using general contrast techniques without specific stains. Figure 2A shows electron-dense subcellular domains in *S. solfataricus*. Figures 2B,C display higher magnifications and detail of a nucleolar-like fibrogranular morphology of subcellular domains in *S. solfataricus*, resembling a typical nucleolar ultrastructure. Despite nucleolar morphology being highly heterogeneous across the ToL, especially in early branching protists, our observations are consistent with a nucleolus that features two main morphological components: a *pars fibrosa* and a *pars granulosa*, which constitute accepted criteria for nucleoli outside amniotes following previous studies (Thiry and Lafontaine, 2005).

To explore which molecular elements underlie the newly discovered nucleolus-like structure, we stained *S. solfataricus* protein extracts using the AgNOR method and subsequently separated them on a SDS-PAGE protein gel. AgNOR-positive bands were excised and sequenced, resulting in 1,618 distinct peptides of which 1,527 exhibited perfect matches (100% similarity) to 376 proteins in the *S. solfataricus* P1 genome, referred to in the following as "precipitated proteins" (Supplementary Tables S1, S2). These included nucleolus proteins, such as: NOP1 (fibrillarin), NOP5, L7Ae, L30Ae, L31Ae, and PUA (pseudouridine synthase), all of which are involved in ribosomal RNA (rRNA) maturation and small nucleolar RNA (snoRNA) metabolism. We also found evidence of RNA polymerase, essential to form nucleolar organizers by initiating rRNA transcription. Gene Ontology (GO) analysis corroborated that "translation", "structural constituent of the ribosome", "rRNA binding", and "unfolded protein binding" were prominent processes among precipitated proteins and significantly enriched (FDR $\leq$ 0.05, Supplementary Table S3). This suggests that proteins in the putative nucleolus-like compartment harbor functions associated with ribosomal expression and RNA binding and are potentially capable of forming macromolecular ribonucleoproteins, which are the core components of non-membranous organelles such as nucleoli and Cajal bodies (Dundr et al., 2000; Pederson, 2011). Of note, as known from eukaryotic nucleoli, not all nucleolar proteins are argyrophilic and thus amenable to AgNOR staining (Sirri et al., 2000). Additionally, not all eukaryotic nucleolar proteins have homologs in Archaea. Consequently, some known AgNOR-positive proteins from Eukarya, such as nucleophosmin and nucleolin were not present in our proteomics analysis, as expected (Lischwe et al., 1979; Hernandez-Verdun et al., 1980).

To obtain a comprehensive view of nucleolar elements in Archaea, we used genomics analysis to complement identified proteins based on AgNOR staining. Using alignment-based approaches, we identified 18 genes related to the KEGG pathway "ribosome biogenesis" (Supplementary Table S4) and a further 41 genes (Supplementary Table S5) identified by the presence of previously reported nucleolus-related domains (Staub et al., 2004). The identified 59 gene homologs encoded for 36 distinct proteins. We propose that these 36 proteins constitute a 'minimal set' of nucleolar elements (Supplementary Table S6). Ten of these proteins were confirmed by AgNOR-stained proteomic sequencing (see above).

In summary, various lines of evidence confirm the presence of nucleolar elements in *S. solfataricus* both *in situ* and *in silico* (Supplementary Figure S6). First, the existence of refringent, electron-dense, and AgNOR-positive nucleolar-like domains is supported by optical and transmission electron microscopy. This observation is consistent with the genome architecture and non-random distribution of 16S/23S rDNA sequences and accumulating rRNA as evidenced by ultrastructural *in situ* hybridization (notably, UISH under denaturalizing conditions can detect rDNA and rRNA). Further, peptide sequencing of AgNOR-stained protein gel bands supports the notion that the silver-stained proteins in the putative nucleolus-like compartments are associated with nucleolus-ascribed structures and functions, such as RNA-protein interactions and pre-rRNA maturation. Further, genomics analysis concludes our understanding of nucleolar elements in *S. solfataricus* by linking silver-stained proteins with a core set of nucleolus homologs, including non-argyrophilic nucleolar proteins that escaped AgNOR-based peptide sequencing.

4. Discussion

Our comprehensive data allowed us to conceptualize a putative ribosome biogenesis pathway in *S. solfataricus*. This is an initial and complementary proposal, which also should be analyzed in light of extensive works on the evolution of ribosome biogenesis in Archaea (Ebersberger et al., 2014; Birikmen et al., 2021). Using the KEEG pathway ko03008 ("ribosome biogenesis in eukaryotes") as a basis and adapting it to an archaeal cell model, we hypothesize that the identified protein homologs co-reside in the putative nucleolus-like domains, which provides additional insights worthy of discussion (Figure 3). For instance, argyrophilic proteins L7Ae and fibrillarin (NOP1) as well as non-argyrophilic EMG1 and NOP2 stand out because they are part of the nucleolar organizer C/D and H/ACA box ribonucleoprotein (Pederson, 2011). This complex directs the highly dynamic post-transcriptional modification of rRNA, in the form of methylations and pseudouridylations (Hernandez-Verdun, 2006; Pederson, 2011). The study of how nucleolar elements move and organize as part of this process has been instrumental in understanding nano-scale nucleic acid-protein interactions and its relation to protein disease and aging research (Jiménez-García et al., 1994; Grummt, 2013). We think that our findings may provide an ancestral view of this kind of nano-scale dynamism that could further contribute to the study of nucleolus biology and related diseases through provision of a minimal model.

Apart from this, the phylogenetic relationship of NOP1 (fibrillarin) and NOP2 deserves further discussion. While NOP2, which codes for archeosine transglycosylase in Archaea is not argyrophilic, it shares a methyltransferase domain with the AgNOR-positive NOP1 (Rodriguez-Corona et al., 2015). Based on this, we hypothesize that during the early stages of nucleolar evolution, argyrophilic proteins may have aggregated around acidic DNA environments, which then served as a subsequent recruitment site for factors involved in post-transcriptional rRNA modification. Many proteins in the putative nucleolus share domains and may perform different functions that became more specialized over time, i.e., adapted further in species with different cellular architectures. Of note, our understanding of how proteins are organized inside the archaeal cell and where cellular processes occur spatially is still limited. For this reason, it caught our attention that protein domains of the signal recognition particle protein SRP19 were present in our data (Supplementary Table S6). In eukaryotes, SRPs are associated with the nucleoli in eukaryotes because SRP-dependent mRNA targeting for

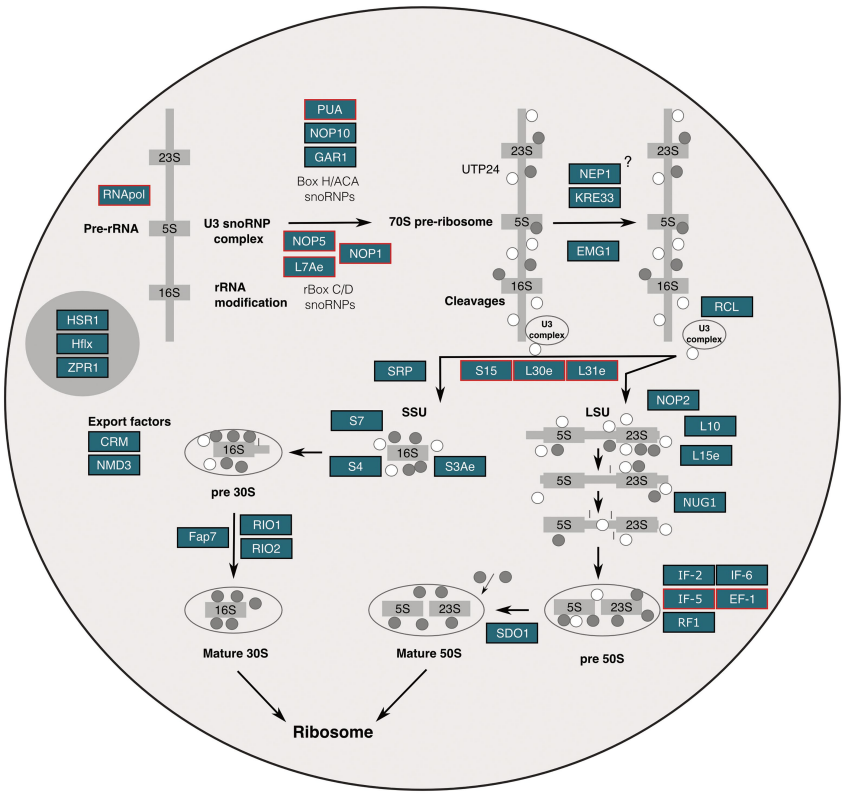

FIGURE 3
Molecular organization of the putative nucleolus of *S. solfataricus* based on ultrastructural, molecular, and genomics evidence. Nucleolar elements identified in the *S. solfataricus* genome through homology search or by means of protein domain prediction are colored in blue boxes; proteins supported by proteomic evidence from sequencing of AgNOR fractions are highlighted in red.

proteins destined to enter the secretory pathway via the endoplasmic reticulum (ER) and subsequent packaging into vesicles in the Golgi apparatus occurs in the nucleolus (Pederson, 2011). However, since Archaea lack an ER and Golgi apparatus, we hypothesize that SRPs are involved in other, potentially similar functions, given their spatial relationship to the nucleolus, which would then precede their secretory signaling functioning, as suggested by our data.

Notably, in contrast to eukaryotic translation where the nucleolus is separated by a nuclear envelope, *S. solfataricus* rRNA transcription and translation occur simultaneously and can be linked spatially to a non-membranous compartment. As a matter for future studies, the accumulation and mobility of proteins putatively inherent to the archaeal cell or genome architecture may result in nucleolus-like multifunctional complexes as subcellular organizers. For instance, if the putative nucleolus is multifunctional (e.g., eukaryotic nucleoli are involved in programmed cell death, metabolic regulation, cell differentiation, stress, and aging), then this may explain the presence of the many archaeal AgNOR-sensitive proteins that we identified that have no apparent functional relation to a eukaryotic nucleolus. The fact that we identified all these elements together in an archaeal nucleolus-like domain through the combination of microscopic and molecular approaches provides an initial understanding for a putative nucleoli in relation to archaeal cell organization. It also opens the door to address outstanding questions, such as how widespread the here-described nucleolus-like structure is across other groups of Archaea or Bacteria, or whether nuclear bodies (e.g., nucleolus, Cajal bodies, etc.) evolutionary predate the presence of a nuclear envelope.

Taken together, we think that our findings may be the beginning of a possible paradigm shift regarding the evolutionary origin of the nucleolus, the nucleus, and the cellular organization and complexity in the prokaryote-eukaryote divide. We posit that the first nucleoli have been discrete fibro-granular and argyrophilic domains whose nature may have been proteinaceous, gene-expression based, subcellular organizers, combining space, structure, and function in the erstwhile randomly distributed prokaryotic cytoplasm.

In support of this, a recent approach using chromosome capture (Takemata et al., 2019; Takemata and Bell, 2021) shows that Crenarchaeotes display a refined mechanism for chromosomal organization by coalescin proteins mediated grouping of distant loci, depending on levels of gene expression. Although we did not identify coalescin in our analysis, a putative archaeal nucleolus relates also to a form of subcellular organizer and chromatin organization. While coalescin is enriched in the so-called B chromosomal compartments that harbor fewer active genes, transcriptionally active A compartments containing rDNA loci are presumably particularly suitable for a nucleolus associated gene expression, organization, and regulation. Analogous to the concept of the eukaryotic nuclear architecture (i.e., Cajal and Polycomb bodies and puffs), gene expression in Archaea

should relate to subcellular structures (like nuclear bodies) arranging genes, RNAs, and proteins, even in the absence of a nuclear envelope. Although past studies (Gaal et al., 2016) claimed that transcriptional active regions in bacteria were a reminiscence of the nucleolus, we think that the search for putative nucleoli should start in Archaea with an integrated approach. As outlined elsewhere (Takemata and Bell, 2021), guiding exploration based on phylogenetics and integrating microscopy, particularly TEM and super-resolution, to genomic, proteomic or transcriptomic approaches, e.g., 3C-Seq mapping, has the potential to provide prolific insights to the cell biology and evolution of nucleolus and ribosome biogenesis from Archaea to Eukarya. The same authors also suggest that a putative nucleolus may be possible in *S. solfataricus*, from a genome architectural point of view.

Despite our diverse lines of evidence, we want to emphasize that many technical limitations are still to be faced. For instance, it remains to be determined whether the here-observed AgNOR-stained subcellular structure(s) colocalize with rDNA/rRNA, due to the cytochemical nature of AgNOR stain that is not compatible to perform colocalization with UISH or antibodies in the same sample. Further, the use of transcription inhibitors such as actinomycin D, could be a greatly complementary approach to assess nucleologenesis, i.e., formation and disruption of nucleoli as inferred from the loss of AgNOR signal. We discern that such functional experiments, in addition to other approaches, such as incorporation of bromouridine to confirm transcriptional activity or the use of specific antibodies and RNA probes to localize proteins and snoRNAs *in situ*, will become an exciting perspective for the evolutionary cell biology community. Of note, actinomycin D reported doses are specific for eukaryotic polymerase I (Sirri et al., 2000), and inhibitors, antibodies, and *in situ* probes are still limited in Archaea research. Particularly, the development of a set of specific antibodies against archaeal ribosomal and nucleolar homologous proteins would be necessary to colocalize each protein.

On the contemporary picture of a two-domain tree of life, Eukaryotes are a branch within the TACK Archaea (Gribaldo et al., 2010). The presence of proto-nucleoli in species of the TACK Archaea suggests that the origin and evolution of the nucleolus traces back through archaeal phylogeny to diverse common ancestors, initially that of Eukarya and TACK-Archaea. It does not escape our minds that the presence or absence of this kind of proteinaceous organelles should be determined in more representatives of Archaea or even Bacteria: a motivation for emergent evolutionary cell biology. By mapping the presence and absence of nucleolar elements (molecular and structural) on the archaeal and bacterial phylogeny, we might be able to propose or reject a gradualist scenario of nucleolar evolution and grasp a more profound understanding of nanoscopic cell architectures.

Data availability statement

All data needed to evaluate the conclusions are present in the manuscript, the Supplementary material, and the References therein. Amino acid translated genes of the genome of *S. solfataricus* strain P1 (GenBank accession number NZ_LT549890.1) are available at https://ftp.ncbi.nlm.nih.gov/genomes/all/GCF/900/079/115/GCF_900079115.1_SSOP1/GCF_900079115.1_SSOP1_protein.faa.gz. All protein features were annotated against the UniProtKB/SwissProt database available at https://ftp.uniprot.org/pub/databases/uniprot/current_release/knowledgebase/complete/uniprot_sprot.fasta.gz. GO annotations were obtained based on UniProt IDs by parsing a GOA gene association file available at http://ftp.ebi.ac.uk/pub/databases/GO/goa/UNIPROT/.

Author contributions

PI-M conceptualized this research under supervision of LJ-G and CV, was involved in all stages of the project, is the main author, and wrote the manuscript. AC contributed to experimental design in genomics and proteomics, supported PI-M in related laboratory procedures, analyzed results from proteomics and genomics, and wrote the manuscript. MM supported PI-M with standardization of *S. solfataricus* cultivation, in Danielle Daffonchio´s laboratory at KAUST. LJ-G holds senior authorship, participated intellectually in the conceptualization and experimental design of this research as doctoral supervisor of PI-M, particularly supported PI-M on the UISH and TEM observations, and wrote the manuscript. The consolidated line of research of LJ-G is the structure, function, and evolution of the nucleolus. CV supported this project financially, delivered infrastructure for its completion, was intellectually involved in all stages of the project, and wrote the manuscript. All authors contributed to the article and approved the submitted version.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

Supplementary material

The Supplementary material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmicb.2023.1075071/full#supplementary-material

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Supplementary Figures

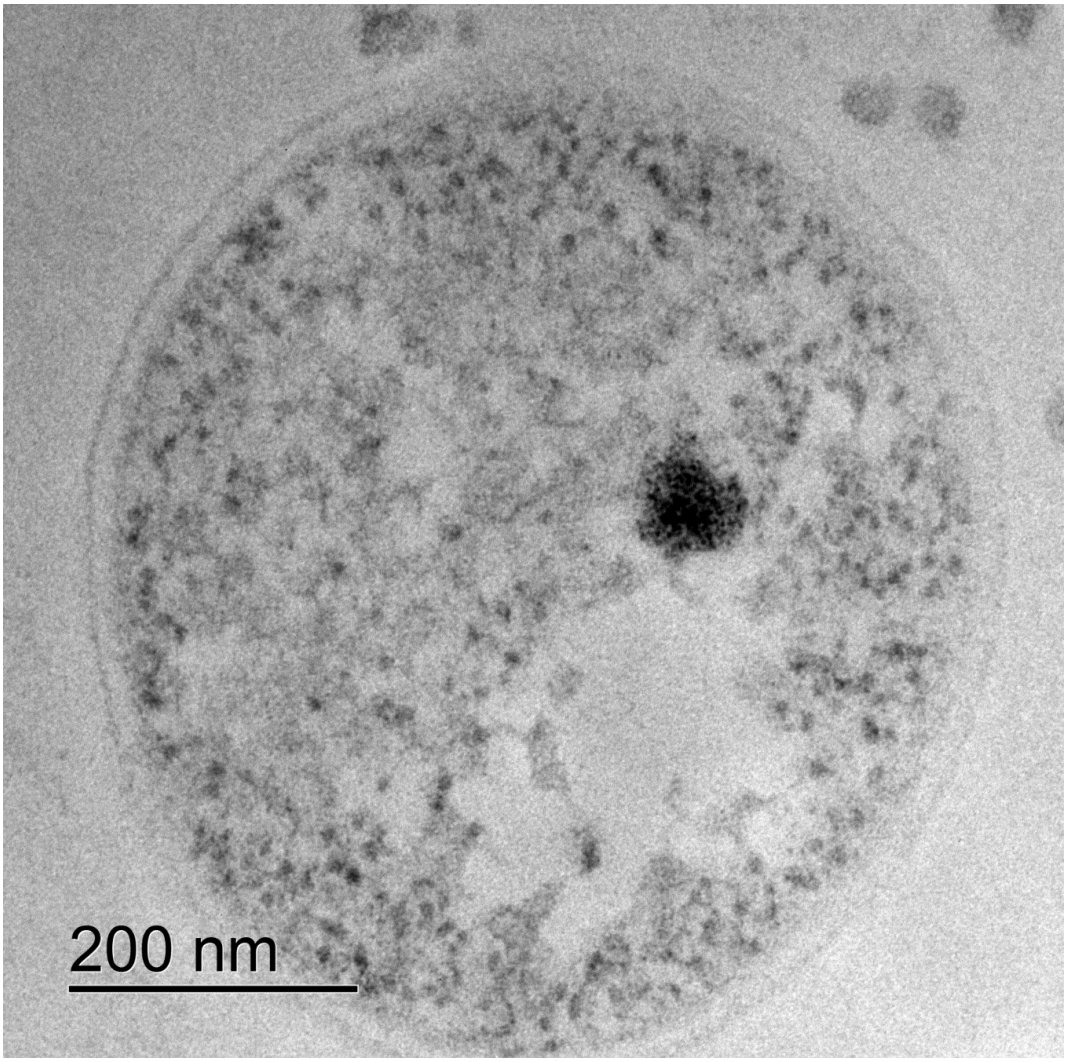

Figure S1. Single *Saccharolobus solfataricus (formerly known as Sulfolobus solfataricus)* cell with a nucleolus-like domain in which a fibro-granular morphology is evident.

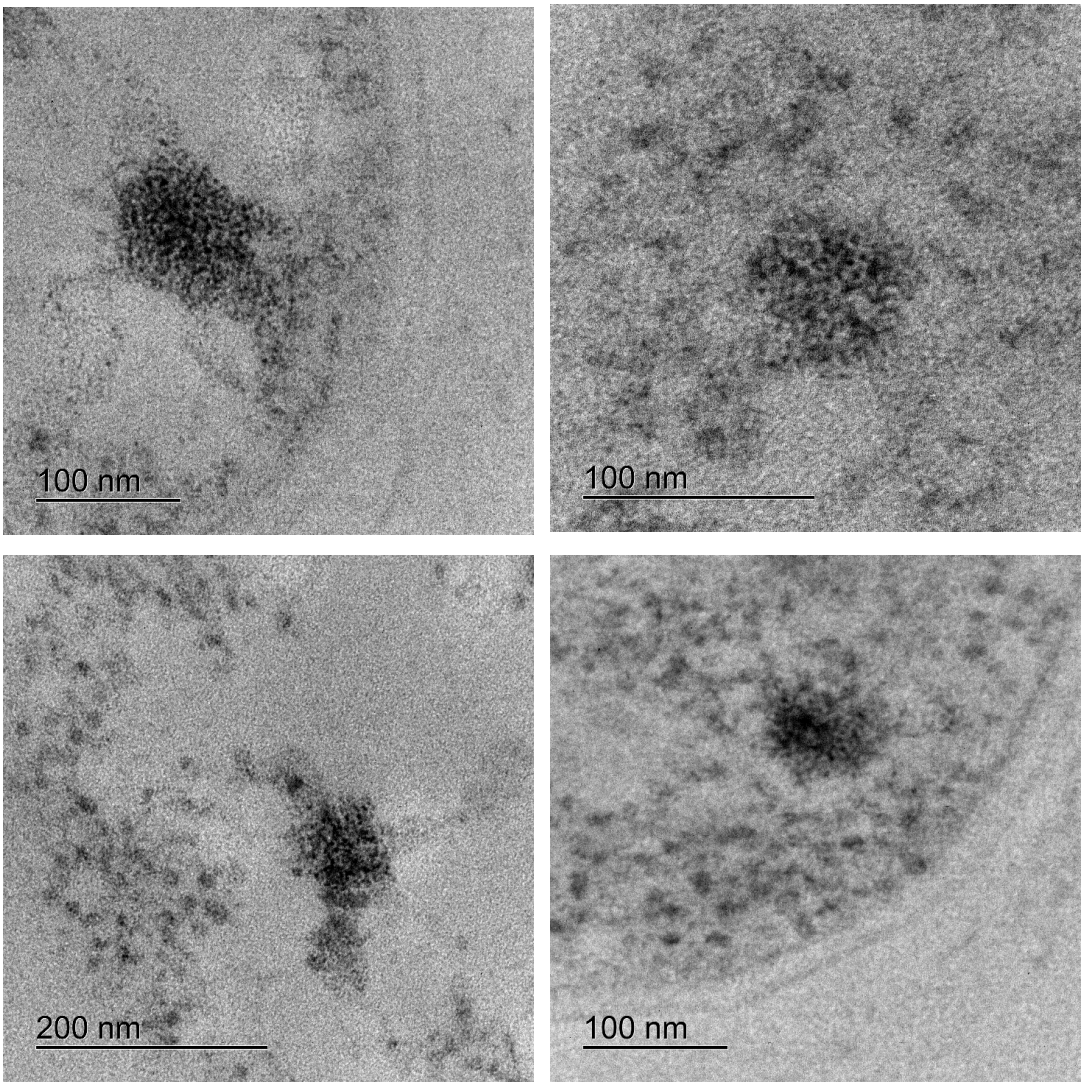

Figure S2. High magnification of four different nucleolus-like domains from four distinct cells of *S. solfataricus*. Granules and fibers are condensed within the respective nucleolus-like domains, contrasting in size and form with the surrounding cytoplasm. All four domains from four distinct cells share a common nucleolar morphology

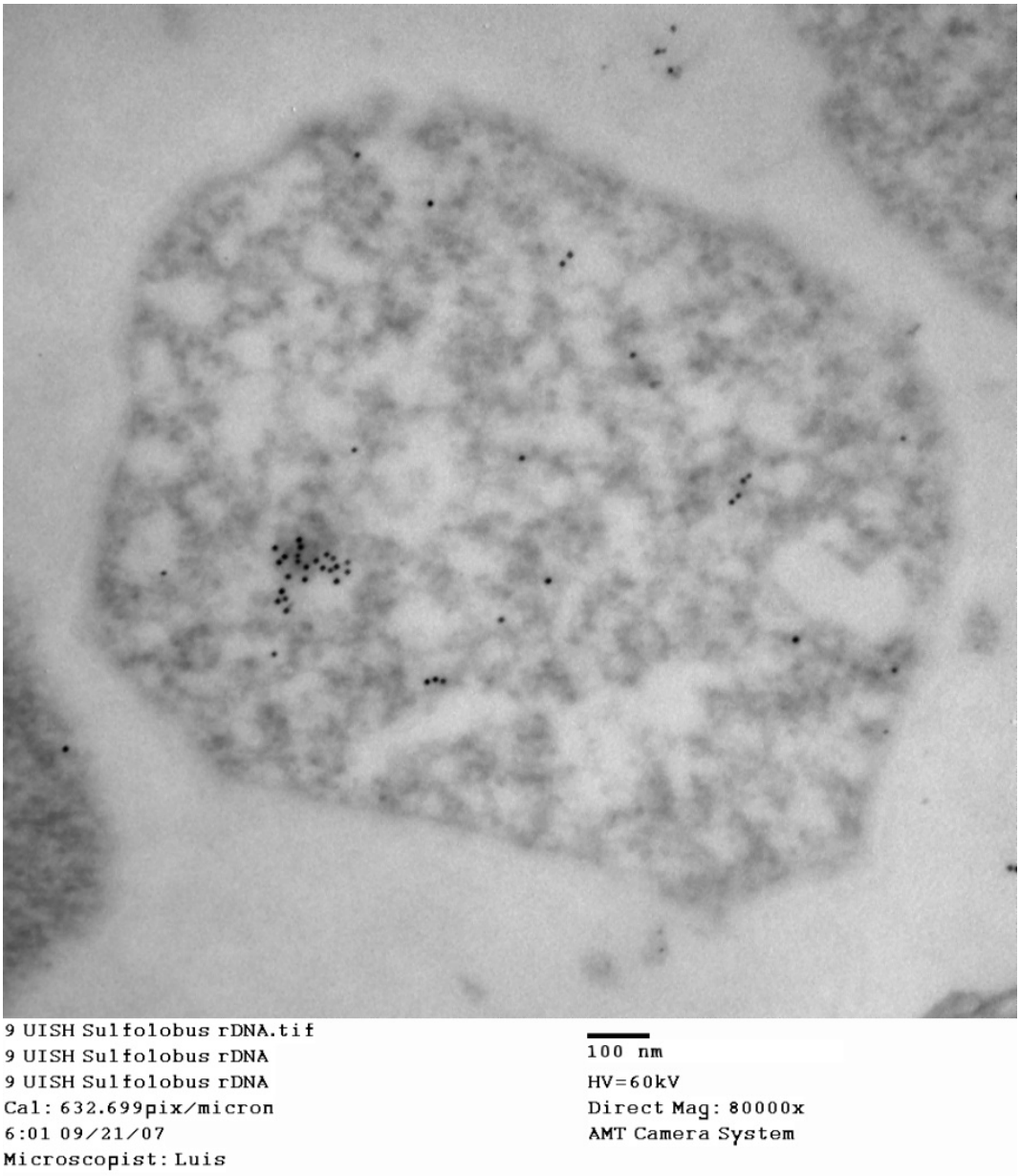

Figure S3. UISH of rRNAs under non-denaturalizing conditions.

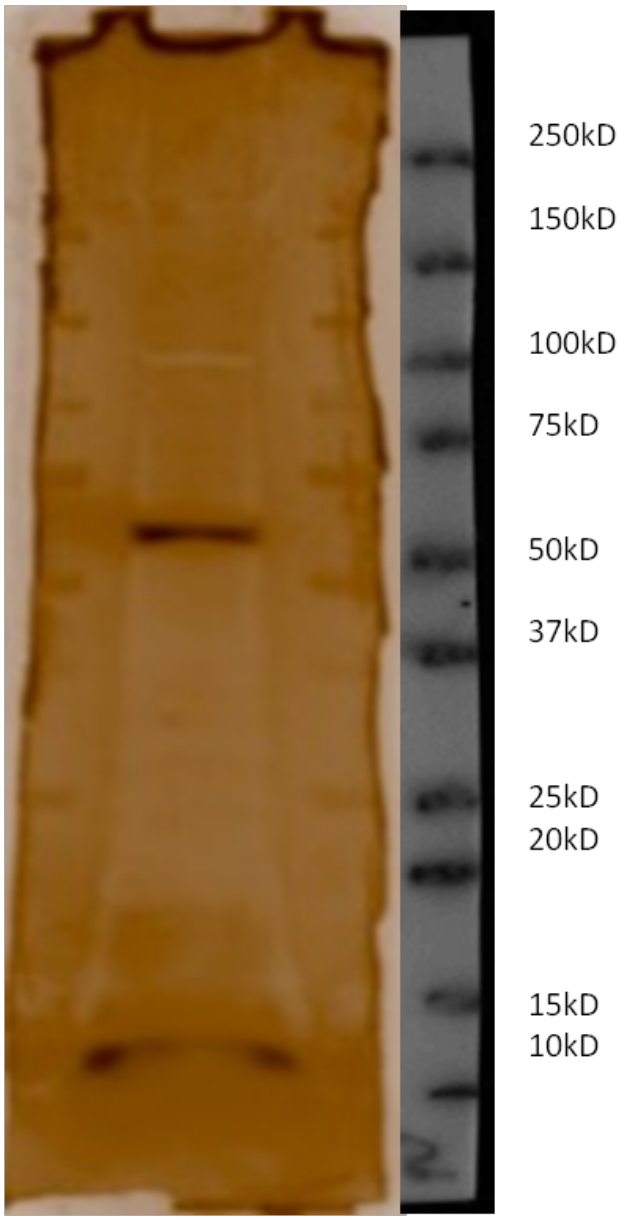

Figure S4. AgNOR banding pattern on SDS-PAGE form *Saccharolobus solfataricus* protein extracts. Black bands show concentration of argyrophilic peptides that were excised and sequenced with ancillary mass spectrometry. To the left is a peptide size reference marker.

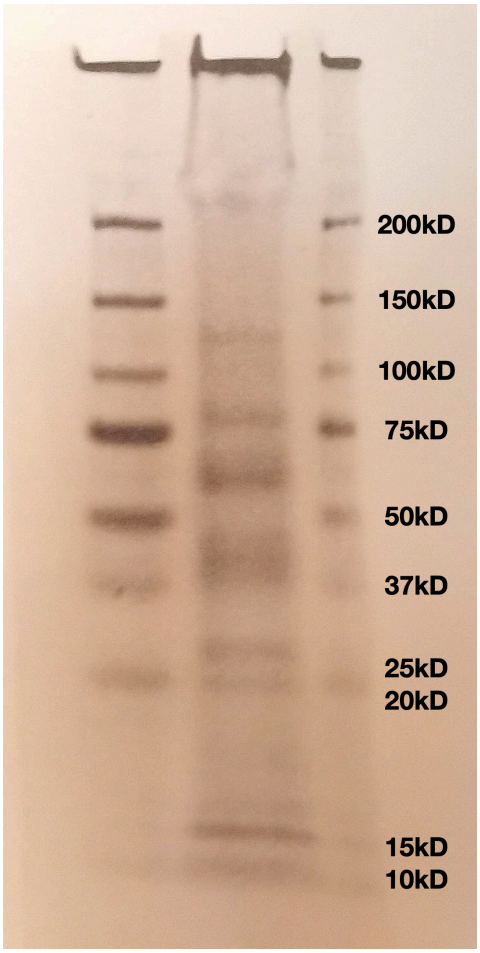

Figure S5. CBB stained banding pattern on SDS-PAGE from *Saccharolobus solfataricus* protein extracts. A diversity of bands is visible in comparison to staining with AgNOR. On both sides is a size reference marker.

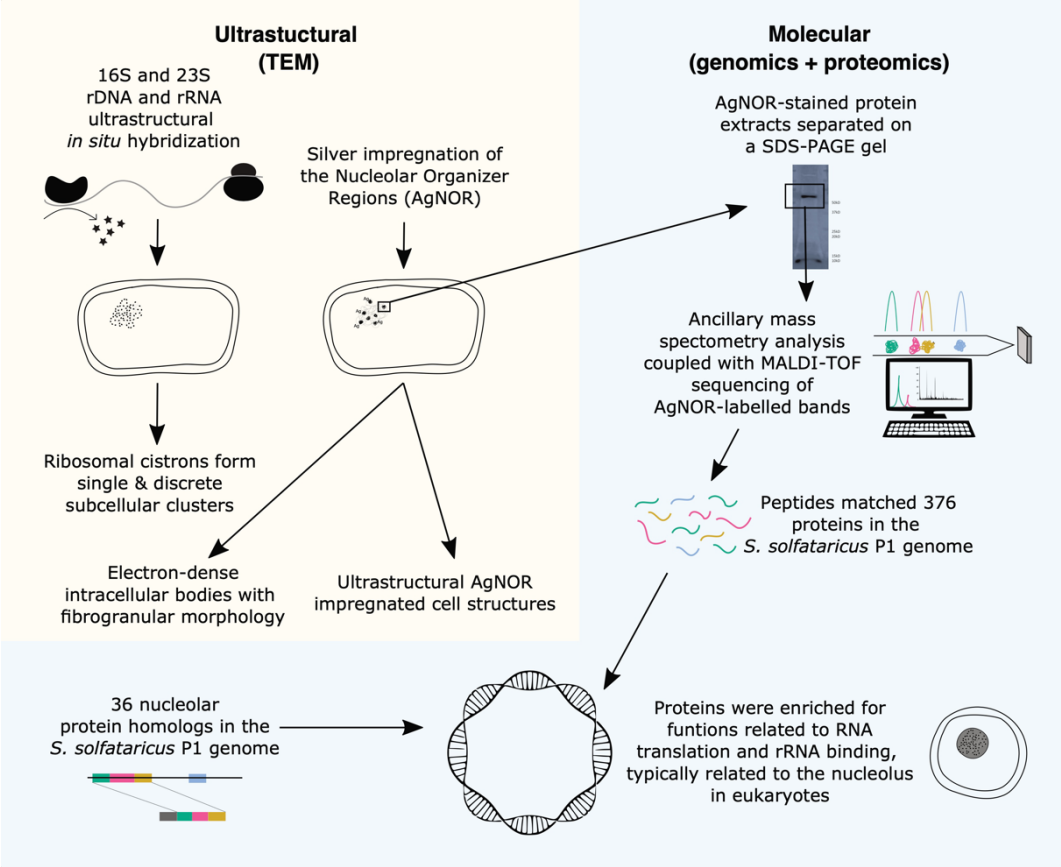

Figure S6. Methodology for Integration of ultrastructural and molecular approaches to assess the presence of a putative nucleolus in an Archaeon. 16S/23S ultrastructural hybridization and AgNOR staining are *in situ* techniques in TEM used to evidence nucleolus-like compartments. In *S. solfataricus* this is characterized by discrete clustering of ribosomal cistrons, fibro-granular morphology, and AgNOR positive discrete subcellular domains. *In vitro* SDS-PAGE analysis of AgNOR proteins with ancillary mass spectroscopy revealed 376 protein matches in the *S. solfataricus* genome, corroborated by the presence of 36 nucleolar protein homologs based on a candidate screen that putatively constitute within the newly described subcellular domain observed by TEM.

Perspectivas generales y conclusión

El descubrimiento del nucléolo en el procarionte *Saccharolobus solfactarius comb. nov. syn. Sulfolobus solfatatricus* ha sido la principal aportación de este trabajo a la ciencia. ¿Por qué es tan relevante?

Consideremos que, más de trescientos años después de la primera descripción del nucléolo por el fisiólogo Fontana, los avances en biología celular, particularmente en microscopía electrónica *in situ* en combinación metodológica con la biología evolutiva, nos permitieron, por primera vez demostrar que: el nucléolo no es una característica única de los eucariontes, y por el contrario existe en el grande e inexplorado dominio Archaea. Logramos probar la hipótesis de trabajo y ahora es momento de construir conclusiones y perspectivas, no sólo con base en los resultados, acaso también en la creatividad que permite una tesis de doctorado.

Entonces, ¿Qué motivaciones aguardan a nuestra curiosidad científica a partir de este descubrimiento?

El nucléolo arqueano abre una nueva línea de investigación para el estudio de estructuras subcelulares en arqueas; su evolución y su morfofisiología. No ha de considerarse al nucléolo de *S. solfataricus* como una curiosidad aislada o una serendipia; pues hemos basado nuestras observaciones en una plataforma metodológica especialmente propicia para el diseño de preguntas sobre el origen y evolución de las estructuras celulares.

Navegar el árbol de la vida con un microscopio sería una metáfora adecuada sobre estos métodos.

Por ello, insisto en la importancia de la biología celular evolutiva, porque nos previene de simplificar o malinterpretar las narrativas históricas de los organelos; aquellas con que hemos de contar los cambios de lo vivo a través del tiempo; una narrativa de la evolución, por ejemplo, la del nucléolo.

En este trabajo, hemos probado que, con base en filogenias resueltas, es posible postular preguntas y experimentos sencillos con gran significado biológico. Nuestros resultados sugieren que el nucléolo debió estar presente en el primer ancestro común de los eucariontes (FECA) y probablemente en el último ancestro común del superphylum TACK archaea y Eucaria (LETackA). Esta conclusión, sobre la naturaleza de ancestros ya extintos, hubiese sido incompleta desde una aproximación exclusivamente molecular, *in silico*. En cambio, fue una tarea parsimoniosa desde la biología celular, y en particular desde la microscopía electrónica, ya que las impregnaciones y tinciones utilizadas son técnicas sencillas, incluso antiguas; no obstante específicas y confiables. Habría que decir sobre el enfoque *in silico*, cuán importante fue para saber que la estructura observada, es en verdad un nucléolo. La sola observación microscópica sin sustento filogenético (genómico y proteómico) probablemente nos habría perdido en el océano de la especulación. Así, para descubrir un nucléolo en las arqueas, fue suficiente una observación dirigida a las células de una especie cultivable del grupo de organismos, que las predicciones por filogenias y estudios moleculares, señalaban para encontrar la evidencia material y visual que anhelamos los científicos.

Por eso, creo que la metodología desarrollada extensamente en el capítulo segundo, que integra biología celular y biología evolutiva, será una herramienta para numerosas preguntas y experimentos que, como éste, sean ontológica- y epistemológicamente bien planteados; y que orienten a la sencillez antes que a la complejidad.

*

En cuanto a la naturaleza misma del nucléolo, ésta no deja de ser enigmática a la luz de su evolución. Por el contrario, los nucléolos de organismos como arqueas y protistas son fascinantes porque pululan de nuevas preguntas sobre la dinámica y la estructura; la forma y la función de los organelos en el contexto de la célula.

Por ejemplo: ¿Cómo se mantiene ensamblado el nucléolo y cuáles son sus fronteras fisicoquímicas? ¿Podremos llegar a observar a través de los microscopios del futuro, la fenomenología *in vivo* del nucléolo en el universo de lo diminuto; donde las propias moléculas establecen la frontera una estructura celular? ¿Podremos darles un significado lógico a nuestras observaciones actuales desde la dicotomía forma-función?

Por ahora podemos, al menos ontológicamente, entender la relevancia científica de estudiar un organelo tan especial como el nucléolo. Empero aún estamos lejos, incluso a la luz de evidencias morfológicas y moleculares; de poder explicar, con modelos y teorías, cuál es en verdad la unidad mínima del nucléolo. Sin embargo, entendemos su relevancia biológica y contruimos sobre el concepto ya casi centenario de organizador

nucleolar que tan elegantemente acuñara Barbara McClincook para nombrar un punto citogenético. Todo comienza por la observación de un punto en el espacio.

Eventualmente la microscopía de súper resolución *in vivo* podría arrojar avances sobre aspectos mas finos. Siempre será imperativo dar significado biológico a las observaciones. Por eso considero que los enfoques comparativos, orientados a explorar más allá de los organismos modelos, son y seguirán siendo sumamente valiosos. Aunque estas cuestiones han sido discutidas en los capítulos segundo y tercero, huelga destacar algunas preguntas; punto de partida para vislumbrar el futuro de los estudios morfofuncionales del nucléolo: ¿Será acaso que las estructuras observadas en arqueas se acercan a un estado lo suficientemente mínimo de un nucléolo para estudiar en ellas la interacción RNA-proteína *in situ*? ¿Hay nucléolos mas básicos o más complejos en otras arqueas o incluso en bacterias?

Por supuesto que si este tipo de estructuras existiesen en las bacterias, datarían el origen del nucléolo en un pasado más pretérito de la evolución. Lo más probable por la distribución de los homólogos nucleolares en el árbol de la vida, sería que las bacterias no ostenten un nucléolo. Sin embargo, la idea del nucléolo mínimo nos obliga a considerar su exploración en todos los procariontes, pues habría que investigar, cómo es la organización espacial de la transcripción y maduración ribosomales en bacterias, y compararla con el nucléolo arqueano.

Otra pregunta legítima es si estos estados nucleolares mínimos corresponden a unidades funcionales. Por eso, no omito mencionar que es preciso confirmar la

actividad transcripcional del nucléolo de *S. solfataricus,* para comenzar una exploración realmente funcional del mismo. Este fue el camino que siguieron otros nucleólogos "históricos" en especies modelo como: *Xenopus leavis, Sacharomyces cerevisae, Caenorabditis elegans, Mus musculus, Arabidopsis taliana* y *Zea mays*. *S. solfataricus* puede convertirse en un modelo para el estudio de nucléolo arqueano en un futuro cercano.

La evidencia actual inclina nuestras sospechas hacia un nucléolo arqueano relacionado con el cistrón ribosómico, como lo demuestran los experimentos de hibridación *in situ*; verbigracia, un nucléolo involucrado desde las arqueas en la transcripción y maduración de los precursores del ribosoma. Por eso, de confirmarse la transcripción ribosomal en la estructura recién descrita, estaríamos frente a un nucléolo activo. En caso contrario pensaríamos que la estructura tal vez precedió a la función transcripcional, y que el proto-nucléolo de arqueas y sus proteínas, aunque homólogas, desempeñan otras funciones que se fueron perdiendo o conservando en el curso de la evolución.

*

A raíz de estas consideraciones, la otrora simplificada arquitectura celular de los procariontes se sacude con la idea de un nucléolo, anterior al origen del núcleo celular. He aquí la relevancia de pensar en un concepto novedoso de organización celular no membranosa en las arqueas.

Entonces, el nucléolo podría considerarse un organizador nanoscópico, de genes y proteínas. Esta es sin duda, una de las perspectivas más interesantes esbozada en el

capítulo tercero y que deriva de la idea de nucléolo mínimo. Brevemente. Si el nucléolo como organizador celular nanoscópico, procura en un mismo sitio, la agrupación de diversos elementos que confluyen en un mismo espacio a raíz de un proceso citogenético (la transcripción de rDNA), entonces el nucléolo es también la manifestación a nivel microscópico de la organización *in vivo* del genoma y por lo tanto un modelo para su estudio.

En este aspecto, no estaríamos hablando solamente de un proceso citogenético sino de un organizador estructural altamente dinámico y bien conservado a lo largo de la evolución. Estamos en un momento en que las discordancias entre nucléolos de diversas especies estudiados experimentalmente brindarán información muy relevante para conceptualizar la forma en que la célula mantiene su arquitectura, incluso en la ausencia de membranas. Además, la idea en biología que la forma sigue a la función; que un proceso da lugar a una estructura o viceversa, constituye una perspectiva adicional que se antoja reflexionar dentro de estos nuevos estudios nucleolares.

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De los resultados y perspectivas planteados anteriormente, se desprende una conclusión general.

El descubrimiento del nucléolo de las arqueas es motivante: cambia paradigamas sobre el origen de los eucariontes y visibiliza cuán necesarios son los enfoques integrativos con propuestas metodológicas sólidas, a través de nuevas disciplinas como la biología celular evolutiva.

Nucléolo proviene del latín que significa nuececilla. ¡Cuántas resignificaciones ha recibido este concepto a lo largo de la historia! Apenas comenzamos a romper las cáscaras más finas de su naturaleza y su origen. El universo celular se abre como lo anticipaban los filósifos a través de una visión cada vez menos especialista y por el contrario más generalista y creativa de la biología. Desde México y Medio Oriente, hemos elegido las vías de la transdisciplina; renovada tracción que parte de preguntas y experimentos sencillos, integradores entre disciplinas; visión crítica para avanzar con parsimonia por caminos más cortos hacia los enigmas de la ciencia.