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Cellularization across eukaryotes: Conserved mechanisms and novel strategies

Brooke McCartney[1] and Omaya Dudin[2]

Abstract

Many eukaryotes form multinucleated cells during their development. Some cells persist as such during their lifetime, others choose to cleave each nucleus individually using a specialized cytokinetic process known as cellularization. What is cellularization and how is it achieved across the eukaryotic tree of life? Are there common pathways among all species supporting a shared ancestry, or are there key differences, suggesting independent evolutionary paths? In this review, we discuss common strategies and key mechanistic differences in how cellularization is executed across vastly divergent eukaryotic species. We present a number of novel methods and non-model organisms that may provide important insight into the evolutionary origins of cellularization.

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Introduction

Multinucleated cells, formed by cell fusion (syncytia) or by nuclear division without cytokinesis (coenocytes), are widely present across eukaryotes. In animals, these cells can either persist during development allowing for cell size increase, efficient activation of gene expression and improved resistance to mechanical constraints and forces [1–7], or be cleaved into individual cells using a specialized form of cytokinesis known as cellularization [8,9]. Here, we aim to review some of the recent discoveries regarding the cellularization process with an emphasis on the similarities and differences in diverse eukaryotic taxa, including animals, close animal relatives, amoebozoans, apicomplexans and plants (Figure 1).

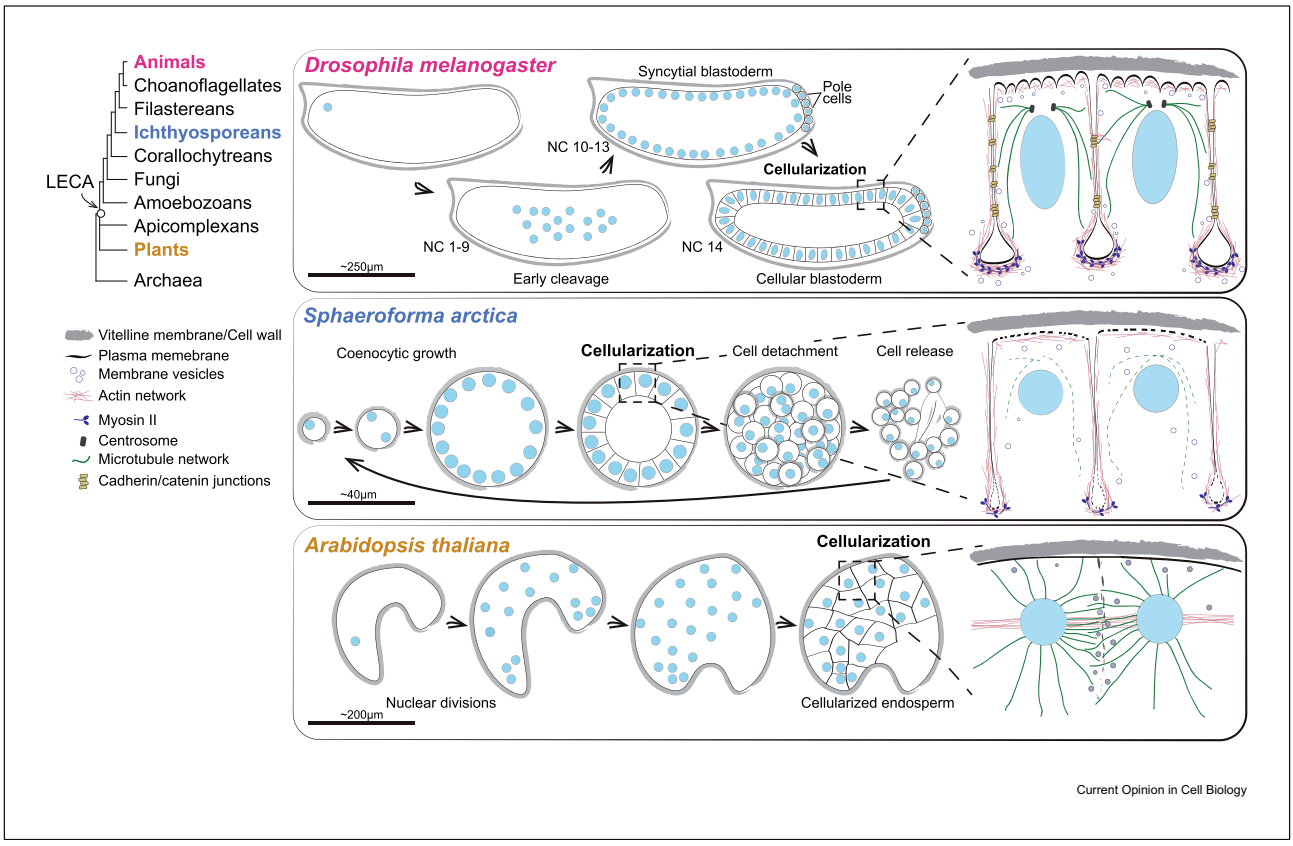
The formation of the syncytial blastoderm during the early development of the fruit fly *Drosophila melanogaster* represents the best-studied model for cellularization; however, such a process is common during the development of animals, plants and unicellular eukaryotes [10–12]. To date, it is still unclear how comparable the cellularization process is across these organisms, or why cellularization has been under selective pressure during evolution. This review aims to provide an overview of the cellularization process across eukaryotic cells that transit synchronously from multinucleated cells to multiple uninucleated cells. We will take advantage of the extensive work done on *D. melanogaster* embryos (recently reviewed in the studies by Hamm et al., Sokac et al. [13,14]) and use it as a benchmark to compare with other insects, flowering plants and protists and detail the key steps of cellularization from nuclear positioning to cytoskeletal and transcriptional regulation. We apologize to our colleagues whose work on the development of animal multinucleated germline cells [15–17], and septation of multinucleated filamentous fungi [18–20] were not included due to space and scope constraints.

Preparing to cellularize: Nuclear positioning and spacing

A universal, necessary precursor to coenocyte cellularization is achieving proper nuclear position within the embryo and spacing between nuclei. In the systems where this has been examined, insects, *Sphaeroforma arctica*, and *Arabidopsis thaliana*, a highly conserved theme is cytoskeletal functions and actin-microtubule (MT) crosstalk.

In the Drosophila embryo, the first nine synchronous nuclear cycles (preblastoderm, NC 1-9) occur without cytokinesis in the central cytoplasm (Figure 1). Although the early fly embryo is by definition a coenocyte, it is conventionally described as syncytial. During these first NCs, the nuclei maintain regular spacing while dispersing along the A–P axis (axial expansion, NC 4-6)

Figure 1



The phylogenetic position (left; LECA = last eukaryotic common ancestor), life cycles (center) and the known cytoskeletal components (right) of three distinct eukaryotic species undergoing cellularization. In *D. melanogaster*, cellularization, which occurs at nuclear cycle (NC) 14, is driven by microtubules, an actomyosin network, extensive plasma membrane remodeling, and cadherin-dependent cell–cell junctions. In *S. arctica*, microtubules coordinate nuclear positioning and actomyosin drives plasma membrane invaginations; however, the subcellular cytoskeletal localization at the membrane remains uncertain (dotted lines). In *A. thaliana*, microtubule-dependent actin asters regulate nuclear positioning whereas a radial microtubule system coordinates cell wall formation around individual nuclei [116].

and migrating to the cortex (NC 7-9). The result is a single layer of uniformly spaced cortical nuclei at NC 10 (Figure 1). Some "yolk nuclei" remain in the central cytoplasm and consequently are not cellularized [21].

Cortical actomyosin contractions generate cytoplasmic flows that propel the nuclei during axial expansion [22,23]. While it was well established that this nuclear movement is tightly linked to the cell cycle [24], recent elegant experiments employing biosensors and optogenetics in live embryos have yielded unprecedented molecular insight [22]. They revealed that local oscillations of Cdk1/PP1 activity begin around the synchronously dividing nuclei in the central cytoplasm. These oscillations spread outward resulting in localized PP1 activity at the cortex, approximately 40 µm above the nuclear cloud. This cell cycle dependent localized PP1 was highly correlated with and required for the Rho activity and localized myosin-2 accumulation necessary for the cortical contractions and cytoplasmic flows that disperse the nuclei [22].

MT-based mechanisms maintain proper nuclear spacing and appear to contribute to both axial expansion and cortical migration [25–28]. A novel cell-free nucleoplasm explant technique was used to demonstrate that nuclear distribution and separation distance is an "intrinsic property" of the preblastoderm nucleoplasm that depends on both central spindle elongation and cytoplasmic actin dependent aster migration [29]. A recent exciting extension of that initial study showed that MT-dependent repulsion is key to nuclear spacing [25]. Their data suggest that interdigitated anti-parallel MTs from neighboring asters re cross-linked by Feo/Prc1 and the kinesin-4 homologue Klp3A. That crosslinking may enable other MT motors to slide the MTs past each other thus separating the nuclei [25,30]. Nuclear migration to the cortex is postulated to be dependent on similar Feo-Klp mechanism [30].

In Drosophila, the cortical arrival of hundreds of nuclei creates the syncytial blastoderm (NC 10-13; Figure 1) and triggers the assembly of an actin "cap" above each

nucleus (reviewed in the study by Blake-Hedges et al. [31]). Disruption of actin via inhibitors or gene mutations produces abnormal nuclear clustering suggesting that actin caps are necessary to maintain spacing. It has long been appreciated that actin cap assembly is dependent on the centrosomes located between the nucleus and the cortex [32,33]. Although the molecular mechanism connecting actin and centrosomes is still unclear, the best candidate is the ELMO/Ced-12-Sponge complex [30]. In the syncytial blastoderm, nuclear spacing and mitotic fidelity are achieved at a cortex increasingly crowded with dividing nuclei in part through the production of "pseudo-cleavage furrows". These dynamic actin-rich plasma membrane invaginations extend starting in prophase and retract during telophase to separate spindles and prevent collisions (reviewed in the study by Schmidt et al. [34]). Interestingly, syncytial cortical divisions are accompanied by a "yo—yo" movement of the nuclei—nuclei are driven apart by elongating spindles during telophase and then restored to their proper positions by Diaphanous (Dia)-dependent cortical F-actin [35]. Such a "viscoelastic feature" results in part from the pseudo-synchronous nature of these cortical divisions that proceed in a wave across the embryo.

Recent work in the cricket, *Gryllus bimaculatus*, has revealed that even among insects with syncytial embryos, there are fascinating differences in how the problem of nuclear distribution and movement is solved [36]. Whereas in Drosophila the nuclei divide synchronously, move in concert, and arrive at the cortex simultaneously, in cricket the nuclei lack cell cycle synchrony, exhibit variability in nuclear movement, and nuclear arrival at the cortex is staggered [36]. Remarkably, these highly variable properties predictably correlate with local nuclear density and movement appears to be coupled to the cell cycle. The variability in the movement of neighboring cricket nuclei is inconsistent with a role for cytoplasmic flows like those in Drosophila. Given the newly described connections between cell cycle synchrony and cytoplasmic flow in flies [22], it is perhaps not surprising that the asynchronous cricket embryo doesn't employ them. Further, in contrast to the nuclear repulsion observed in Drosophila [25], the authors' coupled empirical and modeling approach suggests that a "local, asymmetric, active pulling force on each nucleus" can explain most of the nuclear behaviors in the cricket embryo.

Cellularization also occurs in ichthyosporeans-a lineage of unicellular protists known to be among the closest living relatives of animals [37,38]. In *Sphaeroforma arctica* (Figure 1), the best-studied ichthyosporean model to date, multiple rounds of nuclear division produce multinucleated coenocytes [12,39,40]. These coenocytes then undergo synchronous cellularization producing a transient epithelial-like cell layer [12]. This brief multicellular stage concludes with cell detachment and release to repeat the life cycle. During the pre-cellularization growth and nuclear division phase of *S. arctica* development, actin patches dot the cortex, some of which expand via Arp2/3 creating actin "nodes" [12]. This is followed by the assembly of formin-dependent cortical actin filament networks that separate the nuclei prior to furrow initiation. This network of actin barriers is reminiscent of the transient pseudocleavage furrows in Drosophila which require the formin Dia, as well as the Dia interactors APC2, Rho1, and RhoGEF2 [41—43]. They differ in that the fly metaphase furrows contain both membrane and actin, while the actin barriers in *S. arctica* do not appear to include membrane until the initial phase of invagination begins. In addition, loss of the actin barriers resulting from inhibition of actin, formins, or Arp2/3 did not cause nuclear collisions. Instead, inhibition of MTs produced abnormal nuclear clustering suggesting an MT-only-based mechanism to maintain nuclear spacing. It is tempting to speculate that this system may employ MT sliding like that recently described in the pre-blastoderm fly embryo [25].

In flowering plants, dual fertilization of the oocyte and the endosperm by two independent sperm triggers the development of both the embryonic plant and the endosperm that nourishes it [44—46]. As in the Drosophila embryo, nuclear division in the endosperm proceeds without cytokinesis and the timing of cellularization determines seed size [47—54]. In Arabidopsis, cellularization begins when the endosperm contains approximately 100 nuclei and moves from one end of the tissue to the other, producing a large multicellular area (Figure 1). A new study has yielded an unprecedented look at cytoskeletal dynamics in the endosperm prior to cellularization [55]. Nuclear spacing in the coenocyte appears to be established and maintained by an aster-shaped network of actin cables, the assembly of which is dependent on a co-aligning MT network (Figure 1). Radial MT arrays have been previously described in Arabidopsis and their disruption correlates with uneven nuclear spacing and cellularization defects [56]. Interestingly, Drosophila embryos also form actin-asters during anaphase and telophase in the syncytial blastoderm. The formation of these asters is dependent on centrosomes and MTs, but their role is not known [57].

Cellularization: Initiation, extension, and termination

With the nuclei in proper position, cellularization can commence. Drosophila and *S. arctica* deploy a conserved cytoskeletal tool kit for building new cells in the coenocyte but they do so in distinct ways. In fly, furrow formation uses the remnants of the cycle 13 pseudocleavage furrows [58,59] to generate the "old furrows" that separate adjacent non-daughter nuclei. To separate the daughter nuclei produced in cycle 13, "new furrows"

are established through the action of the centrosomes located above each daughter nucleus and their MT asters that extend toward the membrane. Myosin-2 filaments are initially recruited to the new furrows by a transient tension-driven cortical flow that is biased toward the sites of astral MT overlap [58]. In *S. arctica*, inhibition of MTs produces cells that vary in size and in the number of nuclei, suggesting that MTs may play a role in establishing the sites of furrow formation. But because MTs are required for nuclear spacing, this effect may be a secondary consequence of nuclear clustering. In this case, a non-MT driven mechanism for establishing the sites of furrow formation is needed that could rely on the nuclei themselves, or possibly MTOCs. In both systems, actin assembly is absolutely required for furrow formation: inhibiting actin assembly with Latrunculin, an Arp2/3 inhibitor, or a formin inhibitor eliminates membrane furrow formation in *S. arctica*. Similarly, actin inhibitors or mutants in actin assembly and organization block furrow formation in Drosophila [14]. The rapid creation of hundreds to thousands of new cells creates a tremendous demand for plasma membrane—the cell surface of the fly embryo is estimated to expand by a remarkable 25-fold during cellularization [14]. Fascinating ways to satisfy this demand have been discovered in Drosophila, but as this problem has not been examined in any other system we do not cover it here.

How the force is generated to invaginate and extend membranes is a significant difference between the two systems. The F-actin network separating the *S. arctica* nuclei assembles in the presence of the Myosin inhibitor blebbistatin, but instead of a tight network of actin "hexagons" the actin network appears loose and sinuous consistent with contractility loss [12]. Blebbistatin treatment blocks membrane invagination and causes the striking retreat of membrane furrows that have already initiated, suggesting that actomyosin contractility provides the force for membrane extension. Furrow extension in Drosophila takes advantage of the inverted MT basket surrounding each nucleus that positions MTs parallel to the plane of the growing furrow (Figure 1). Here, the MT motor Pavarotti-KLP (a mitotic kinesin-6) provides the force to pull the membrane between the nuclei in the first, "slow phase" of furrow extension [60,61].

Drosophila myosin-2 does not provide the driving force for furrow initiation and extension as it does in *S. arctica* or during conventional cytokinesis in many, but not all organisms [12,62,63]. However, both direct and indirect fine-tuning of myosin-2 activity play a critical role in Drosophila cellularization. The basal ends of the furrows create an interconnected array of actomyosin rings that change shape as the furrows extend [14]. Initiated as circular rings, they become rigid hexagons throughout the slow phase. As the final fast phase begins, the actin network again transforms into circular rings that will constrict to close the basal ends of the new cells. Myosin-2 activity shapes the initial circular rings, is silenced during the hexagonal slow phase, and is reactivated in the fast phase to shape and ultimately participate in the closure of the re-circularized rings [14]. The timing of myosin-2 reactivation is critical: premature constriction of the rings traps the nuclei in their grip resulting in catastrophic cellularization failure. Krueger et al. (2019) elegantly demonstrated that the actin network is not competent to respond to myosin-2 activation during the hexagonal phase. This resistance to contractility is the result of the synergistic activity of actin crosslinkers, maternally provided Cheerio (fly Filamin) and transiently expressed Bottleneck, that promote the rigid hexagonal F-actin network and may block actin filament sliding [64]. Surprisingly, re-circularizing the network and enabling actomyosin contractility requires fimbrin, a third actin crosslinker [64]. While some crosslinkers curtail contractility, their functions are supported by the action of RhoGAPs (Cumberland GAP [65] and GRAF, [66]) that suppress Rho1 activity upstream of Myosin-2 activation during cellularization. Interestingly, myosin-2 is only required for the first phase of ring closure when the hexagons are re-circularized and constriction begins. The rapid ring constriction characterizing the second and final phase of ring closure is myosin-2 independent and instead is driven by cofilin and F-actin disassembly [67] similar to ring constriction during budding yeast cytokinesis [68].

Little is known about the mechanics of cellularization initiation, extension and termination in non-animal species, but the role of myosins is an emerging theme. Through unknown mechanisms, myosin-2 drives furrow initiation and extension in *S. arctica* while maintaining open rings to navigate the nuclei [12]. Chytrid fungi have myosin-2 that may play a role in their cellularization [69]. The class VI-like myosin, MyoJ, drives basal constriction in the apicomplexan parasite *Toxoplasma gondii* but is not required to complete their specialized cellularization (endodyogeny) that occurs within a host vacuole [70,71]. Even less is known about the cytoskeletal mechanisms governing plant endosperm cellularization. While cellularization defective mutants have been identified [72], some of which support a role for MTs [49,73], these have not shed much light on cytoskeletal mechanisms.

An important feature of cellularization is the establishment of cortical domains required for epithelial polarity and developmental coordination. In animals, including fly embryos, these domains are enriched with conserved polarity factors such as par proteins, cell adhesion molecules, and lateral membrane components scribbled and Disc large 1 (Dlg1) (reviewed in the study by Schmidt et al. [34]). In *Drosophila*, the scribble/Dlg module orchestrates the initial formation of the cadherin/catenin-

based cell-cell junctions (basal junctions) concomitant with furrow canal formation and growth [74–76]. Although not directly needed for the formation or maintenance of the furrow canals [77], basal junctions are thought to maintain the specific composition of the furrow canal throughout its progression [77] and are essential for subsequent activation of the key Notch developmental pathway [78]. In ichthyosporeans including *Sphaeroforma*, par proteins, cadherins, scribble, Dlg, and notch proteins have not been identified [79,80] but multiple β- and α-catenin homologs are present and highly expressed during cellularization [12]. These observations strongly suggest that ichthyosporeans rely on a divergent program that may include cadherin-independent catenin-based junctions to establish polarity and provide structural integrity to the furrow. This hypothesis is consistent with previous work identifying catenin-based cell junctions during the aggregative multicellular life stage of the slime mold *Dictyostelium discoideum*, which also lacks cadherins [81,82]. One key question remains, however: did the animal cortical polarity pathway evolve gradually with catenins having the ancestral role followed by increased cell-junction complexity? Or did ichthyosporeans and amoebozoans evolve independent polarity programs with analogous cell-adhesion structures?

Triggering cellularization

In *Drosophila*, cellularization is concomitantly triggered by multiple processes including cell cycle progression, maternal product degradation and zygotic genome activation (ZGA) making it difficult to assess the specific contribution of each process (reviewed in the studies by Sokac et al., Hamm et al., Schulz et al. [14,83,84]). Nonetheless, extensive work has shown that cellularization relies on the timely degradation of maternal products by RNA binding proteins (RBPs) such as Smaug [85], a poorly defined developmental oscillator that measures the time interval between fertilization and NC14 [86,87], Cdk1-dependent mitotic waves ensuring synchronized nuclear positioning along with ploidy [22,88,89], and a critical set of both maternally provided and early zygotic genes [14]. The initial machinery required for cellularization, including actin network components (*Myosin-2* (*Zip*), *Anillin, and Dia*), major regulators of cell polarity (*Rho1, Rok, RhoGEF2*) and membrane trafficking (*Rab8, Rab5, Rab11, Pld*), are all maternally supplied [14]. However, their function is locally orchestrated by a small set of early zygotic genes (*Bnk, Dunk, Nullo, Slam, Sry-*α) expressed shortly before the extensive ZGA [14]. Although the regulation of these genes is poorly understood, their activation depends on the developmental oscillator, the key ZGA activator named Zelda (*Zld*) and the nuclear-to-cytoplasmic (N/C) ratio [13,89–92]. Most of the above-mentioned genes have been shown to be conserved and expressed during the early embryonic development of various insects arguing for a single evolutionary origin in this lineage [93,94]. Nonetheless, key differences have also been reported, including various patterns of blastoderm formation and diverse adoption of signaling pathways, implying a certain capacity for adaptation [95–97].

In flowering plants and unicellular eukaryotes, the pathways triggering cellularization remain mostly unknown. *Arabidopsis* endosperm cellularization only occurs after a defined number of nuclear divisions [98] and depends on the tight regulation of the major plant regulator auxin [99]. This suggests the presence of at least two regulators, a plant-specific switch reliant on auxin and a possibly ancestral mechanism conditional on nuclear ploidy and/or cell cycle progression. In ichthyosporea, cellularization occurs in parallel with an extensive transcriptional wave of cytoskeletal and cell adhesion genes [12], but the mechanisms triggering such a wave are still unknown. Previous work has shown that, as in insect embryos, the periodicity of the nuclear division cycles during the coenocytic growth of *S. arctica* is maintained by a timer which is independent of cell volume and growth rate [40]. Moreover, *S. arctica* cells exhibiting increased nuclear number-to-volume ratio, caused by nutrient limitation [40] or by sedimentation selection [100], cellularize earlier, arguing for a possible link with the N/C ratio. This nuclear control of cellularization has also been shown in earlier diverged unicellular species. For instance, in the multinucleated life stage of apicomplexan parasites also known as Schizonts, nuclear division occurs first asynchronously but is completed with a final synchronous cycle coinciding with cellularization [101]. Such development in parasites undergoing schizogony implies again that a nuclear-constrained and cell cycle-regulated signal may be in play [101,102]. This nuclear restricted regulation has also been shown to occur in the single-celled amoebozoan slime mold *Physarum polycephalum*, where nuclei are proposed to act like mobile processors promoting spatio-temporal function in absence of membrane compartmentalization [103]. Altogether, these studies demonstrate that although the pathways triggering cellularization seem to be species-specific, they all share an intimate relationship with nuclear number and position. Such a local regulation at the level of single nuclei represents an interesting parallel with a local nuclear community effect previously reported in *Drosophila* [86]. Recent work has shown that nuclear communities can sense the N/C ratio and may influence neighboring nuclei in a Cdk1-dependent manner [89], suggesting that nuclei may represent a signaling center around which spatio-temporal regulation of cellularization across species may have evolved.

Outlook

Cellularization represents an exciting process relying on a sophisticated interplay between cytoskeletal dynamics, structural coordination, and transcriptional regulation. As reviewed here, despite being present

across many species spanning the eukaryotic tree of life, cellularization seems to employ distinct species-dependent mechanisms, even between the closest relatives discussed here; *Drosophila* and the cricket *G. bimaculatus*. This suggests that cellularization has probably evolved independently multiple times, nonetheless, two mechanisms were repeatedly employed for faithful cellularization across species: i) the cytoskeleton (be it actin, microtubules or both) for partitioning multinucleated cells into individual ones, and ii) the nuclei as sites of transcription and local signaling, and as a means of counting. As these features were present in the last common ancestor of all eukaryotes, it suggests that multinucleated cells may have used the ancestral cytokinetic machinery and gradually increased its complexity by integrating the capacity to synchronize the cell cycle, coordinate cytoskeletal components and establish overall structural integrity. This idea raises its own questions: why did multinucleated cells repeatedly emerge in the first place, what are the selective pressures driving this, and how did the ancestral cytoskeletal and transcriptional pathways adapt to it? All these fundamental questions have yet to be addressed.

Extensive work is still needed to support the convergent nature of cellularization. The renewed enthusiasm for non-model organisms and the extensive efforts to develop genetic tools represent an exciting path for the future. For instance, the recent development of Agrobacterium-mediated transformation of the chytrid fungus *Spizellomyces punctatus* is a significant step in the right direction [104]. Indeed, Chytrid fungi, which undergo cellularization to produce flagellated zoospores, have both animal/amoeboid and fungal characteristics at different points in their life cycles placing them in a unique position to investigate the evolutionary origin of cellularization. Similarly, a new method for stable transfection in another animal relative undergoing cellularization occasionally, the corallochytrean *Corallochytrium limacisporum* [105], will enable the investigation of an exciting novel species for future comparisons.

Aside from the evolutionary origins of cellularization, several mechanistic questions remain unanswered, even in model systems such as *Drosophila*. For example, the spatio-temporal order of the triggering events (i.e. transcriptional activation, local nuclear signaling, and readout of the N/C ratio) is not known. For that, the emergence of novel single cell and single nucleus sequencing methods will be useful [103,106,107]. Also, long-term and low-phototoxic imaging together with novel optogenetic methods to locally activate or degrade specific genes [108–110] will reveal the mechanisms of spatial coordination, and the different compensatory checkpoints present across the AP axis. Moreover, the recent development of expansion microscopy methods will also be beneficial in characterizing the ultrastructural dynamics of cellularization across many species [111,112]. The mechanical forces locally required to complete cellularization are not well understood, but the innovative use of injected magnetic beads to assess those forces is already generating novel insight into the mechanics of cellularization [113–115]. Taken together, we believe that these new methods and novel organisms promise to yield exciting discoveries about the origins and mechanisms of cellularization for years to come.

Author contributions

Both authors contributed equally to the conceptualization, content and drafting of this review.

Conflict of interest statement

Nothing declared

Data availability

No data was used for the research described in the article.

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