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Supporting Online Material

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Fossilized Nuclei and Germination Structures Identify Ediacaran "Animal Embryos" as Encysting Protists

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Globular fossils showing palintomic cell cleavage in the Ediacaran Doushantuo Formation, China, are widely regarded as embryos of early metazoans, although metazoan synapomorphies, tissue differentiation, and associated juveniles or adults are lacking. We demonstrate using synchrotron-based x-ray tomographic microscopy that the fossils have features incompatible with multicellular metazoan embryos. The developmental pattern is comparable with nonmetazoan holozoans, including germination stages that preclude postcleavage embryology characteristic of metazoans. We conclude that these fossils are neither animals nor embryos. They belong outside crown-group Metazoa, within total-group Holozoa (the sister clade to Fungi that includes Metazoa, Choanoflagellata, and Mesomycetozoea) or perhaps on even more distant branches in the eukaryote tree. They represent an evolutionary grade in which palintomic cleavage served the function of producing propagules for dispersion.

The ~570-million-year-old phosphorites of the Ediacaran Doushantuo Formation in southern China contain globular fossils showing palintomic cleavage (cell division not accompanied by cytoplasmic growth). These have been interpreted as embryos of some of the earliest animals (*1*–*8*) and alternatively as giant bacteria (*9*). Preservation of internal cellular structures, such as nuclei, within Doushantuo fossils (*5*, *8*, *10*) may provide further clues to their biological nature. Such structures, however, are frequently discounted as features of taphonomic degradation (*9*, *11*, *12*).

To resolve the issue, we investigated ~450 Doushantuo embryo-like fossils using synchrotron-radiation x-ray tomographic microscopy (srXTM), a nondestructive technique for three-dimensional imaging internal structure at micrometer resolution (*13*). Fourteen specimens preserve a distinct class of subcellular bodies—one per cell—that are potentially identifiable as preserving evidence of nuclei (Figs. 1 and 2 and figs. S1 to S4). The specimens are referrable to *Tianzhushania* [senior synonym of *Megasphaera*, *Parapandorina*, and *Megaclonophycus* (*3*, *6*, *14*)] and *Spiralicellula* (*15*). The nucleus-like bodies fulfill relevant criteria for biogenicity: Their occurrence is consistent and repeated (12 of the 14 specimens have one such body in each cell); they are regularly positioned in the cells within any single individual (central to the cells in four of the specimens, peripherally in the others); they have a consistently globular shape; and the volumetric ratio between bodies and cells corresponds to that of nuclei and cells in eukaryotes (fig. S6 and table S1). Furthermore, one specimen (Fig. 2 and fig. S1, D to H) has two elongated and one dumbbell-shaped nucleus-like body, suggesting that they are in the process of division.

The cell content surrounding the nucleus-like bodies is generally homogenous but sometimes with a finely granular fabric of dispersed less-dense objects about 1 to 3 μm in size (for example, dark spots in the peripheries of Fig. 1, F to I). This fabric may reflect the bacterial replacement of cytoplasm seen in taphonomic experiments with modern embryos (*10*). The outer parts of the nucleus-like bodies are preserved as a fabric of euhedral apatite crystals (Fig. 1C), a few micrometers in size, or of botryoidal void-filling growth (Fig. 1G). Internally, there is usually a spheroidal body, about 20 to 50 μm in diameter (Fig. 1, B and C, and figs. S1H and S4, C and L). In most cases, this body is positioned eccentrically, so that it appears to merge with the surrounding matrix, which has a similar fabric (Fig. 1C). The fossilized fabric is similar to that of the cytoplasm, although it may differ slightly in x-ray attenuation. The spherical bodies resemble nucleoli (*8*), but given the void-filling character of the surrounding fabric, they are more likely to represent shrunken nucleoplasm.

The nucleus-like bodies meet the criteria for structures present in the living organism. They are unlikely to be taphonomic artifacts, and we therefore identify them as nuclei. The nuclear envelope is not fossilized (the eukaryote nuclear envelope consists of two lipid bilayers only a few nanometers thick, with miniscule fossilization potential), but evidence of the presence of a nucleus is preserved by the mold of its external morphology. This interpretation is inconsistent with a proposed hypothesis that the microfossils are giant bacteria (*6*, *9*, *14*, *16*, *17*). It is consistent with a eukaryote affinity, but this does not necessarily imply that the Doushantuo fossils represent embryos of animals. None of the putative metazoan characters identified in the cellularly preserved Doushantuo fossils (*1*, *4*, *5*, *8*) are metazoan synapomorphies [supporting online material (SOM) text]. Some are taphonomic artifacts, but the rest are holozoan symplesiomorphies or

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homoplasies. Our analysis reveals developmental stages that preclude the postcleavage embryonic development of animals, indicating that the fossils represent neither crown-group metazoans nor multicellular stem-group metazoans.

Any reinterpretation of the Doushantuo fossils must account for the facts that (i) the palintomic cluster of cells (*Parapandorina*) was enclosed within a multilayered envelope with tuberculate surface texture (*Megasphaera*), which was itself enveloped in a process-bearing cyst wall (fig. S5), diagnostic of the senior synonym *Tianzhushania* but usually not preserved in phosphatized specimens (*3*, *6*, *7*); (ii) a similar structure envelops the helicospiral (*18*) and the irregularly shaped fossils (Fig. 3) (*19*) from the same deposits; and (iii) the palintomic cleavage does not appear to involve cellular to tissular differentiation or germ-layer formation but merely results in aggregates of thousands of cells (*5*, *19*).

Fossils with the characteristic *Megasphaera* envelope (the inner, tuberculate wall layer of *Tianzhushania*) may deviate from the normal globular morphology (Fig. 3A) by attaining irregular shape, projecting finger-like protrusions that sometimes branch (Fig. 3, B and C) (*19*, *20*) or forming peanut-like shapes (Fig. 3, D to J). These contain hundreds of thousands of tightly packed cell-like bodies, about 1 to 10 μm in size, some with zones of different cell morphology (Fig. 3, F, G, I, and J) but no structures resembling coordinated cell layers or differentiated tissues. In most of these specimens, the marginal regions show semi-isolated or isolated bodies consisting of one or a few cells (Fig. 3I) embedded in a matrix continuous with the outer surface. The largely identical envelope in well-preserved specimens (Fig. 3, A, C, and D) is a strong indication that the nonglobular morphs belong to the same taxon as the normal, globular, *Tianzhushania*.

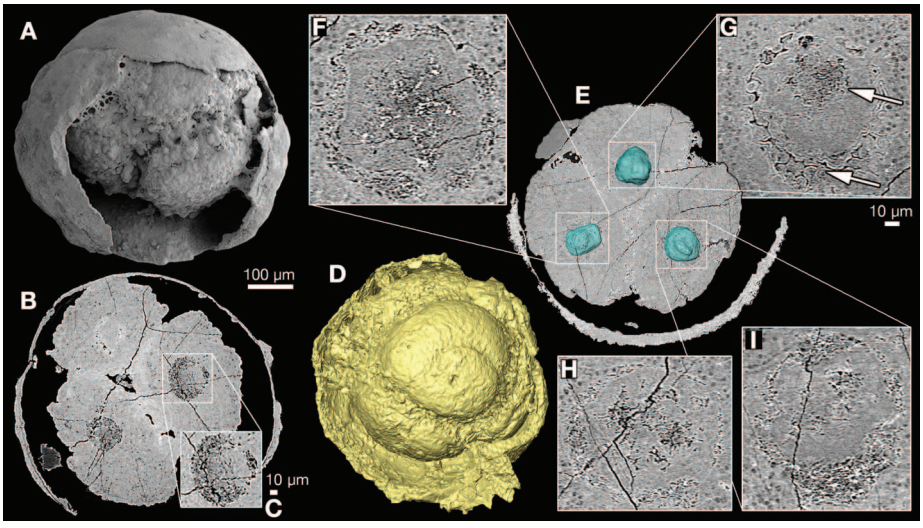

Fig. 1. *Tianzhushania* and *Spiralicellula* from the Ediacaran Doushantuo Formation, Datang Quarry, Weng'an, Guizhou Province, China. In the srXTM slices, lighter tones reflect higher x-ray attenuation than do darker ones. (**A** to **C**) *Tianzhushania*, four-cell stage, SMNH X 4403. (A) SEM picture shows partly preserved smooth envelope and three cells. [(B) and (C)] srXTM-sliced specimen with three nuclei. (**D** to **I**) *Spiralicellula*, four-cell stage; SMNH X 4404; srXTM tomograms. (D) Surface rendering shows helicospiral twist of cell. (C) Slice through plane with three nuclei (blue, surface rendered). [(F) to (I)] Enlargements of slices through nuclei. (G) Excentral globular body in nucleus (top arrow) and void-filling botryoidal apatite in nuclear periphery (bottom arrow) (movie S4).

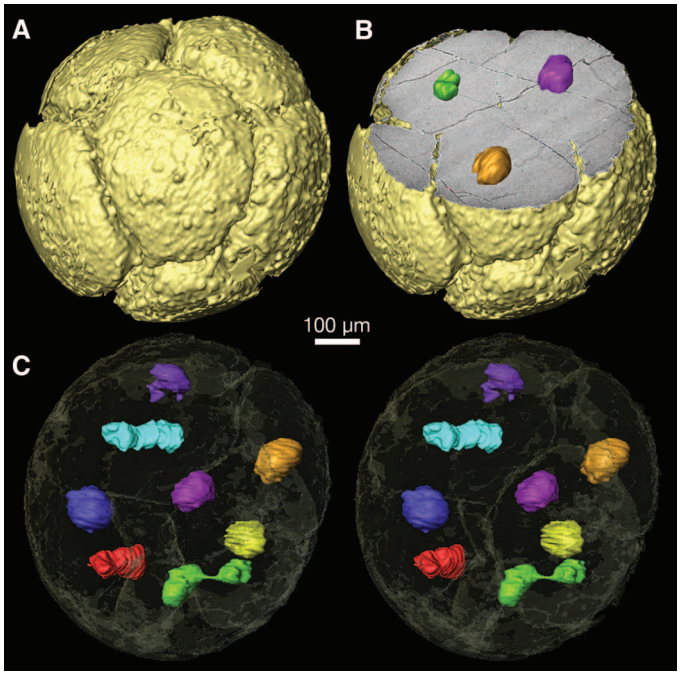

Fig. 2. *Tianzhushania* from the Ediacaran Doushantuo Formation, Datang Quarry, Weng'an, Guizhou Province, China; eight-cell stage; SMNH X 4405. (**A**) Surface rendering shows six cells. (**B**) Same orientation, slice through plane with three nuclei (surface rendered). (**C**) Stereo-pair of transparent rendering of cells with opaque surface rendering of nuclei, showing one dumbbell-shaped (green), two elongated (red and turquoise), and five globular nuclei (fig. S1, D to H, and movies S1 and S2).

The introduction of *Spiralicellula* into the class of Doushantuo embryo-like fossils raises further difficulties for the animal embryo interpretation. The internal bodies in *Spiralicellula* are coiled helicospirally (*15*), and the shape of the whorls suggest that the body is elongated, vermiform. Such a cell phenotype is inconsistent with blastomeres in a developing embryo. Nonetheless, our data show that they are nucleated cells (Fig. 1, D to I), and we interpret their shape to mean that the individual propagules produced by palintomic division in *Spiralicellula* were elongated or amoeboid. *Spiralicellula* is otherwise indistinguishable from *Tianzhushania* in terms of the pattern of equal cell division, taphonomy, and preservation.

"Helical embryos" described from the Doushantuo (*18*) occur within an envelope that in all morphological details, except for a helicospiral groove or row of radial canals, correspond to the envelope seen in *Tianzhushania* (*14*). They were interpreted as late-stage (post-blastula) embryos, suggesting that the later stages of the early cleavage embryos in the Doushantuo had finally been found (*18*). Although there are cleavage products composed of thousands of cells (*5*, *19*), there is no evidence that *Megaclonophycus* developed into the helical forms. The inverse interpretation—that the helical forms represent the unicell stage of the *Spiralicellula* forms with helicospirally coiled vermiform cells during early cleavage—is more compatible with the available evidence.

The combined developmental evidence from the fossils indicates a life cycle (Fig. 4) in which *Tianzhushania* and *Spiralicellula* represent mother cells, enlarged by hypertrophic growth, that encysted within a multilayered envelope (*Megasphaera* and helical forms, respectively) and directly, or after a resting period, began a process of coordinated mitotic palintomic cleavage (*Parapandorina–Megaclonophycus*). Eventually, germination occurred, during which the outer envelope wall ruptured, and the more pliable inner wall with its content emerged in finger-like protrusions, typically forming peanut-like objects. The cells resulting from the cleavage process escaped as propagules, probably through dissolution of the inner wall. The part of the life cycle

from propagules to the next round of hypertrophic growth is unknown.

This sequence of development is incompatible with the embryonic development of metazoans (and with the life cycle of modern giant bacteria), and the integrity of the nuclei during division (Fig. 2) is also inconsistent with the open mitosis characteristic of metazoans (*21*). The developmental pattern is more compatible with nonmetazoan holozoans. In the mostly parasitic Mesomycetozoea, cells commonly grow hypertrophically and encyst, whereupon they divide, usually palintomically, to form an aggregate of cells similar to a cleavage embryo (*22*–*24*). The size of the cysts varies from a few micrometers (*22*) to greater than a millimeter (*25*). These cells are at some stage released into the environment as asexual propagules (endospores). Mesomycetozoean propagules may be arranged in zones of different maturation within the cyst (spore), with the release-ready individuals outermost (*26*), a phenomenon observed also in *Tianzhushania* (Fig. 3J). The propagules are released through perforation or rupture of the wall of the cyst, often distended to peanut-like shape (fig. S7) (*25*, *27*) or through germinal tubes (*22*, *27*). Both of the latter morphological features are present in *Tianzhushania*, and the presence of a rarely preserved external wall in *Tianzhushania* (*3*) suggests a similar process of morphogenesis. In the mesomycetozoean *Ichthyophonus*, the peanut shape results when the cell mass of propagules—still enveloped by the inner part of the cyst wall—protrudes through an opening in the outer cyst wall (fig. S7) (*27*). The helicospiral cell shape in *Spiralicellula* is incompatible with cleaving blastomeres in a multicellular embryo but rather suggests coiled vermiform cells. This might be compared with the free living, presumably saprotrophic, mesomycetozoean *Corallochytrium* living in coral reef lagoons; the released propagules of this organism are vermiform, or "limax-shaped," having sinusoidal movements (*28*).

Characters available for phylogenetic assessment are insufficient to decide whether or not *Tianzhushania* and *Spiralicellula* belong within the Mesomycetozoea. Convergence of morphological and anatomical features is common among Eukaryota (*29*). Multicellularity by means of retention of cell contact during cleavage has been achieved independently a number of times (*30*), and palintomy is a recurrent theme in eukaryotes (*31*–*34*). Most instances of eukaryote palintomy differ in various respects from the pattern seen

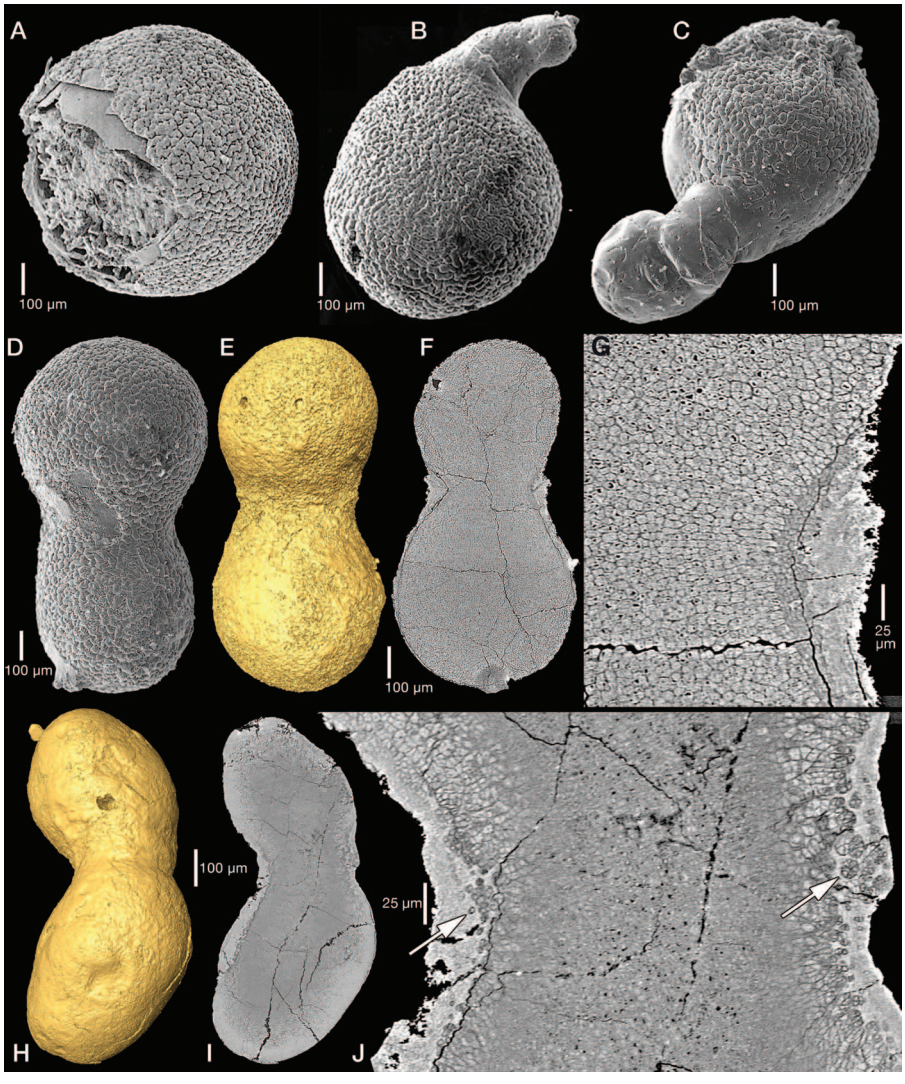

Fig. 3. *Tianzhushania* from the Ediacaran Doushantuo Formation, Datang Quarry, Weng'an, Guizhou Province, China. (**A**) Regular and (**B** to **J**) irregular forms, the latter interpreted to be in the germinating stage: MESIG 10022 [(A) SEM micrograph]; MESIG 10023 [(B) SEM micrograph (*19*)]; MESIG 10024 [(C) SEM micrograph (*19*)]; MESIG 10021 [(D) SEM micrograph]; SMNH X 4447 [(E) to (G) srXTM renderings]; SMNH X 4448 [(H) to (J) srXTM renderings]. (A) Surface of regular globular specimen shows envelope structure, to be compared with the similar envelope structure in (B) to (D). [(B) and (C)] Germinating specimens show protruding tubes and envelope structure. (D) Peanut-shaped specimen shows envelope structure. (E) Isosurface rendering of peanut-shaped specimen. (F) Orthoslice through (E). (G) Detail of approximate level in (F), showing cellular units. (H) Isosurface rendering of peanut-shaped specimen. (I) Orthoslice through (H). (J) Detail of approximate level in (I), showing cellular units. There is a progressive individuality of cellular units toward the periphery, including detachment of single- and oligocellular units (arrows).

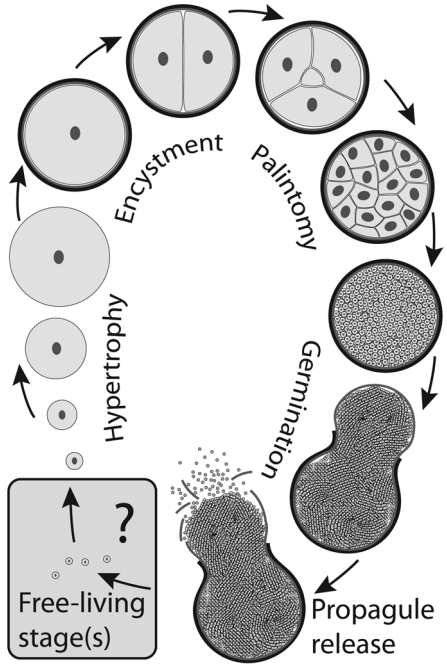

Fig. 4. Proposed life cycle of *Tianzhushania* through hypertrophic growth of mother cell, encystment in multilayered wall, palintomic cleavage resulting in a tightly packed mass of pre-propagules, germination by opening of outer cyst wall, and release of propagules by degradation of inner cyst wall. Shown is the role of the outer and inner cyst walls in forming the peanut-shaped germination stages (see also modern mesomycetozoean examples in fig. S7). The outer cyst wall (seldom preserved) is indicated in black; the inner cyst wall dark is indicated in gray.

in the Doushantuo fossils [for example, opalinids are multinuclear (*32*)]. Only volvocalean embryos show so many rounds of palintomy, but the resulting blastomeres are connected by a system of cytoplasmic bridges (*35*) that are not present in the fossils. The combination of palintomy within a multilayered cyst wall and peanut-shaped germination stages as seen in the fossils conforms to the pattern seen in nonmetazoan holozoans; nonetheless, there are no discrete characters in the Doushantuo fossils that are uniquely holozoan. The "animal embryos" likely represent nonmetazoan holozoans or possibly even more distant eukaryote branches.

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Supporting Online Material

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From Flat Foot to Fat Foot: Structure, Ontogeny, Function, and Evolution of Elephant "Sixth Toes"

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Several groups of tetrapods have expanded sesamoid (small, tendon-anchoring) bones into digit-like structures ("predigits"), such as pandas' "thumbs." Elephants similarly have expanded structures in the fat pads of their fore- and hindfeet, but for three centuries these have been overlooked as mere cartilaginous curiosities. We show that these are indeed massive sesamoids that employ a patchy mode of ossification of a massive cartilaginous precursor and that the predigits act functionally like digits. Further, we reveal clear osteological correlates of predigit joint articulation with the carpals/tarsals that are visible in fossils. Our survey shows that basal proboscideans were relatively "flat-footed" (plantigrade), whereas early elephantiforms evolved the more derived "tip-toed" (subunguligrade) morphology, including the predigits and fat pad, of extant elephants. Thus, elephants co-opted sesamoid bones into a role as false digits and used them for support as they changed their foot posture.

The enlarged radial sesamoid bones of giant panda forefeet (*1, 2*) are classic examples of evolutionary exaptation (*3, 4*): co-option of old structures for new functions. It is less widely recognized that such "sixth toes" or "false thumbs" have evolved convergently in numerous tetrapods, such as moles and frogs (*5, 6*). They exist in numerous mammals in a less enlarged state, variably called the prepollex/prehallux (here called predigits), radial/tibial sesamoids, or other terms (such as falciform, accessory scaphoid, or navicular). Whether these sesamoids are ancestrally or convergently evolved in various tetrapod clades remains to be determined. The latter seems likely, given the absence of similar sesamoids in most fossil outgroups, yet a cartilaginous nodular precursor cannot be excluded. Regardless, enlarged sesamoids are quite prominent in both the manus (forefeet) and the pedes (hindfeet) of elephants, where they have been mistaken for sixth digits or otherwise presumed to play a role in foot support (*7*–*9*). Indeed, the recent discovery that moles have developmentally switched their radial sesamoid (prepollex) to a digit-like identity (*10*) intimates that elephants and other species may have done the same. Here, we report a multidisciplinary anatomical, histological, functional, and phylogenetic analysis (*11*) of the predigits in elephant feet. We hoped this would illuminate how elephants evolved their characteristic subunguligrade (nearly "tip-toed," with only distal toes contacting the ground) foot posture and function, as compared with the plesiomorphic plantigrade ("flat-footed," with wrists/ankles contacting the ground) foot posture in many other tetrapods.

In 1710, Blair (*7*) provided the first detailed osteological description of elephants, concluding that they have six toes. The "sixth toes" (medialmost position; corresponding to digit zero) were later identified as the enigmatic prepollex

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