Nuclear envelope budding is an evolutionary conserved phenomenon

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Keywords: Nuclear transport, electron tomography, budding, vesicles, exosomes

Running title: Electron Microscopy of Nuclear Budding

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Abstract

Eukaryotic cells are defined by the compartmentalization of the cytoplasm into organelles, the largest of which is the nucleus, which contains the cellular DNA. Transport into and out of the nucleus is highly regulated and is traditionally thought to occur solely through nuclear pores. However, a small number of papers has repeatedly shown vesicular budding from the nuclear envelopes in different organisms. We used electron microscopy to identify such nuclear envelope budding events in a human cell line, *Caenorhabditis elegans* worms, the two yeasts *Saccharomyces cerevisiae* and *Schizosaccharomyces pombe* and the parasitic protist *Trypanosoma brucei*. Progressing to electron tomography, the finer details of the 3D architecture of such budding events was revealed. We summarize all the organisms in which this mode of translocation over the nuclear envelope has been observed and conclude that this may be a fundamental, evolutionary conserved mechanism of transport inside eukaryotic cells.

Introduction

The nucleus is the most prominent organelle of the eukaryotes, enclosing most of the cell's genetic material within their double lipid bilayer, the nuclear envelope. These two membranes were the evolutionary step between prokaryotes and eukaryotes that pose limitations for certain molecules to be in contact with the DNA. As the nuclear envelope is not permeable for most of the molecules inside the cells, special structures on their surfaces, called the nuclear pores, exists for highly selective translocation over the membranes (Watson, 1954). Small molecules can passively penetrate the nuclear pores from the cytoplasm to the nucleus and vice versa, while bigger molecules require an energy-dependent transportation mechanism to enter or leave the nucleus. Different types of proteins are required for guiding the larger sized molecules through

the pores, forming specific complexes for importing or exporting their cargoes (importins and exportins respectively) (Chook et al., 2001; Hoelz et al., 2011).

Since the discovery of the nuclear pores in 1950, this transporting system has been accepted as the only known way of communication between the cytoplasm and the nucleoplasm (Kohler et al., 2007). However, in virus-infected cells, an alternative method of nuclear export has been described. Herpes simplex virus, which are replicated in the nucleoplasm, are released into the cytosol via outwards budding of the nuclear envelope (Schwartz et al., 1969; Hagen et al., 2015; Fradkin et al., 2016; Wang et al., 2018). Since 1955, there have been many observations and reports suggesting that these nuclear budding events probably occur in healthy cells as well and different interpretations of their mechanism have been discussed (Gay, 1955; Hadek et al., 1962; Afzelius, 1963; Elston et al., 1965; Longwell et al., 1965; D. Szollosi, 1965; Aldrich et al., 1970; Dickinson et al., 1970; Gullvåg, 1970; Mepham et al., 1970; Dickinson, 1971; Hochstrasser et al., 1987; M. S. Szollosi et al., 1988). These observations were made between a range of organisms using mostly cells that are under differentiation (embryonic cells) and various terminologies were given to describe the events (Table 1). The most recent reports were made in healthy developing *Drosophila melanogaster* embryos and in the sea urchin gastrula (Hatch et al., 2012; Speese et al., 2012; Jokhi et al., 2013; LaMassa et al., 2018).

This poses the question: is nuclear envelope budding an alternative way within eukaryotic cells for the transport of material through the nuclear envelope specifically during viral infections and in the development of some organisms, or is it instead a universal mechanism found in most/all eukaryotes? In this study, we examine nuclear envelopes in a human cell line (*Homo sapiens*), the worm (*Caenorhabditis elegans*), the budding yeast (*Saccharomyces cerevisiae*), the fission yeast (*Schizosaccharomyces pombe*) and the protozoan African sleeping sickness parasite

(*Trypanosoma brucei*), to see if there is an evolutionary conserved alternative path of nuclear import and export, besides the tightly regulated traffic over nuclear pores, in eukaryotes. We use cells that are matured and grown under normal conditions to investigate if these events are only present during cell differentiation or are independent of the organism's developmental cell stage.

Materials and Methods

Preparation of cell cultures and animals

Human mast cell line 1 (HMC-1; (Butterfield et al., 1988) cells were cultured in Iscove's modified Dulbecco's medium (IMDM) and treated as described previously (Zabeo et al., 2017). Procyclic *T. brucei* strain 427 was cultured in SDM-79 media with 20% fetal bovine serum. Cultures were prepared and maintained at a concentration of 5×10^5 and 1×10^7 cells per milliliter (Höög et al., 2010). Logarithmically growing wild type cells of the fission yeast *S. pombe* were grown at 30^0C as described (Höög et al., 2007; Zabeo et al., 2017). The *C. elegans* wild type reference strain was the Bristol variety, N2. The worms were cultured on normal growing media plates (NGM plates) and the *E. coli* strain OP50 was used as a food source. The worms were maintained at the optimal temperature of 25 ^{0}C (Sulston et al., 1988; Svensk et al., 2016). Wild type cells of *S. cerevisiae* (S228C background) were cultured and maintained in YPD media (Hill et al., 2016).

High-pressure freezing of cells and animals for electron microscopy

All samples used in this study has been prepared using high-pressure freezing followed by freeze substitution. HMC-1 and *S. cerevisiae* cells were loaded into membrane carriers and were high-pressure frozen in a Leica EM PACT1 (Leica microsystems, Wetzlar, Germany). *T. brucei* and *S. pombe* were loaded into carriers and were high-pressure frozen in a Leica EM PACT2. *C.*

elegans samples were loaded into aluminium specimen carriers and were high-pressure frozen in a Wohlwend Compact 3 (M. Wohlwend GmbH, Sennwald, Switzerland). Yeast paste from wild type *S. cerevisiae* cells was used as a cryoprotectant. A short freeze substitution protocol was applied, using 2% uranyl acetate (UA) for one hour (Hawes et al., 2007; Zabeo et al., 2017). Samples were then embedded in K4M or HM20 resin.

All samples were sectioned in 70 nm thin sections and placed on either copper slot grids or mesh grids. Sections were stained using 2% UA and Reynold's lead citrate (Reynolds, 1963).

Preparation of cells for electron tomography

For tomography, *S. pombe* cells were high-pressure frozen and fixed through freeze substitution with anhydrous acetone containing 0.25% UA, 0.1% dehydrated glutaraldehyde and 0.01% osmium tetroxide (OsO_4). Serial semi thick sections of about 210-250 nm (*S. pombe*) and 350 nm (*T. brucei*) thick sections were cut and samples were treated as described before (Höög et al., 2007; Höög et al., 2014).

Image acquisition and electron tomography

All thin sections were imaged at 120 kV either on a LEO 912 OMEGA (Zeiss, Krefeldt, Germany) equipped with a 2k x 2k VELETA Olympus CCD camera or on a Tecnai T12 transmission electron microscope equipped with a Ceta CMOS 16M camera (FEI Co., Eindhoven, The Netherlands). Tilt series of serial sections of *T. brucei* samples were acquired using the serialEM software on a Tecnai TF30 300 kV microscope equipped with a Gatan OneView 4K camera (FEI Co., The Netherlands). For the *S. pombe*, digital images were taken every 1.5^{o} over a $\pm 60^{o}$–65° range operating a Tecnai TF20 electron microscope (FEI Co., Eindhoven, The Netherlands). The tomograms were acquired with the use of the serialEM

(Mastronarde, 2005). The tomographic reconstruction was performed with the use of the IMOD software package (Kremer et al., 1996).

Phylogenetic tree

A phylogenetic tree was made showing all the organisms where nuclear budding events have been observed. The tree was generated based on the NCBI taxonomy browser for scientific names and visualized by the EvolView online software.

Results

Electron microscopy pictures revealed nuclear budding events in HMC-1 cells

To examine if nuclear budding occurs in human cells, we imaged 122 thin sections of human mast cells (HMC-1) containing a nucleus using transmission electron microscopy. The HMC-1 nucleus has a diameter of ~8 μm. Therefore, the 122 thin sections observed here corresponds to approximately one nuclear volume (8 μm/70 nm thick sections = 114 serial sections equals one nucleus). Three morphologically different categories of budding events were observed (Figure 1A-C; n=122 sections). The most commonly observed morphology was outwards directed buds (Figure 1A). In some cases, the electron dense material is clearly between the two membranes with the outer leaftlet expanding towards the cytoplasm. In other occasions, both the outer and inner membrane leaflets are expanding towards the cytoplasm with material partially wrapped by the nuclear envelope bud (Figure 1A). The second form of nuclear envelope extensions observed was material found in between the nuclear membranes with no clear protrusion in either direction (Figure 1B). Lastly, budding events with a general morphology of the nuclear envelope expanding towards the nucleoplasm were also observed (Figure 1C). None of these nuclear budding events are resembling nuclear envelope and ER contact sites (Figure S1). The budding

events were found in 11.5% of HMC-1 cells with some thin sections of nuclear envelopes containing more than one bud. The frequency of the different bud morphologies is displayed in Figure 1D. In order to investigate if nuclear budding events are evolutionary conserved inside animal somatic cells we progressed to examine the nuclear envelope of the nematode *C. elegans* in a similar manner.

Nuclear budding in the nematode *C. elegans*

Whole *C. elegans* specimens were high pressure frozen and sectioned for electron microscopy. Images were taken of somatic cells containing a nucleus (n= 138). This number represents approximately a total nuclear volume of five nuclei. 12.3% of these images contained a nuclear envelope budding event. The majority of the nuclear budding events recorded in *C. elegans* have the general morphology of the outwards protruding buds described for the HMC-1 cells (Figure 2A-C). Only one event fell in the third category of the inward nuclear envelope buds as described before (Figure 2D). In these budding events, the material was visible between the membranes, with one occasion where the material appeared to be surrounded by a membrane (Figure 2C). Similar to the HMC-1 cells, some of the nuclear sections contained more than one budding event. Having established that nuclear envelope budding is present in somatic animal cells, we progressed to study species from other kingdoms of life.

Nuclear outwards budding events are also present in the fungi *S. cerevisiae*

The fungi *S. cerevisiae* and their nuclei were visualized in a total of 161 thin sections. This number of sections corresponds to a total nuclear volume of five nuclei. All the five nuclear budding events recorded in *S. cerevisiae* have the general morphology of the outwards protruding buds as described for the HMC-1 cells (Figure 3A-C). No other categories were found

(Figure 3D). In these budding events, the electron dense material was not always visible but, in some occasions, part of the nucleoplasm seemed to be surrounded by the bud (Figure 3C). *Schizosaccharomyces pombe*, another well-studied organism from the Fungi kingdom but with different properties than the *S. cerevisiae*, was further investigated.

Nuclear budding events in *S. pombe*

The fission yeast *S. pombe* was sectioned and 101 images were observed. This number corresponds to a total nuclear volume of four nuclei. In these images, 7 nuclear budding events were observed, of which most had the outwards budding morphology (Figures 4A, 4C) with one of them being positioned in between the membranes (Figure 4B). Two of the events had a strikingly bigger size compared to the rest and an extra membrane layer surrounded the electron dense material (Figure 4B). The morphology of the budding events varied a lot, with some buds having small sizes with not so obvious electron dense material and others being a lot bigger with an extra membrane surrounding the material.

The nuclear budding events are conserved between mammals and protozoa

To investigate if nuclear budding events also occur in a unicellular organism of the protozoa sub-kingdom, thin sections of *T. brucei* cells were prepared as described before and 119 nuclear slices were observed. This number corresponds to a total nuclear volume of approximately four nuclei. Nuclear budding events were also observed in these samples. All the budding events found in the *T. brucei* nuclei had the outwards protruding morphology (Figure 5A-C). In the majority of the visualized events, the electron dense material was easily observed and had a distinct round morphology (Figure 5C). In order to investigate the 3D structure of these budding

events, electron tomography was performed on *T. brucei* as well as the yeast *S. pombe* samples, another well-studied representative of the fungi kingdom.

Electron tomography shows the 3D architecture of nuclear budding events in *S. pombe* and *T. brucei*

Using electron tomography, a better z-resolution can be achieved and the detailed membrane morphology of the nuclear budding events could be revealed. Therefore, montaged dual-axis electron tomograms of large volumes of high-pressure frozen fission yeast and *T. brucei* cells were acquired, including approximately three and one complete nuclear volumes respectively (Figure 6 A and D; n=1 *S. pombe* budding event; n=2 *T. brucei* budding events). In *S. pombe*, one event of nuclear envelope budding was visualized. The membrane-enclosed vesicle had a nucleoplasm similar texture and was located in between the two nuclear lipid bilayers (Figure 6B). The budding vesicle dimensions were 88 nm length by 149 nm width. The 3D model of this nuclear envelope budding event shows that only the outer envelope is being extended around the vesicle.

In electron tomograms of *T. brucei*, two events of nuclear budding were seen (Figure 6E, 6G). The most progressed budding event had very distinct electron densities at the neck of the nuclear envelope (Figure 6G, white arrow). The buds had dimensions of 78 nm length by 75 nm width and 94 nm length by 69 nm width respectively. Similarly, only the outer nuclear envelope was extended to surround a particle/vesicle surrounded by an electron dense material, the presence of a membrane surrounding the transported material could not be ascertained (Figure 6 F, 6H).

Thus, we conclude that nuclear envelope budding was found in between 3 and 12% of the thin sections observed in all species examined, spanning from human cell line to the protist *T. brucei*.

The nuclear envelope buds were similar in all cells but displayed a greater variability and larger size in HMC-1 cells (Figure 1 and S2).

Discussion

We have shown that nuclear budding events can be seen in cells of all of the examined species, stretching from animals, over fungi into protozoa (In total 540 thin sections have been observed). The frequency of these events corresponds to approximately 2.7 times per nucleus. This shows that the nuclear envelope budding is an evolutionary conserved feature of eukaryotes, even though the structure of such budding events appears to change between species. For example, in human cells, we saw three different kinds of nuclear envelope extensions, described as inwards protruding, outwards protruding and particles in between the double lipid bilayers. The size of these nuclear envelope buds had only small variations between the species with HMC-1 cells having relatively bigger sized buds (Figure S2).

Even though not widely discussed as a means of nuclear transport, there is a trail through the literature of these events occurring in animals, plants and protists. Figure 8 shows a phylogenetic tree of all the species where nuclear budding events have been observed. Including the species in our study, these events have now been reported in 18 different species across the eukaryota domain. This is the first time all these observations from 1955 until now have been gathered and presented as an evolutionary conserved process. The two fundamental questions are now: what do they transport and where does it go? In a relatively recent study in *Drosophila melanogaster* embryos, it has been shown that ribonucleoproteins (RNPs) are being exported through nuclear envelope budding (Speese et al., 2012). That groundbreaking study strongly supports the hypothesis that nuclear envelope budding facilitates the transport of genetic material from the nucleus to the cytoplasm (Gay, 1955; Hadek et al., 1962; Afzelius, 1963; Longwell et al., 1965;

D. Szollosi, 1965; Aldrich et al., 1970; Gullvåg, 1970; Mepham et al., 1970; Hochstrasser et al., 1987). Alternative theories argue that these budding events may be a membrane storage mechanism that the cell will use in later stages of its development or that they play a role in the formation of the endoplasmic reticulum (ER) and secretion granules (Gay, 1955; Dickinson et al., 1970). An alternative, maybe more far-fetched, interpretation was that these budding vesicles enclosing genetic material might be incipient mitochondria. That theory was based on the increased number of mitochondria in the cytoplasm of pollen grains during the mitotic period that these budding events were observed (Mepham et al., 1970). The exact role of these events is not yet determined so none of the above theories can be ruled out. However, viruses are known to high-jack the cells own functions to produce new virions (Davey et al., 2011). The fact that viruses such as herpes simplex, Rous sarcoma and SV40 use this mechanism to exit the nucleus (Epstein, 1962; Elston et al., 1965; Darlington et al., 1968; Hagen et al., 2015; Fradkin et al., 2016; Roller et al., 2017), and its evolutionary conservation, are strong arguments for that this mechanism of transport over the nuclear envelope has an important function inside eukaryotic cells.

That leaves us with the question where are these vesicles transported? Some of our images shows clearly that the vesicle formed by the budding event will have a double bilayer (Figure 2C, 4B, 6B). Yet, we do not commonly observe double membrane vesicles in the cytoplasm of these cells (Höög et al., 2007), indicating that these vesicles could be either delivered fast to the plasma membrane, fused with another compartment, demembranated in the cytoplasm, or that we have failed to note their presence. A number of cryo-electron microscopy studies of exosomes, small vesicles released from cells into the extracellular space, have shown a small fraction of the population to have double and even triple bilayers (Poliakov et al., 2009; Yuana et al., 2013;

Höög et al., 2015; Zabeo et al., 2017). In particular, our previous study of HMC-1 cell derived exosomes showed vesicles with double membrane bilayers 100-200 nm in size, similar to the size of nuclear budding events measured in most of these cells (Zabeo et al., 2017). This suggests that vesicles formed by nuclear envelope budding might be secreted. However, all previous studies of nuclear envelope derived vesicles argue that the enclosed material should be released into the cytoplasm at some stage but identification of the released material once in the cytoplasm has not yet been possible. This obviously limits our ability to understand the function of these budding events. An interesting suggestion on how they are transferred and released into the cytoplasm is that of the connection with the endoplasmic reticulum (ER) (Hadek et al., 1962; Gullvåg, 1970). The idea in this case was that once the buds are located between the two nuclear membranes, they travel through the ER cisternae from where they are later released into the cytoplasm as ER budding vesicles. As the ER is a continuation of the outer nuclear membrane and well known for the vesicles it releases (Shibata et al., 2010; Phillips et al., 2015), this might be a theory worth considering.

Many of the studies of nuclear envelope budding so far has been made on cells that were found in developing organisms. Based on these studies, it was assumed that these budding events are important during specific developmental stages of the cells (D. Szollosi, 1965; Dickinson, 1971; M. S. Szollosi et al., 1988; LaMassa et al., 2018), none of these studies looked for events of nuclear envelope budding in mature organisms. In our experiments, we examined adult worms and cell culture cells under normal conditions. Observing frequent budding events under these conditions implies that these nucleo-cytoplasmic interactions are also important after the organisms are fully developed and might be part of normal cellular activity. This mode of transport across the nuclear envelope may have different cargo and function depending on the

developmental stage of the cell. We conclude that this is a fascinating area of conserved cell biology that has been largely ignored to date and we hope this paper encourage to new studies in the field.

Acknowledgements

This project was funded by the Swedish Research Council Grand (2015-00560) and the Knut and Alice Wallenberg Foundation (grand KAW 2012.0284, KAW 2014.0275) to Richard Neutze, the Swedish Research Council Young Investigator Grant (2015-05427) and Knut and Alice Wallenberg grant to Prof. Thomas Nyström and Johanna Höög.

All authors would like to thank Prof. Marc Pilon for providing the *C. elegans* worms and for contributing intellectually to the manuscript writing. We would also like to thank Prof. Thomas Nyström for providing the *S. cerevisiae* strain.

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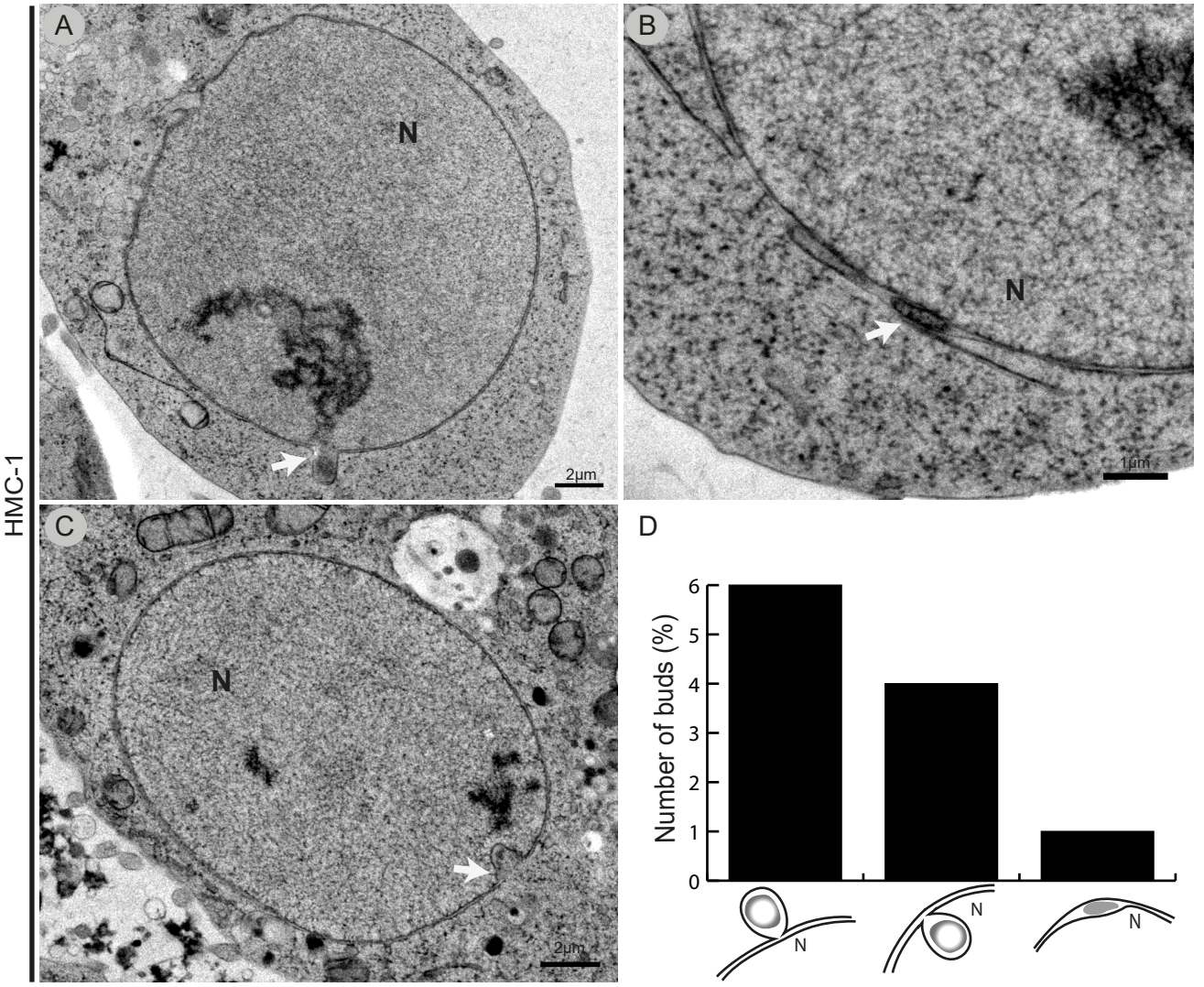
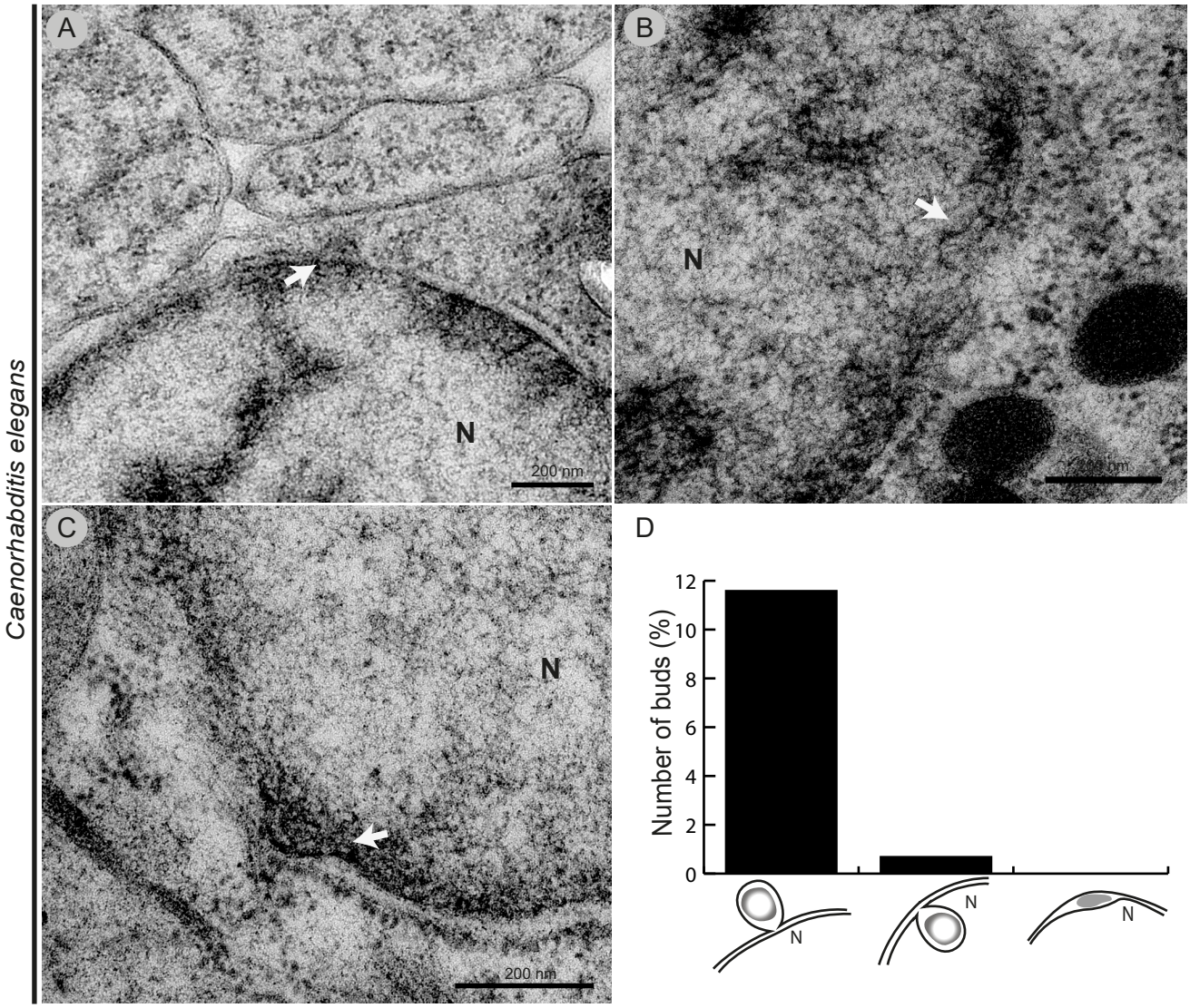
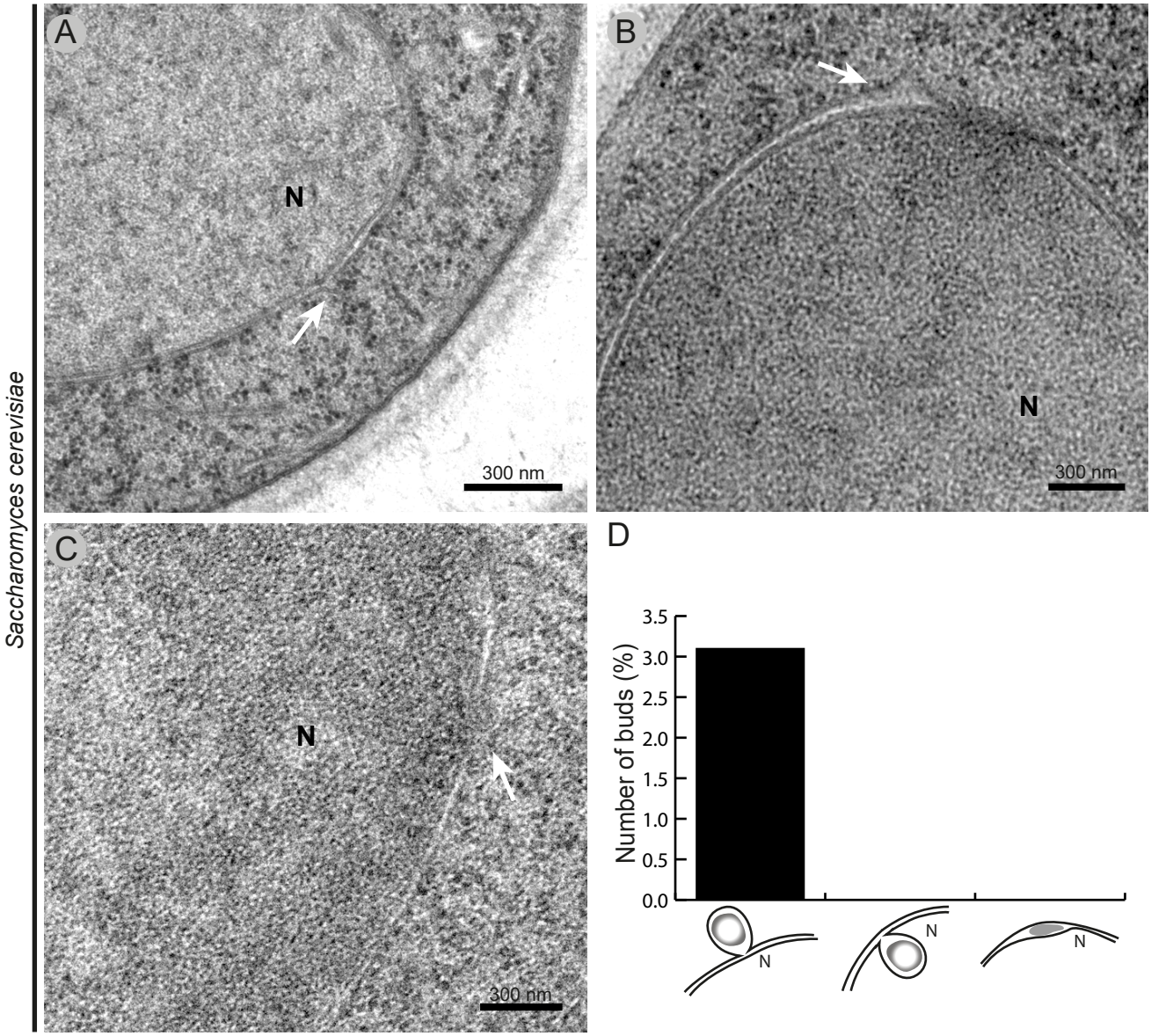
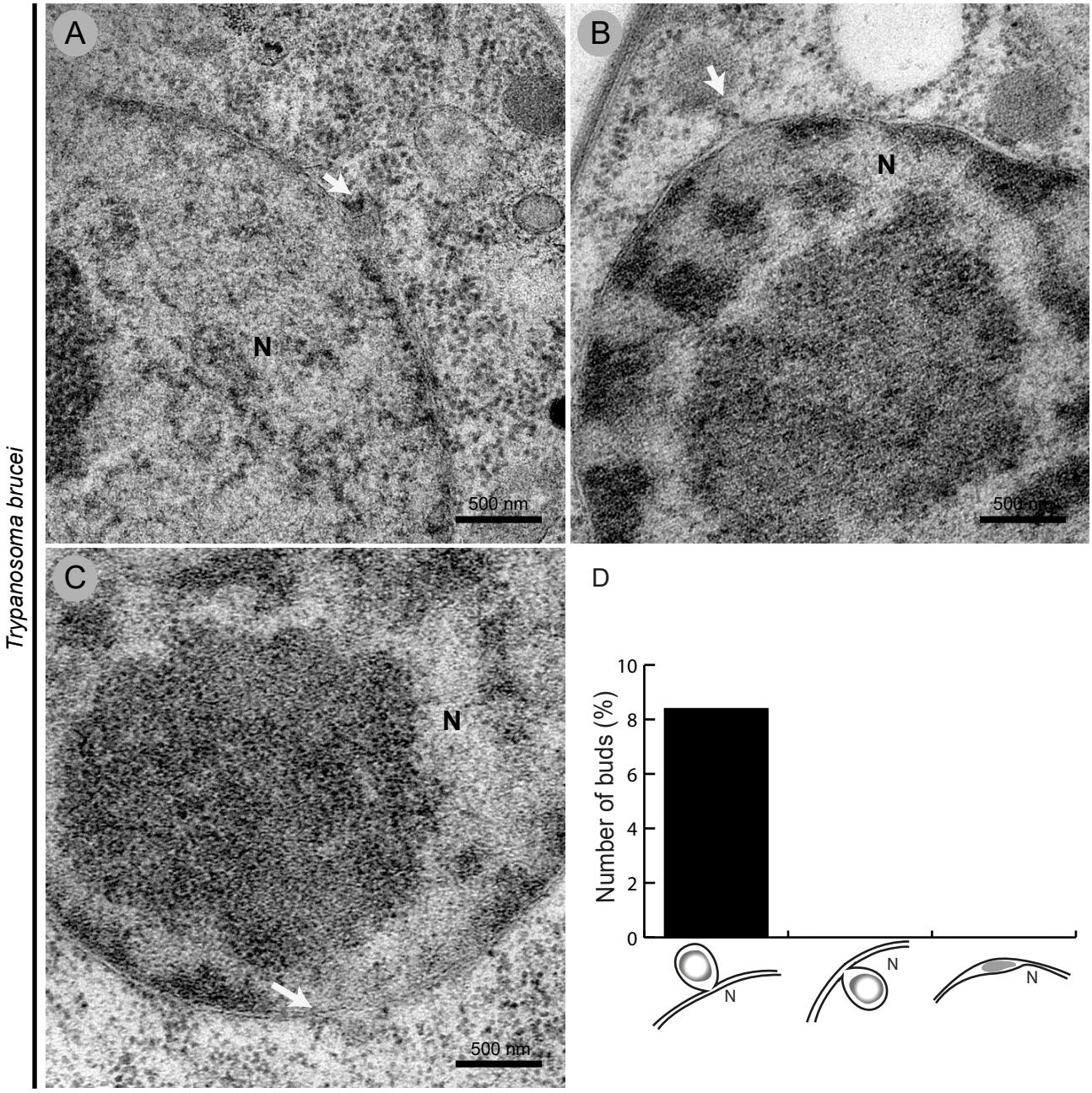
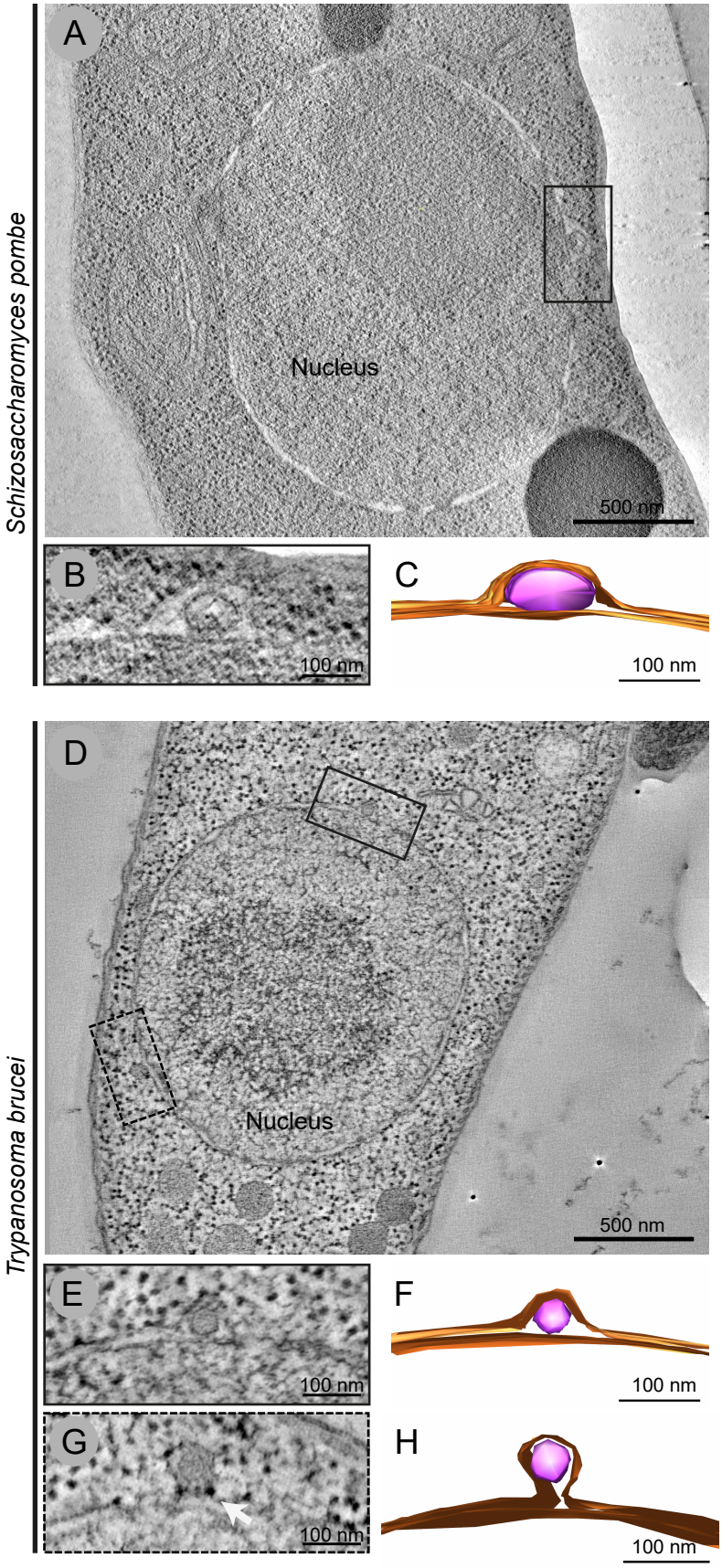
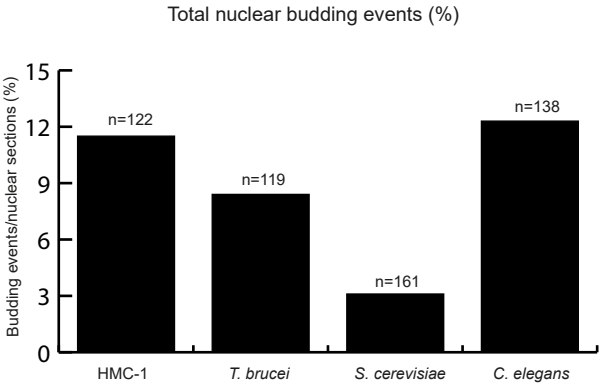
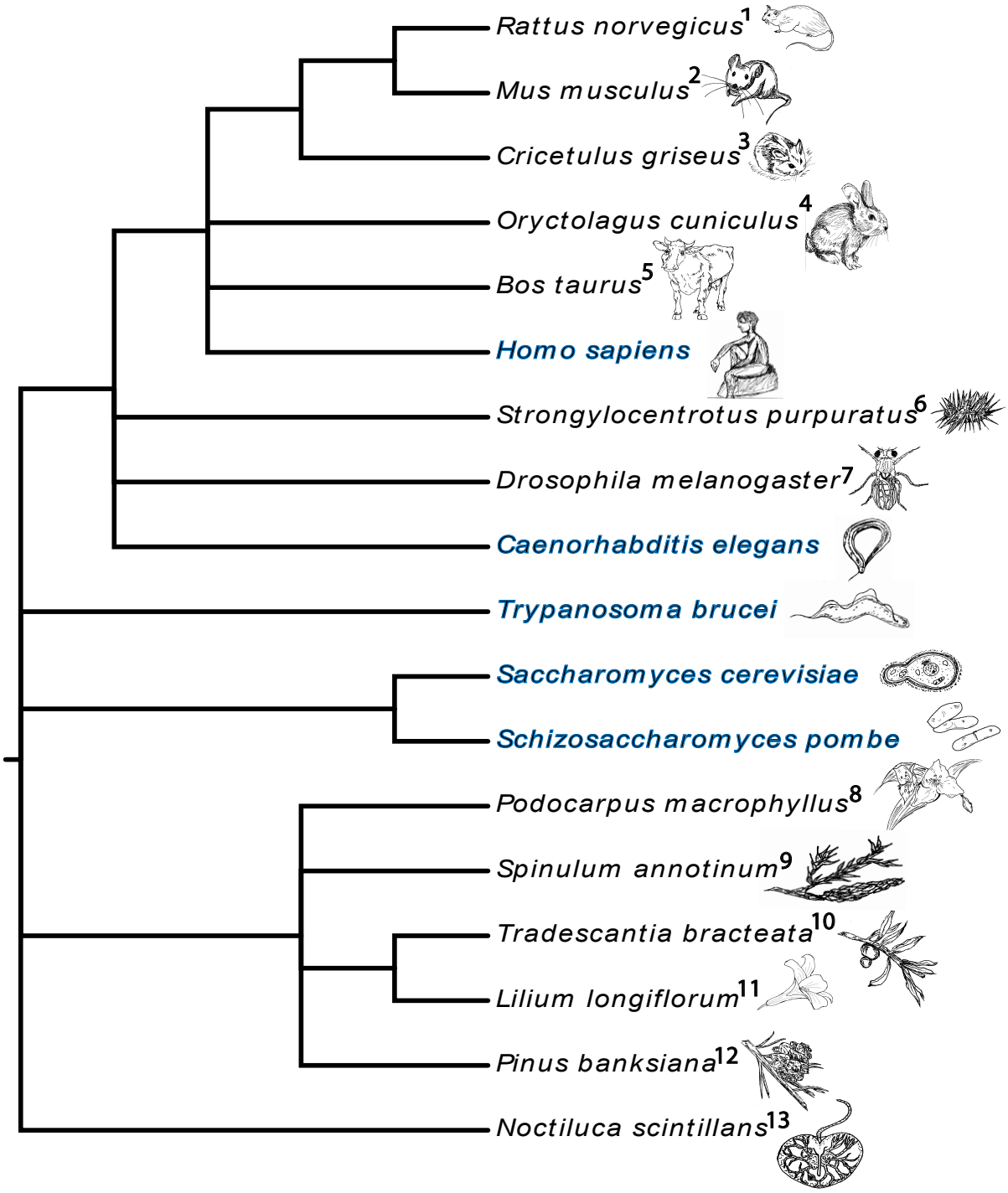

HMC-1
A
N
2μm
B
N
1μm
C
N
2μm
D
Number of buds (%)
0
1
2
3
4
5
6
N
N
N


Caenorhabditis elegans
A
N
200 nm
B
N
C
N
200 nm
D
Number of buds (%)
12
10
8
6
4
2
0
N
N
N


Saccharomyces cerevisiae
A
N
300 nm
B
N
300 nm
C
N
300 nm
D
Number of buds (%)
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
N
N
N


Trypanosoma brucei
A
N
500 nm
B
N
500 nm
C
N
500 nm
D
Number of buds (%)
10
8
6
4
2
0
N
N
N


Schizosaccharomyces pombe
A
Nucleus
500 nm
B
100 nm
C
100 nm
Trypanosoma brucei
D
Nucleus
500 nm
E
100 nm
F
100 nm
G
100 nm
H
100 nm


Total nuclear budding events (%)
Budding events/nuclear sections (%)
15
12
9
6
3
0
n=122
n=119
n=161
n=138
HMC-1
T. brucei
S. cerevisiae
C. elegans

Phylogenetic tree of species with observed nuclear budding events