

REVIEW ARTICLE

Evolutionary Cell Biology of Proteins from Protists to Humans and Plants

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Keywords

Cell function; eukaryote; evolution; molecular biology; protozoa; ultrastructure.

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ABSTRACT

During evolution, the cell as a fine-tuned machine had to undergo permanent adjustments to match changes in its environment, while “closed for repair work” was not possible. Evolution from protists (protozoa and unicellular algae) to multicellular organisms may have occurred in basically two lineages, Unikonta and Bikonta, culminating in mammals and angiosperms (flowering plants), respectively. Unicellular models for unikont evolution are myxamoebae (*Dictyostelium*) and increasingly also choanoflagellates, whereas for bikonts, ciliates are preferred models. Information accumulating from combined molecular database search and experimental verification allows new insights into evolutionary diversification and maintenance of genes/proteins from protozoa on, eventually with orthologs in bacteria. However, proteins have rarely been followed up systematically for maintenance or change of function or intracellular localization, acquirement of new domains, partial deletion (e.g. of subunits), and refunctionalization, etc. These aspects are discussed in this review, envisaging “evolutionary cell biology.” Protozoan heritage is found for most important cellular structures and functions up to humans and flowering plants. Examples discussed include refunctionalization of voltage-dependent Ca^{2+} channels in cilia and replacement by other types during evolution. Altogether components serving Ca^{2+} signaling are very flexible throughout evolution, calmodulin being a most conservative example, in contrast to calcineurin whose catalytic subunit is lost in plants, whereas both subunits are maintained up to mammals for complex functions (immune defense and learning). Domain structure of R-type SNAREs differs in mono- and bikonta, as do Ca^{2+} -dependent protein kinases. Unprecedented selective expansion of the subunit a which connects multimeric base piece and head parts (V0, V1) of H^{+} -ATPase/pump may well reflect the intriguing vesicle trafficking system in ciliates, specifically in *Paramecium*. One of the most flexible proteins is centrin when its intracellular localization and function throughout evolution is traced. There are many more examples documenting evolutionary flexibility of translation products depending on requirements and potential for implantation within the actual cellular context at different levels of evolution. From estimates of gene and protein numbers per organism, it appears that much of the basic inventory of protozoan precursors could be transmitted to highest eukaryotic levels, with some losses and also with important additional “inventions.”

BASIC PRINCIPLES

For the evolution of complex eukaryotic cells, increase of genetic information and its sequestration into a nucleus was a prerequisite. This, paralleled by increased

cytoplasmic complexity, has been facilitated by increased energy supply enabled by symbiotic nidation of endocytosed bacteria performing oxidative phosphorylation (Hedges 2002). Although Lynch and Marinov (2016) recently argued that acquirement of mitochondria did not

Table 1. Changes of defined proteins and/or reassignment to variable functions during evolution

Proteins essentially maintained in structure, function, and localization
EF-hand and C2 domain Ca ²⁺ -binding proteins (CaBPs: CaM), clathrin, COPs, GTPases, IP ₃ R (lost in some taxons), Ca ²⁺ -ATPases/pumps (PMCA, SERCA), SNAREs (incl. Syntaxin, SNAP-25), NSF
Expansion of subfamily members by ohnologs
SNAREs, actin, tubulin, H ⁺ -ATPase SU-a
Proteins with characteristics changing between subfamily members
Stomatin/flotillin in ciliates
Proteins with changing intracellular localization
Centrin (spindle cap, in plasmodesmata of plants); Ca ²⁺ -influx channels: CaM-controlled influx channels incl. voltage-gated Ca ²⁺ channels (in cilia of ciliates, in neurons, lost in plants)
Proteins with specific, evolutionarily variable domain numbers
CaBPs with C2 domain motifs: PL-C, synaptotagmins with variable number of C2 domains
Proteins with occasional extra-domains
R-SNAREs (longin-type “synaptobrevins” in ciliates and plants)
Proteins with incorporated regulating sequences
Ca ²⁺ -dependent protein kinases (CDPK, with CaM-like domain) in ciliates and plants
Proteins lost during evolution
CRCs type IP ₃ R and RyR-LPs lost in apicomplexan parasites (malaria) and in plants
Proteins further differentiating during evolution
CRCs type RyR-LP or RyR: only RyRs in higher animals
Loss of a subunit
CaN: SU-A lost in plants
Refunctionalization of proteins
η-tubulin in ciliates: engaged in basal body formation; proteins engaged in cytokinesis recruited for biogenesis of the contractile vacuole complex
Functionalization of proteins with originally unknown function
GPI anchor of vsAGs in protozoa vs. animal forms
Proteins newly “invented” during evolution
Examples: animals: keratin, cyclic hormones (e.g. oxytocin); plants: cellulose synthase
Proteins under discussion for ciliates and other protozoa
Examples: apoptosis proteins ciliates: programmed nuclear death, trimeric G-proteins

Note that indications given do not reflect all details which are to be retrieved from the text.

boost biogenetic capacity in eukaryotic cells, the advantage may have been the provision of ATP at defined intracellular sites near mitochondria, just as discussed for the involvement of Ca²⁺ signaling also in early eukaryotic evolution (below). This was paralleled by efficient phagocytosis of nutrient bacteria for intracellular digestion. Archaea, particularly of the “TACK” superphylum (Lokiarchaeota), recently come into the limelight of eukaryote evolution, since they share important molecules with eukaryotes (Guy and Ettema 2011; Koonin and Yutin 2014) and anticipated the following aspects: protein-bound DNA (nucleosomes), provision of cytoskeletal elements, protein modifications, and endo-/phagocytosis capability (Ammar et al. 2012; Spang et al. 2015). Such archaeobacteria may have provided a “mother-cell” for endocytotic uptake and symbiotic integration of specific eubacteria, together with uptake of specific eubacterial genes. The most essential components of the cytoskeleton, actin, and tubulin in polymerizable form are thought to originate from stages preceding the last common eukaryotic ancestor (Koonin and Yutin 2014), followed by accessory and regulatory proteins (Wickstead and Gull 2011). Partial compartmentalization is observed already in multiple prokaryotic lineages (Diekmann and Pereira-Leal 2013), as are some prokaryote cell fusions (Martijn and

Ettema 2013) and extensive lateral gene transfer (Abby et al. 2012; Katz 2012), to mention just some aspects of most likely importance for early eukaryote evolution.

With increasing complexity, this prokaryote-to-eukaryote transition required control by a plethora of key molecules some of which are addressed in this review. Some concern Ca²⁺ which steadily leaks into cells and is transformed from toxic agent into a useful second messenger. The cell has to remain flexible, as a fine-tuned machine, under steadily changing conditions. Each cell may be optimally organized in the respective environment, as pointed out for organismic life by environmentalist Jakob von Uexküll already in the 19th century. Nevertheless, terms such as “lower” and “higher” eukaryotes may be used to indicate evolutionary successions. However, “low” does not necessarily mean primitive; for this, we will find ample testimony when we trace the “evolutionary career” of individual proteins. As summarized in the Tables 1, 2, we see proteins maintained throughout evolution, but often with more or less changes in structure, function, and intracellular localization. In this sense, “evolutionary cell biology” a term propagated by Lynch et al. (2014) should follow the key goal to provide cell biology with an evolutionary perspective. This is the perspective of this article.

Table 2. Similarity (of molecular structure) and comparison of intracellular localization of selected proteins in Monokonta and Bikonta

Molecule	Occurrence in		Comparison Mono-/Bikonta	
	Monokonta	Bikonta	Similarity	Localization
CaM	+	+	Yes	Variable
CaN	+ (SU-A and SU-B)	+ (<i>Paramecium tetraurelia</i>) – Plants: SU-B only	Yes	Variable
GTPases	+	+	Yes	Comparable
Q-SNAREs	+	+	Yes	Identical
R-SNAREs				
“Brevins”	+	– (<i>P. tetraurelia</i>)	na	na
Longins	+/- (Rare in mammals)	+ <i>P. tetraurelia</i> , plants	Mostly diff.	na
Synaptotagmin	+	+ Plants (not in <i>P.t.</i>)	Yes	na (plants)
eSyntag	+ <i>Monosiga brevicollis</i> + Mammals (rare)	+ <i>P. tetraurelia</i>	Variable	Unknown
PMCA	+	+	Yes	Identical
SERCA	+	+	Yes	Identical
Flotillin/Reggie	+	+	Variable	Variable
Centrin	+	+	Yes	Different
Actin	+	+	Yes	Variable
Tubulin	+	+	Yes	Similar
CDPK	–	+ Ciliates, plants	Different	Unknown
V-dep.Ca ²⁺ channels	+	+ <i>P. tetraurelia</i> + <i>Chlamydomonas</i> – Plants	Identical? na na	Different Identical na
TRP channels	+	– Ciliates + <i>Chlamydomonas</i>	na na	na na
IP ₃ R	+	+ <i>P. tetraurelia</i> – Plants	Yes na	Similar
GPI proteins	+	+	Similar anchor	Different

Note that this table gives only a gross outline; for details, see text.

n.an/na = not analyzed/not applicable or not analyzed; *P.t.* = *Paramecium tetraurelia*.

There is a common molecular heritage

Widely different genes from different bacteria must have been collected by early eukaryotes; in the end (archae) bacterial genes contribute by ~37% to mammalian gene sequences (McFall-Ngai et al. 2013). Moreover, gene transfer occurs frequently between different levels of evolution (Soucy et al. 2015), and this is an ongoing process up to vertebrates (Crisp et al. 2015). Altogether ~28% of genes are all-eukaryotic (McFall-Ngai et al. 2013). This makes this review feasible. It starts at the level of protists (and occasionally of bacteria), meaning mainly protozoa and fewer taxons of algae, the green counterparts.

Protozoa already contain a considerable inventory of genes/proteins and protein assemblies which can be followed up to multicellular organisms. There are reasons to assume that the last eukaryotic common ancestor already possessed such organelles (Brooks and Wallingford 2014). Two main evolutionary lineages are frequently envisaged; depending on the occurrence of single cilia (or flagella) or of ciliary (flagellar) pairs, organisms are subdivided—whether right or wrong remaining debatable—into mono- or bikonta. On this basis, one can ask—beyond genomics—two fundamental questions. First, which changes in function and localization may

have occurred during evolution? Second, are there consistent differences between the main lineages of eukaryote evolution, such as mono- and bikonts? As summarized in Table 1, widely different mechanisms result in changes during evolution. According to Table 2, about two-thirds of the molecules analyzed look rather similar the two lineages, only about one-third being considerably different, but with considerable changes in intracellular localization.

Proteins mandatory for basic eukaryotic functions are in particular focus. Cytoskeletal elements, like microtubules and microfilaments, have been inherited from prokaryotes (Pilhofer et al. 2011), to which, in eukaryotes, accessory proteins and motor proteins have been adopted (Wickstead and Gull 2011). This was required for intracellular transport, together with motor proteins whose number is identical in *Tetrahymena thermophila* and in *Homo sapiens* (Eisen et al. 2006). This was complemented by molecules for vesicle budding and other proteins mediating specificity of membrane-to-membrane interactions, docking, and final fusion. All this is mediated essentially by coatomer proteins (COPs), SNARE proteins (soluble N-ethylmaleimide-sensitive factor attachment protein receptors) and GTPases (monomeric low-molecular weight GTP-binding/GTP hydrolyzing proteins) (Bonifacino and

Glick 2004; Grosshans et al. 2006; Mizuno-Yamasaki et al. 2012; Rothman 2014; Südhof 2014).

SNAREs are integrated in membranes by a single carboxy-terminal hydrophobic stretch (Rothman 2014; Südhof 2014). They contain a SNARE domain around central layer of a typical aminoacids, Arg and Gln, and, therefore, are called, R- and Q-SNAREs, respectively. This R/Q situation, however, is not fully maintained, from *Paramecium* on up to highest species (Plattner 2010a). GTPase effects are regulated by proteins with antagonistic, activating, and inhibiting effects still to be studied in protists.

Establishment of a simple intracellular trafficking system is considered an early achievement of eukaryote evolution (Dacks and Field 2007), and this may have included SNAREs and GTPases. Remarkably, for early eukaryotes a set of ~20 SNAREs has been extrapolated (Kloepper et al. 2008). Although this is well below values found in protists and in any other organism (Plattner 2010a), this may have allowed for increased energy input by phagocytosis, as found already in Archaeota (Koonin and Yutin 2014) and further differentiation of lytic compartments and recycling vesicles. In addition, H⁺-ATPase/pumps and Ca²⁺-regulating proteins had to be established, as to be demonstrated. Some of these components have been available already in bacteria. Since the eukaryotic cell, therefore, had not to start from zero, but from a collection of prokaryotic capabilities, emerging eukaryotic complexity is not a problem of a square-the-circle; rather, evolution of early eukaryotic cells may largely have profited from the accumulation of genes from bacteria encoding some putative precursors/homologs. Gene duplication, as seen in *Paramecium* (Arnaiz et al. 2007), and diversification have been an additional driving force.

Most salient features of endocytosis are the eventual use of clathrin, together with adaptor proteins, as a molecular filter which also causes bulging of a vesicle domain to be internalized, in conjunction with dynamin—a member of a GTPase superfamily—that helps pinch off a vesicle (Hinshaw 2000; Rothman 2014). Such components occur already in protozoa, specifically in *Tetrahymena* (Briguglio et al. 2013; Elde et al. 2005). Surprisingly dynamin has precursors in bacteria (Bramkamp 2012). Also unexpectedly, coated pits in *Paramecium* are sites of constitutive internalization of glycosylphosphatidylinositol (GPI)-anchored proteins (Flötenmeyer et al. 1999), just as in mammalian cells (Langhorst et al. 2008; Veith et al. 2009).

In summary, key players in early eukaryotic cell activity most likely must have included cytoskeletal elements and motor proteins, SNAREs, GTPases (monomeric GTP-binding/hydrolyzing proteins), and their modulators, as well as key elements required for endocytosis. SNAREs serve for attachment and final fusion of distinct organelles (Rothman 2014; Südhof 2014) specified by organelle-specific Rho-type GTPases (Mizuno-Yamasaki et al. 2012). Together with components mandatory for endocytosis, also available at the protozoan level, this appears as a basic heritage transmitted from early to most

recent eukaryotes. They all serve for increasing complexity of vesicle trafficking up to mammalian and plant cells.

The time frame available for changes during eukaryote evolution

Some molecular phylogeny studies support the subdivision of multicellular organisms into two main branches (Hedges 2002) which, by most researchers, are called monokonts and bikonts, respectively. Among monokonts, choanoflagellates are rather close to the roots of animals, to which myxamoebae, like *Dictyostelium*, are less close (Katz 2012). Unfortunately, still now much less experimental and molecular data are available for choanoflagellates than for myxamoebae. Among bikonts, we find Alveolata, including ciliates. Both lineages appear approximately of the same age, with common ancestors ~1.5 billion years ago (Hedges 2002). As evidenced also in this review, there are not only some specific differences in molecules between unikonts and bikonts, but also some common features are observed at the molecular level, thus potentially reflecting their common origin. Further on, some proteins or protein subunits may have been lost in specific unikonts or bikonts, respectively.

Frequently, proteins are predicted by database mining, as is currently intensified with choanoflagellates (Cai et al. 2015; King et al. 2008). Together with myxamoebae (*Dictyostelium discoideum*), choanoflagellates (e.g. *Monosiga brevicollis*) play a pivotal role, as testimony for metazoan evolution along the unikont lineage, from Porifera (sponges) up to man (Carr et al. 2008; King et al. 2008; Ruiz-Trillo et al. 2007). There is some consent that myxamoebae have evolved before divergence of animals (Williams 2010; Williams et al. 2005). For the bikont lineage, ciliates are frequently considered a model. Species mainly used experimentally are *Paramecium tetraurelia*, *T. thermophila*, and *Tetrahymena pyriformis*. Bikont evolution is thought to have proceeded from green flagellates, such as *Chlamydomonas reinhardtii* (Jékeley 2007), and further on via complex Charophyta-related algae (McCourt et al. 2004; Umen 2014) to angiosperms (flowering plants) (Magallón et al. 2013). Recall that protozoa and the unicellular algae under consideration here are jointly called “protists.”

It appears, however, that the evolutionary scale envisaged up to now may possibly have been underestimated, since recent estimates based on the constantly low mutagenesis rate in *P. tetraurelia* argue for much older origin of ciliates, that is, 1,500 Myr (McGrath et al. 2014). Remarkably this is also the time range assumed for formation of multicellular organisms (Alegado and King 2014) and even for mono-/bikont separation (Hedges 2002). These remarkable discrepancies and uncertainties justify to have another look at the problem, and analysis of closest living relatives is the only chance to have a glance at a likely molecular inventory of progenitors under discussion.

Questions envisaged in this review

This situation implies several major aspects: On one hand, there is sufficient capacity to maintain a substantial percentage of the old genetic inheritance, including functionally most important functions, and on the other hand, innovation could take place. This includes increasing epigenetic control, including tissue- and stage-specific expression profiles, the latter also in protists (considering, e.g. cell duplication and nuclear death). During evolution, new domains may be included in proteins, domains may be rearranged or transferred, translation products can be modified and targeted to other sites of the cell, etc. Whenever function and localization is scrutinized, the question emerges whether the respective gene products have maintained their basic molecular structure and function as well as intracellular localization up to the highest levels of eukaryotes. This question is the hard core of this review which is mainly based on the authors experience with *Paramecium* and on work with mammalian cells. As far as data are available, this question is pursued from low up to highest levels of evolution. Many other teams have substantially contributed to cell biology and evolutionary aspects of protists, with widely scattered literature, which called for an overview.

SPECIFIC CONSERVATION AND CHANGES DURING EVOLUTION

Among others, the following examples are discussed in this review, as summarized in Tables 1, 2: (i) Some proteins maintain function and intracellular localization from low to high levels of eukaryotic evolution. Examples of considerable conservation are GTPases, calmodulin (CaM), PMCA-type Ca^{2+} -ATPase/pumps (maintenance during evolution, with a PMCA precursor in bacteria), and SERCA-type Ca^{2+} pumps. (ii) Some proteins eventually change their intracellular position, for example, voltage-dependent Ca^{2+} -influx channels in cilia of ciliates, but presence in neurons and absence in mammalian cilia and in plant cells. (iii) Some genes/proteins undergo an unprecedented expansion of paralogs/ohnologs, as occurring in *Paramecium* with actin and with the α -subunit (SU- α) of H^+ -ATPase. (iv) A domain may be added to a protein, for example, integration of a (longin-) domain in ciliates and plant R-SNAREs. Another example is the integration of a CaM sequence in "Ca²⁺-dependent protein kinases" (CDPK) in ciliates and in plants; here, CDPKs substitute for Ca²⁺/CaM-activated protein kinase ("CaM-kinase"). (v) A protein subunit (SU-A) may be lost, accompanied by refunctionalization of SU-B of phosphatase 2B (calcineurin, CaN). Both SUs occur in ciliates, but SU-A is lost in plants where a SU-B derivative is refunctionalized to cope with ionic stress. (vi) Some molecules are lost, as seen with Ca²⁺-release channels, type IP₃ receptor; these are maintained from mono- and bikont protozoa up to man, but lost in plants. (vii) Ryanodine receptor-type Ca²⁺ channels (RyR) have *bona fide* precursors in ciliates (RyR-like proteins, RyR-LP), although with lower molecular weight.

(viii) Altogether many new types of ion channels emerge during evolution. (ix) Members of the SPFH (stomatin, prohibitin, flotillin, bacterial HflK/C) superfamily are engaged in scaffolding membrane microdomain with specific proteins. HflK precursors occur in bacteria, and SPFH members can vary during evolution, with either stomatin- or flotillin/reggie-related characteristics dominating in closely related ciliates. More defined orthologs are found up to man and angiosperms. (x) A variety of proteins are newly generated as evolution goes on.

These examples stand for many others to be discussed. In summary, during evolution only part of the proteins analyzed in some detail remain unchanged in structure, function, subcellular localization (Tables 1, 2), and interaction with partner proteins. Beyond this, also some considerable differences in the evolutionary development may be noticed between protozoans, metazoans, and plants.

Conservation of structure, function, and localization

To summarize, there is a variety of proteins which essentially maintain their basic structure during evolution. Among them are some of the CaBPs, specifically those with C2 domains (synaptotagmin, PL-C) and EF-hand-motifs (CaM), together with some proteins containing an additional part with acidic aminoacids (e.g. centrin). The Ca²⁺-ATPases/pumps (PMCA, SERCA) and IP₃R are other examples. The group of essentially conserved proteins also encompasses proteins that are more directly involved in vesicle trafficking, such as clathrin and adaptor proteins (APs) together with coatomer proteins (COPs), all required for vesicle budding, furthermore, GTPases, most SNAREs including syntaxins, SNAP-25 and synaptobrevins (aside from "longin"-types), as well as the SNARE-specific chaperone, NSF. CaN is widely conserved from some protozoa to mammals, but undergoes considerable change in plants, to mention just one example of severe changes.

Ca²⁺-ATPases/pumps

Ca²⁺-ATPases/pumps have evolved in two forms, that is, PMCA and SERCA, both with 10 transmembrane domains and formation of a phospho-intermediate (P-type ATPases) during their primary active Ca²⁺ transport activity (Brini and Carafoli 2009; Bublitz et al. 2011; Palmgren and Nissen 2011). PMCA's have a precursor in bacteria to counteract the permanent leakage of Ca²⁺ from the outside (Domínguez et al. 2015). Generally, they bear a carboxy-terminal autoinhibitory Ca²⁺/CaM-binding domain, as demonstrated in mammals (Carafoli 1994; Lopreiato et al. 2014). This exhibits only small variability, when ciliates, myxamoebae, and plants are compared with mammals.

In *Dictyostelium*, both PMCA and SERCA are described as regulators of [Ca²⁺]_i (Schlatteer et al. 2004). In *Paramecium*, these pumps would be too sluggish to mediate rapid Ca²⁺ downregulation, for example, after massive exocytosis stimulation, thus stressing the importance of Ca²⁺ binding to high-capacity CaBPs (Plattner 2017a,b).

Since PMCA's working kinetics is rather sluggish, as found also in mammalian cells, PMCA was considered to

serve mainly for household activity (Brini and Carafoli 2011). In *Paramecium*, balance calculations suggest that both P-types pumps can regulate $[Ca^{2+}]_i$ at best on a ~60-min time scale (Plattner 2017a,b). This makes both CaBPs and antiporter systems important for rapid $[Ca^{2+}]_i$ down-regulation after stimulation. In line with this, more recent considerations addressing mammalian systems stress the relevance of PMCA for local $[Ca^{2+}]_i$ fine-tuning in subplasmalemmal microdomains (Lopreiato et al. 2014).

In contrast to the PMCA, the SERCA pump has no CaM-binding domain; it serves for the refilling of the ER, the main Ca^{2+} store in animal (Vandecaetsbeek et al. 2011) and plant cells (Liang and Sze 1998). A SERCA-type activity has been recorded in *Dictyostelium* (Schlatterer et al. 2004). Thus, *D. discoideum* is endowed with both types of Ca^{2+} pumps, SERCA and PMCA (Wilczynska et al. 2005). Similarly both pumps occur in *P. tetraurelia* (SERCA [Hauser et al. 1998, 2000; Kissmehl et al. 1998], PMCA [Elwess and Van Houten 1997]). In *Paramecium*, SERCA occurs in the ER from where it is also exported to alveolar sacs (Hauser et al. 1998, 2000), the cortical Ca^{2+} stores (Länge et al. 1995; Stelly et al. 1991) from where Ca^{2+} is released during exocytosis stimulation (Hardt and Plattner 2000). This situation strongly recalls the sarcoplasmic reticulum of muscle cells.

In summary, the basic aspects of PMCA and SERCA function appear rather strictly maintained throughout evolution in both mono- and bikonts.

CaBPs

Let us anticipate some general aspects of CaBPs. About 3,500 CaBPs are known from animals and ~800 from plants (Morgan et al. 2004; Williams 2006). Among CaBPs, there are some with high-affinity/low-capacity characteristics, for example, with EF-hand domains (CaM, centrin, calcineurin B) and some with C2 domains (PL-C, synaptotagmin, copines). CaM possesses four EF-hand domains and is highly conserved, with a homolog already in some bacteria (Swan et al. 1987). There are 68 CaBPs in eubacteria, an unknown number in protozoa, and finally 2,540 in animals and 499 in plants (Morgan et al. 2004; Williams 2006). C2 domains mediate Ca^{2+} -dependent binding of the respective CaBP, or rather of the respective C2-domain, to membranes (Südhof 2013). The number of C2-type CaBPs, which are not known from bacteria (Domínguez et al. 2015), has been reported as 242 in plants and 762 in animals (Morgan et al. 2004; Williams 2006).

Other CaBPs are devoid of such specific domains; rather, they contain acidic stretches serving for rapid binding of Ca^{2+} with low affinity but high capacity. This applies to luminal CaBPs contained in Ca^{2+} stores, with lectin (sugar binding) functions, such as calreticulin and calnexin (Zhou et al. 2013). Both these CaBPs have been identified in *Dictyostelium* (Fajardo et al. 2004; Müller-Taubenberger et al. 2001) and in *Monosiga* (Cai 2008), whereas information from *P. tetraurelia* is preliminary (Plattner et al. 1997b). An additional group of CaBPs of ciliates contains

high-affinity sites together with low-affinity binding sites (Gogendeau et al. 2008; Kim et al. 2002). This combination of low-affinity and high-affinity binding is also a feature of centrin, also in ciliates (Kim et al. 2002).

In summary, CaBPs diversified enormously during evolution, but functionally important domains are retained.

CaM

CaM with its 4 EF-hand Ca^{2+} -binding motifs serves for widely different functions not only in man (Chin and Means 2000) and plants (Poovaiah et al. 2013) but also down to protozoa (Saimi and Kung 2002) including choanoflagellates (Cai 2008). CaM modulates several cation influx channels from ciliates (Brehm and Eckert 1978; Preston et al. 1991; Saimi and Kung 2002) to human brain (Findeisen et al. 2013; Levitan 1999). In both these cases, Ca^{2+} -influx channels are first activated and then rapidly deactivated by the forming Ca^{2+} /CaM complex. CaM can thus be considered a flexible channel subunit (Saimi and Kung 2002). The role of these Ca^{2+} channels has been documented primarily by electrophysiology (Saimi and Kung 2002) and most recently been supported by molecular biology (Lodh et al. 2016).

A conserved function of CaM is its participation in the assembly of docking/fusion sites for dense core-secretory vesicles at the cell membrane in *Paramecium* (Kerboeuf et al. 1993), that is, at sites where CaM is enriched according to immune labeling (Momayezi et al. 1986). Similarly, by binding to synaptobrevin, CaM is essential for the assembly of neurotransmitter release sites in mammalian neurons (Quetglas et al. 2002).

CaM is an activator of CaN, not only in higher eukaryotes but also in ciliates (Kissmehl et al. 1997) and in myxamoebae (Aichele and Mutzel 2001; Hellstern et al. 1997). Already in protozoa, the CaN-A subunit contains a CaM-binding domain. This ensemble, together with the modulating effect of CaM on cation influx channels (Saimi and Kung 2002), therefore, may represent an old functional heritage of eukaryotic cells, yet executed at different sites of the cell. Another conserved function of CaM is the regulation of the PMCA-type Ca^{2+} -ATPase/pump. For CaN, see below, for synaptotagmin.

So far, in choanoflagellates, EF-hand CaBPs are known only from genomic analyses (Burkhardt 2015). Beyond CaM, mammalian cells possess additional high-affinity/low-capacity CaBPs with four EF-hands. Also the myxamoeba, *D. discoideum* expresses not only calmodulin (André et al. 1996) but also some other developmentally regulated forms of CaBPs (Loomis 2014; Sakamoto et al. 2003). From lower eukaryotes on, cells contain CaM-binding proteins. This holds for *Dictyostelium* (Catalano and O'Day 2008), *Tetrahymena* (Gonda et al. 2000; Nagao and Nozawa 1985), and *Paramecium* (Chan et al. 1999) and is continued up to plants (Bouché et al. 2005) and mammals (Bhattacharya et al. 2004).

All this forms part of an extensive Ca^{2+} -based signal transmission machinery occurring, with variations on the basic theme, from bottom-to-top of eukaryote evolution. Thereby, CaM is one of the most important Ca^{2+} -

dependent regulatory proteins with high conservation throughout evolution. Its effects are achieved by conformational change which alters the characteristics of binding to target molecules.

CaN

Such conservation is only partially observed with CaN (protein phosphatase 2B, PP2B). CaN is a dimeric Ser/Thr phosphatase of little substrate specificity (Klee et al. 1998; Rusnak and Mertz 2000) and accordingly with widely different functions, as outlined by Fraga et al. (2010). CaN consists of a catalytic SU-A (CaN-A) and a smaller regulatory SU-B which possesses four EF-hand Ca^{2+} -binding motifs (Guerini 1997). Both subunits have been identified in *P. tetraurelia* (Fraga et al. 2010; Kiss-mehl et al. 1997). Fourteen isoforms of the catalytic SU-A are encoded by seven subfamily genes, each with two rather similar members, that is, ohnologs (Fraga et al. 2010). This is a most convincing example of ohnolog formation. In contrast, only two very similar genes are found for SU-B which—rather surprisingly—encode an identical protein. Also the binding site for SU-B in SU-A is very similar in the paralogs/ohnologs. This is an extreme example of selective restriction of variability of one SU of a protein complex. In contrast to the situation in *Paramecium*, *Dictyostelium* expresses two distinct forms of CaN-B (Aichem and Mutzel 2001).

In *Paramecium*, CaN exerts an effect on stimulated exocytosis (Momayezi et al. 1987), probably by affecting Ca^{2+} dynamics (Fraga et al. 2010). Its effect on exocytosis performance has been repeatedly documented also in metazoan cells (Sim et al. 2003). This includes regulation of ion channels in mammalian cells (Herzig and Neumann 2000). Remarkably, in immunogold labeling experiments with *P. tetraurelia*, coated pits (“parasomal sacs”) are heavily labeled with gold particles (Momayezi et al. 2000). These findings agree with observations, in neuronal cells, that CaN/PP2B activates retrieval of synaptic vesicle membranes by dephosphorylation of dynamin (Lai et al. 1999; Marks and McMahon 1998) which once had been called a “pinchase.” We expect many more functions for CaN also in *Paramecium*, possibly including regulation of cation influx channels, as amply documented for CaN in metazoan cells (Herzig and Neumann 2000; Rusnak and Mertz 2000). In *Dictyostelium*, its presence (Dammann et al. 1996) and widely diversified functions (Weissenmayer et al. 2005) suggest that dimeric CaN can be considered an old heritage. Remarkably, SU-A is lost in plants.

Copines

These are a family of Ca^{2+} -dependent membrane binding proteins endowed with a C2 domain (Tomsig and Creutz 2002). They have been detected not only in *Paramecium* (Creutz et al. 1998) and in *Dictyostelium* (Damer et al. 2005), but they also occur in plants (*Arabidopsis*) and in animals up to mammals (Creutz et al. 1998; Tomsig and Creutz 2002). In *Dictyostelium*, they are localized to the plasma membrane as well as to endocytotic and phagocytotic membranes, in addition to the contractile

vacuole system. Downregulation of one of the six copine genes affected biogenesis and function of the contractile vacuole (Damer et al. 2007), whereas the function of copines in other systems is not established.

The tandem of GTPases and SNAREs

When the (ultra)structural appearance of ciliate and mammalian dense core-secretory systems is compared, they evidently look very different (see below). GTPases are monomeric GTP-BPs, activated by GTP hydrolysis. For membrane trafficking, Rab-type GTPases are in focus. These are required for binding organelle-specific antigens. Thus, in cooperation with SNAREs, GTPases mediate specific vesicle/membrane interactions (Grosshans et al. 2006; Stenmark 2012). For instance, early endosome antigen EEA1 binds a Rab-type GTPase designated as Rab5. Other textbook examples include Rab7 for late endosomes and Rab11 for recycling endosomes. This situation has allowed one to address the specific functional topologies of different GTPases with green fluorescent protein (GFP) fusion proteins in *T. thermophila* and *P. tetraurelia* (Bright et al. 2010). Only a few lineage-specific paralogs have evolved for use in special organelles, such as the contractile vacuole complex of ciliates. Remarkably, GTPases are abundant already in the Lokiarchaeota-type archaeobacteria (Spang et al. 2015), where they contribute to the genome by 1.7%, as compared with 1.2% in *D. discoideum*, 0.6% in *H. sapiens*, 0.55% in *T. thermophila*, and 0.3% in *Arabidopsis thaliana*.

SNAREs and GTPases have been identified, localized, and eventually functionally probed in different protozoa. This includes SNAREs (Plattner 2010a,b) and GTPases in *Paramecium* (Peterson 1991; Saito-Nakano et al. 2010) as well as GTPases in *Tetrahymena* (Bright et al. 2010). The number of SNAREs identified by genomic analysis in *T. thermophila* and *P. tetraurelia*, 88 and 229 (Saito-Nakano et al. 2010), respectively, may well reflect the whole genome duplications in *P. tetraurelia* (Aury et al. 2006) which can serve for amplification effects, rather than for refinement in signaling. The number of Rab-type GTPases is generally said to increase with organismal complexity, culminating with 36 members in *A. thaliana* and 67 in *H. sapiens* (Rojas et al. 2012). However, according to Bright et al. (2010), the numbers reported for *T. thermophila* are 56, 54 for *D. discoideum*, 57 for *A. thaliana*, and 63 for *H. sapiens*. Thus, molecules directing vesicle trafficking seem to have been diversified already at an early stage.

Remarkably, GTPases are encoded already by a number of bacteria where they participate in the regulation of protein and DNA synthesis and also of fine-tuned different pathways, depending on the cell's energy status (Kint et al. 2014). Some pathogenic bacteria are able to inject GTPases into host cells, thus manipulating trafficking and overall function (Ham et al. 2011). An alternative pathogenic strategy aiming at inhibiting fusion with phagolysosomes is the expression of inhibitory SNARE-like proteins by the bacterial invaders (Shi et al. 2016; Wesolowski and Paumet 2010).

Database analysis of choanoflagellates and related filasterians has revealed a variety of molecules essential for neurotransmission in metazoans (Burkhardt 2015; Burkhardt et al. 2014). This includes R- and Q-SNAREs (e.g. synaptobrevin, syntaxin, SNAP-25 [Cai 2008]) and SNARE assembly proteins (e.g. Munc18 and complexin) as well as Homer (able to bind to cortical Ca^{2+} stores). One of the rare experimental analyses with choanoflagellates has applied fluorescence anisotropy, calorimetric, and crystallographic analyses for verifying the interaction of SNARE with Munc proteins (Burkhardt et al. 2011).

More intensive experimental and domain analysis of SNAREs and associated proteins in *P. tetraurelia* has revealed 26 syntaxin isoforms with differential localization comparable to mammalian cells (Kissmehl et al. 2007), together with the SNARE chaperone, NSF (Froissard et al. 2002; Kissmehl et al. 2002), and SNAP-25 (Schilde et al. 2008). All representatives of the synaptobrevin family were found to be of the “longin” type, that is, with a longin domain distal to the SNARE domain (Schilde et al. 2006). This corresponds to the situation in plants where all R-SNAREs then known were “longins” (Lipka et al. 2007; Uemura et al. 2005). In contrast, R-SNAREs of choanoflagellates are of the conventional type (Cai 2008). In summary, there are specific differences maintained in the mono- and bikont lineage, respectively.

R- and Q-SNAREs have been found in different eukaryotic phyla. Essentially, the inventory of SNAREs is high already in unicellular (“lower”) eukaryotes, not only in ciliates (Plattner 2010a,b) but also in myxamoebae and chlorophyceae (Sanderfoot 2007). This abundance not only with SNAREs (including those involved in the secretory pathway) but also with GTPases (Bright et al. 2010; Guerrier et al. 2017).

In the myxamoeba *Dictyostelium* (Kienle et al. 2009) and in various unicellular organisms, SNAREs have been characterized on an informatic level, with the result that their number is only slightly higher in “higher” than in “lower” eukaryotes (Sanderfoot 2007), including *Dictyostelium* and *Chlamydomonas*. It is feasible to assume that Ca^{2+} -driven, SNARE-mediated membrane fusion also occurs in choanoflagellates inferred from the presence of complexin (Yang et al. 2015). This molecule binds to the SNARE complex like a clamp which is overridden when Ca^{2+} binds to synaptotagmin after stimulation (Martin et al. 2011). Accordingly, SNAREs and a synaptotagmin-LP should be found in these cells and, in fact, stochastic data on SNAREs can be retrieved from the databases of choanoflagellates (Burkhardt 2015; Burkhardt et al. 2011, 2014; Kienle et al. 2009). SNARE proteins have been analyzed for structure, function, and localization in more detail up to mammals (Rothman 2014; Südhof 2014) and flowering plants (Lipka et al. 2007; Sanderfoot 2007; Sansebastiano and Piro 2014; Uemura et al. 2004; Vedovato et al. 2009). Disregarding closely similar ohnologs within subfamilies, the number of SNAREs in *P. tetraurelia* is comparable to that in

H. sapiens (Plattner 2010a) and in *A. thaliana* (Lipka et al. 2007).

In conclusion, SNAREs belong to the protein repertoire of all eukaryotes.

Examples of minor changes of proteins during evolution

Proteins combining characteristics of different subfamilies

Members of the stomatin-prohibitin-flotillin (reggie)-HflK bacterial protein (SPFH) superfamily form microdomains in different membranes of multicellular animals (Browman et al. 2007; Lapatsina et al. 2012) as well as in angiosperms (Daněk et al. 2016). Their occurrence in low evolutionary levels is surprising, as the prototype, reggie (flotillin) has been detected by upregulation during neuronal regeneration, as reviewed by Stuermer (2010). There are homologs in different animal phyla (Rivera-Milla et al. 2006) and even in bacteria (Hinderhofer et al. 2009). When we analyzed the possible occurrence of members of the SPFH superfamily in ciliates, we realized that they may be closer to stomatin in *P. tetraurelia*, but closer to reggie/flotillin in *T. pyriformis* (Reuter et al. 2013). Occurrence of flotillin 1 (reggie 2) and of flotillin 2 (reggie 1) is reported from database search in choanoflagellates (Burkhardt 2015; Burkhardt et al. 2014), thus suggesting a widespread presence in eukaryotes.

Proteins with specific, but quantitatively variable functional domains

In contrast to EF-hand CaBPs, C2 domain containing CaBPs are not known from bacteria (Domínguez et al. 2015; Morgan et al. 2004; Williams 2006), but they have been identified from protozoa on. A C2 motif is a β -barrel with a Ca^{2+} -binding loop that, upon Ca^{2+} binding, can sink into a lipid bilayer. This takes place with synaptotagmin, for example, during membrane fusion which is, thus, enabled (Rizo et al. 2006; Südhof 2013, 2014). However, the number of C2 domains per synaptotagmin molecule may differ, as found in multicellular organisms and in ciliates. Most mammalian synaptotagmin species have two C2 domains (Rizo et al. 2006), as do plants (Craxton 2004, 2007), but exceptions with four such domains have been described as “enhanced Syntags” (eSyntag) (Martens and McMahon 2008; Min et al. 2007). No synaptotagmin or related proteins have been found in the *D. discoideum* genome (Barber et al. 2009), in contrast to a more recent report (Zhang et al. 2011) although this refers to abortive literature citations. Only eSyntag-like genes have been found in the genomes of *M. brevicollis* (Barber et al. 2009) and of *P. tetraurelia* (R. Kissmehl and H. Plattner, unpubl. observ.; Plattner 2010b)—functional verification still to be accomplished. These phenomena may be paradigmatic for domain shuffling—an important mechanism of evolution (Di Roberto and Peisajovich 2014; Marsh and Teichmann 2010). There is no mono-/bikont consistency in C2 domain bearing synaptotagmin and related proteins.

Ca²⁺ signaling, a revolution in early eukaryote evolution, required new “inventions”

During evolution of the eukaryotic cell, two basic revolutions came into play. One was the well-known exploitation of O₂ for energy conservation. Another one—although less reflected—was the recruitment of Ca²⁺ as a universal second messenger (Berridge 2006; Berridge et al. 2003; Clapham 2007) and a mandatory factor specifically for membrane-to-membrane interactions (Carafoli and Krebs 2016; Marchadier et al. 2016; Plattner and Verkhatsky 2015, 2016). Here, the crucial problem was the antagonism between ATP-based energetics and the low solubility of Ca²⁺ phosphates. Thus, the given establishment of ATP as universal energy carrier has forced cells to keep [Ca²⁺]_i low. While this is true already of bacteria (Domínguez et al. 2015), evolution of large eukaryotic cells required small, short signals strictly restricted in time and in space to strategic sites. This has the favorable side-effect of low-energy expenditure of ATP-based regulation (Plattner and Verkhatsky 2016). To achieve this, Ca²⁺-release channels (CRCs) had to be “invented,” which had to be locally restricted to strategic sites, in order to produce short and site-specific signals. This, together with Ca²⁺-influx channels in the plasmamembrane and Ca²⁺-binding proteins (CaBPs) of different kinetics at specific sites in the cytosol can not only effectuate efficient local signaling, but it can also facilitate [Ca²⁺]_i downregulation with little energy consumption (Plattner and Verkhatsky 2016). Signaling can be executed by high-affinity/low-capacity CaBPs (Zhou et al. 2013). For instance, calmodulin (CaM), endowed with four EF-hands along a central α-helical link, performs a conformational change when activated by Ca²⁺, and after binding to a target molecule, initiates a site-specific effect (Fernandes and Oliveira-Brett 2017; Park et al. 2008). By the same token, low-affinity/high-capacity CaBPs can serve as immobile Ca²⁺ buffers by inactivating Ca²⁺ signals which, thus, become even more topologically restricted. The intracellular contribution of different CaBPs is particularly well analyzed in some nerve terminals (McMahon et al. 2016), but much less in protozoa (Plattner 2014, 2015b; Plattner and Verkhatsky 2015).

Clearly, in principle, proteins engaged in Ca²⁺ signaling occur in all eukaryotes, but with considerable variability. This is particularly true of CRCs, as summarized recently (Plattner and Verkhatsky 2015, 2016).

Change of localization and function of proteins during evolution

Three examples are selected for a discussion about proteins whose intracellular localization changes during evolution, that is, centrin, voltage-gated Ca²⁺ channels and SNAREs (with a side glance at calmodulin).

Centrin

Centrin is a good example of topological variability. Centrin is the name-giving component of the centrosome of the mammalian cytoskeleton. This molecule also occurs,

although at different sites, in ciliates, choanoflagellates, and all animal phyla, but not in angiosperms (Hodges et al. 2010). It is found in the centrosomes of green algae, such as *Chlamydomonas* (Azimzadeh 2014). In myxamoebae (*Dictyostelium*), centrin is represented by two centrin-related paralogs (Mana-Capelli et al. 2009) without stringent association with the centrosome (Levy et al. 1996). Altogether the occurrence of centrioles/centrosomes is quite variable during evolution (Azimzadeh 2014; Hodges et al. 2010). Centrin molecules are able of self-assembly (Yang et al. 2006) and formation of filamentous aggregates which can also result in binding specific proteins (Martinez-Sanz and Assairi 2016). In *Paramecium*, centrin makes an elaborate cortical contractile network, called the “infraciliary lattice” (Beisson et al. 2001; Gogendeau et al. 2008), whose negative charges enable centrin to act as an immobile Ca²⁺ buffer (Sehring et al. 2009). Alternatively, it builds a Ca²⁺-sensitive contractile stalk in the ciliate *Vorticella* (Buhse et al. 2011; Levy et al. 1996). Due to its high Ca²⁺-binding capacity, this “infraciliary network” acts as a powerful immobile Ca²⁺ buffer when cells are flushed with Ca²⁺ (Sehring et al. 2009), with the result of strong contraction. In fact, the availability of dual, widely different Ca²⁺-binding constants of centrin in different organisms including protists (Levy et al. 1996; Zhang and He 2012) enables centrin to act as a high-sensitivity/low-capacity as well as a low-sensitivity/high-capacity CaBP.

In higher plants, in the absence of a centriole/centrosome, centrin is not detectable in the spindle poles (Del Vecchio et al. 1997). However, centrin occurs in plasmodesmata where it may regulate permeability (Blackman et al. 1999). This can be considered an alternative to the Ca²⁺-sensitive open/closed state of metazoan gap junctions—a pathway of intercellular communication (Zhou and Jiang 2014).

In many organisms, uni- or multicellular, centrin also forms part of ciliary and flagellar basal bodies (Carvalho-Santos et al. 2011), which evidently is not the case in organisms lacking such structures (*Dictyostelium* and angiosperms). Quite in contrast to this, in mammalian cells centrin is required for centriole duplication (Salisbury et al. 2002) and, thus, exerts a vital function.

To sum up, the distribution of centrin in different organisms and within different cell types varies widely (Bhattacharya et al. 1993). Evidently this variability does not follow a clear monokont/bikont scheme, although centrin is substituted for by related proteins with cytoskeletal functions.

Centrin is present in basal bodies and in centrosomes of animals (Vonderfecht et al. 2012), thus reflecting the ability of their interconversion (Bornens and Azimzadeh 2007; Wang et al. 2011) that had been recognized already more than a century ago (Henneguy-Lenhossék theory). Several groups of unicellular eukaryotes do not form a centriole/centrosome, including some Alveolata (*Tetrahymena*, *Paramecium*, Apicomplexa) and green flagellates (*Chlamydomonas*) (Azimzadeh 2014; Hodges et al. 2010; Zhang and He 2012), although they possess centrin

(Hodges et al. 2010). In *Dictyostelium*, a quite unusual centrin with low sequence homology is associated with the nucleus in an unusual form (Gräf et al. 2004). Strikingly, there is no centrosome in choanoflagellates, and the flagellar basal bodies serve as microtubule organizing centers during nuclear division (Karpov 2016).

The situation with centrin is particularly diverse when cytospindles in animal cells, from ciliates up to mammals, are compared with those in plant cells. To comprehend this, one has to recall again the similarity of ciliary/flagellar basal bodies and centrioles—both structures being absent from plant cells. In ciliates, centrin is required for basal body formation (Stemm-Wolf et al. 2005), as reviewed by Pearson and Winey (2009), just as in metazoans (Salisbury et al. 2002; Tassin et al. 2016). Nevertheless, in ciliates, the alter ego of basal bodies, the centriole, does not contain any centrin (Hodges et al. 2010). In the present context, one should recall that centrin in ciliates additionally forms an extensive contractile cortical filament network (Beisson et al. 2001), intermingled with actin (Kissmehl et al. 2004). The situation in choanoflagellates is like in metazoans (Hodges et al. 2010). In angiosperms, the microtubule organizing centers of spindle pole caps are devoid of centrin (Hodges et al. 2010). Rather, centrin in plants is enriched in the cell plate during cell division (Del Vecchio et al. 1997).

Besides this gross line of evolutionary development, centrosomes exhibit considerable inconsistency with regard to their name-giving molecular constituent, centrin. As a nucleator for microtubules in centrioles, pole caps, and ciliary/flagellar basal bodies, the widely distributed tubulin isoform, γ -tubulin, is a much more wide-spread determinant (Firat-Karalar and Stearns 2014). This also holds for a variety of protists (Klotz et al. 2003).

Another interesting aspect is the similarity of the immune synapse of lymphocytes and the docking sites of trichocysts in *Paramecium* cells (Griffiths et al. 2010). Here, microtubules serve for transport of trichocysts (Platner et al. 1982) and of lytic granules, respectively, to the cell membrane. For the formation of microtubule organizing centers in lymphocytes, centrin is transported along the cell membrane (Yuseff et al. 2011). In contrast, centrin is present at the self-assembly sites of this microtubule subpopulation in *Paramecium*, that is, the ciliary basal bodies (Beisson et al. 2001). In both cases, secretory vesicle transport takes place in the plus-to-minus direction of microtubules, whereas in most secretory cells, this transport starts at microtubules emanating from a centrosome located at the cytocenter and, thus, goes in opposite direction.

In summary, apart from centrin, there is hardly any other protein with comparable variability in localization and function during evolution.

Voltage-dependent Ca^{2+} channels for ciliary activity

In ciliates, voltage-dependent Ca^{2+} -influx channels are restricted to the ciliary membrane where they are activated upon depolarization to induce ciliary reversal, as outlined in Fig. 1. This is no more so in metazoan cilia

which have acquired other types of ciliary channels which even may not be important for the modification of ciliary activity (Malicki and Johnson 2017); see Fig. 2, 3. In vertebrates, voltage-gated Ca^{2+} channels are transferred to motor endplates and to synaptic membranes of neurons of the central nervous system (Atlas 2013; Yamashita 2012). Partial sequencing in *Paramecium* revealed sequence homology (Yano et al. 2013), albeit full sequencing, eventual subtyping, and reconstitution studies are still missing.

In cilia of unicellular organisms, some basic $[Ca^{2+}]_i$ in cilia is required for normal activity which increases severalfold during stimulation (Salathe 2007). In ciliates, mechanical stimulation generates an action potential by activation of mechanosensitive Ca^{2+} channels in the somatic (nonciliary) cell membrane and a receptor potential by activation of voltage-gated Ca^{2+} channels (Naitoh and Eckert 1969). Insight into their localization has been achieved by comparative electrophysiology of *Paramecium* cells before and after deciliation (Dunlap 1977; Machemer and Ogura 1979), as well as by proteomic analysis of purified ciliary membranes (Yano et al. 2013) and by partial gene sequencing and cloning (Lodh et al. 2016). Localization and basic mechanism appear similar for flagella of *Chlamydomonas* (Beck and Uhl 1994; Fujiu et al. 2009) and for cilia in ctenophores, the comb jellies (Tamm 2014). Consequently, upon depolarization, ciliates and ctenophores perform ciliary beat reversal, whereas *Chlamydomonas* responds by changing wave form of the flagellar beat (Fujiu et al. 2009). Detailed molecular characterization of voltage-dependent Ca^{2+} channels in cilia and flagella of lower eukaryotes still awaits scrutiny. Remarkably, these channels are inactivated by formation of a Ca^{2+} /CaM complex in both *Paramecium* (Saimi and Kung 2002) and neurons of the human brain (Findeisen et al. 2013; Levitan 1999).

In metazoans, there exist immotile primary cilia in the anlagen of some tissues, such as nephrons of the kidney, and in the motile cilia of ciliated epithelia, such as trachea, oviduct and ependymal cells lining the ventricular system of brain and spinal cord (Doerner et al. 2015; Yano et al. 2013). See Fig. 2, 3 for comparison of primary and epithelial cilia. In this context, see discussion about TRP-type channels, below.

In ciliated epithelia, intraciliary $[Ca^{2+}]_i$ increase accelerates ciliary beat by hyperpolarization due to the opening of ATP-sensitive K^+ -channels (Ohba et al. 2013). With this respect, there is some similarity to hyperpolarization-induced (although not ATP mediated) accelerated forward swimming in *Paramecium* (Preston et al. 1992; Van Houten 1998). Epithelial cilia of mammals are generally devoid of voltage-dependent Ca^{2+} channels. Only ependymal cilia contain such channels, but here they are preferably enriched in the nonciliary membrane, with only small effect of their activation on ciliary beat; therefore, they presumably serve for secretory and differentiation activities (Doerner et al. 2015; Pablo et al. 2017). Also polycystin-type channels are no more considered important for ciliary activity in animal cells—aside from the passive movement of the primary cilium (Battle et al. 2015).

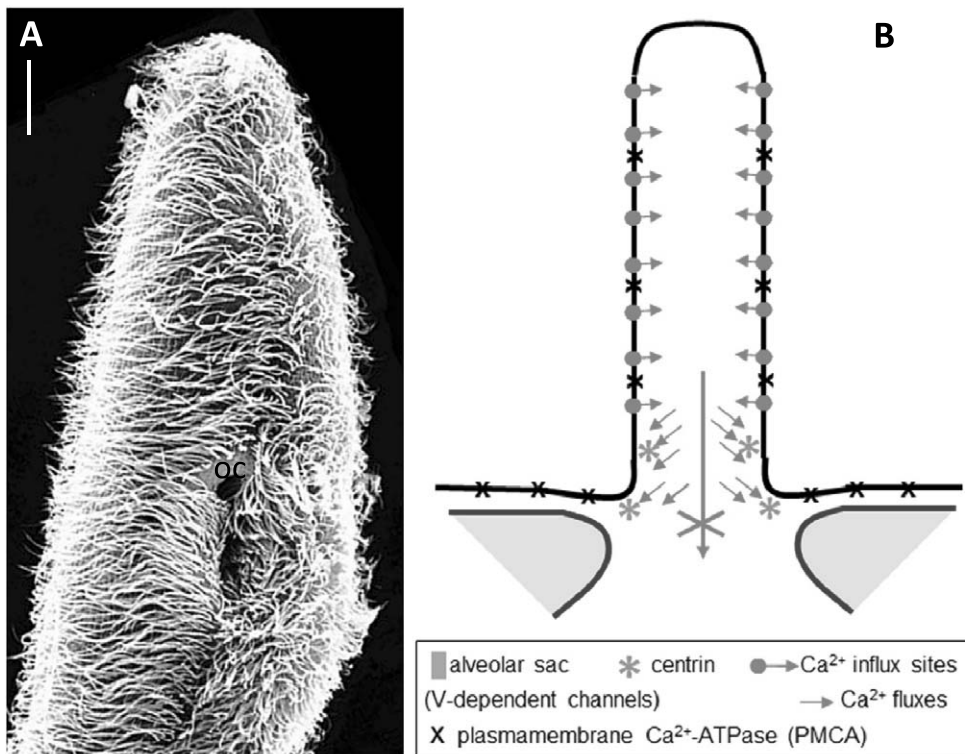


Figure 1 Cilia in *Paramecium tetraurelia*, scanning electron micrograph (A) and scheme (B) showing the distribution of PMCA and restriction of voltage-dependent Ca^{2+} channels to cilia. Upon depolarization, Ca^{2+} enters cilia, thus inducing ciliary beat reversal. Subsequently centrin, localized at the basis of cilia, rapidly binds Ca^{2+} , thus inhibiting spillover into the cell soma. Also note that alveolar sacs are not involved. (A) Courtesy by J. Hentschel. Scale bar in (A), 10 μm .

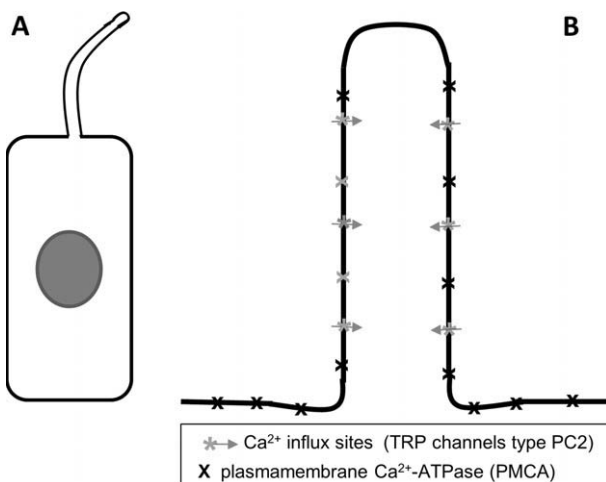


Figure 2 The primary cilium is located apically in some developing epithelia (A) and contains PMCA and TRP-type channels (B). For details, see text.

Altogether ciliary mechanosensation in mammals remains little understood (Malicki and Johnson 2017).

During evolution, ciliary channels of ciliates have been substituted for by transient receptor potential (TRP) channels in mammalian primary cilia (Battle et al. 2015) and in

epithelial cilia (Lorenzo et al. 2008)—a legacy from early unikont precursors (Cai et al. 2015). The types of cation channels regulating ciliary activity vary greatly between the different types of cilia, in protozoan and metazoan cells. Substitution of Ca^{2+} -dependent and CaM-sensitive Ca^{2+} -influx channels during evolution by TRP-type channels may increase responsiveness to widely different stimuli. Taken together, this is a clear case of replacement of a protein by other ones during evolution. In summary, voltage-dependent Ca^{2+} channels, once governing ciliary activity in protists, do occur in mammalian nerves including neurons of the central nervous system where they contribute to long-term potentiation, that is, learning (Atlas 2013; Yamashita 2012). This is quite a remarkable “career.”

Deviating features and functions of calmodulin and SNAREs

During evolution, CaM has acquired some additional functions. In mammalian nerve terminals, a spillover of Ca^{2+} during activation activates CaM in the cytosol and thereby a Ca^{2+} /CaM-activated protein kinase (“CaM-kinase”), as summarized by Swulius and Waxham (2008). Nerve terminals contain this CaM kinase (DeLorenzo et al. 1979), which facilitates the release of neurotransmitter vesicles from F-actin by phosphorylation of synapsin. This facilitates the refilling of the subplasmalemmal store of vesicles releasable by exocytosis (Chi et al. 2003; Swulius and

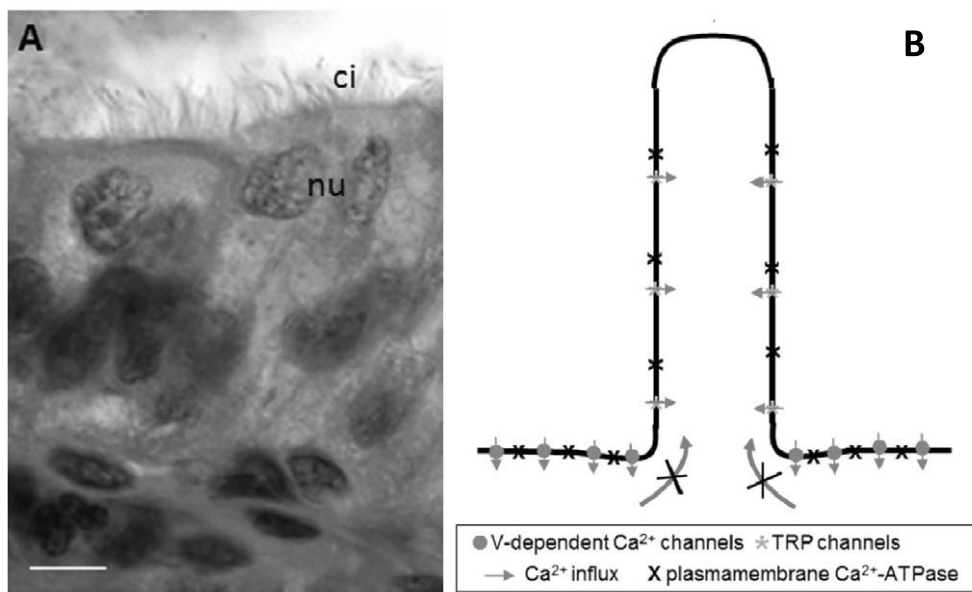


Figure 3 Ciliated epithelium (mouse oviduct, fixed and hematoxylin/eosin stained). Cilia (ci) shown in (A) are working independently (B) of any eventual voltage-dependent Ca^{2+} channels in the nonciliary membrane. Any Ca^{2+} signals eventually arising in the cell body do not remarkably affect ciliary activity. Note occurrence of TRP channels and PMCA in the ciliary membrane. nu = nuclei. (A) Courtesy by J. Hentschel. Scale bar in (A), 10 μm .

Waxham 2008). A comparable function is not known from other dense core-secretory vesicle systems, including protozoa. Both types of secretory systems are also (ultra)structurally quite different (Fig. 4) although they both contain fast releasable secretory vesicles.

A set of SNAREs comparable to that in protozoa and animals is also present in angiosperms (Lipka et al. 2007; Sanderfoot 2007; Uemura et al. 2004; Vedovato et al. 2009). However, R-SNAREs are preferably of the longin type. A longin domain must be an old heritage, as it is found in Lokiarchaeota, together with the transport machinery called ESCRT ("endosomal sorting complexes required for transport"), hypothetical vacuolar fusion domain protein and with BAR/IMD superfamily proteins (important for membrane deformation) (Spang et al. 2015). Thus, these archaeobacteria already were prepared for vesicular traffic (which they do not have).

Expansion of protein inventory by subfamily/ohnolog encoding genes

This phenomenon is most pronounced in *P. tetraurelia* (Aury et al. 2006) and documented in some detail for actin, SNAREs, and SU-a of the H^+ -ATPase.

Actin

Remarkably actin and actin-like proteins (LPs) are found already in Lokiarchaeota-type archaeobacteria (Spang et al. 2015), whereas these cells contain no tubulin.

Paramecium actin genes, *PtAct*, include an unusual number of subfamilies (and accordingly of ohnologs), resulting in a great number of *PtAct* proteins and of actin-

like (or actin-related) proteins (Sehring et al. 2007a). Depending on definition, representatives include 14–17 and 3–6, respectively, complementary forms. Four of the *PtAct* gene products encode ohnologs with differential subcellular localization, which also holds for the translation products of the other subfamilies. Numerous *PtAct* paralogs are situated along the cell surface. An exchange of *PtAct* isoforms is particularly impressive during maturation of the food vacuole, that is, phagolysosomes (Sehring et al. 2007b); see Fig. 5. *PtAct4* does not only occur in the cortex but is also required for initiating formation of phagosomes and of the cleavage furrow (Sehring et al. 2010). In *Paramecium*, para-ohnologs evidently may serve for vesicle fusion or budding as well as for cyclosis and to regulate access of vesicles to other membranes. Although specific effects still have to be scrutinized, differentiation of this kind appears without precedent in eukaryotic cells.

A second look upon SNAREs

As mentioned, SNAREs are abundant in *Paramecium*. In *P. tetraurelia*, many of the R- and Q-SNAREs represent ohnologs, mostly with only 15% or less nucleotide difference between the ohnolog genes (Kissmehl et al. 2007; Schilde et al. 2006). This low difference makes it difficult, or even impossible to assign to such SNAREs specific intracellular localization by monospecific antibodies. This difficulty also holds for any attempt to assign specific functions via posttranslational silencing by competitive nucleotide sequences. In these cells, ~70 SNARE-type genes have been identified by database screening (Klopper et al. 2007) which is more than in man. Even if one disregards genes with only $\leq 15\%$ difference, this would

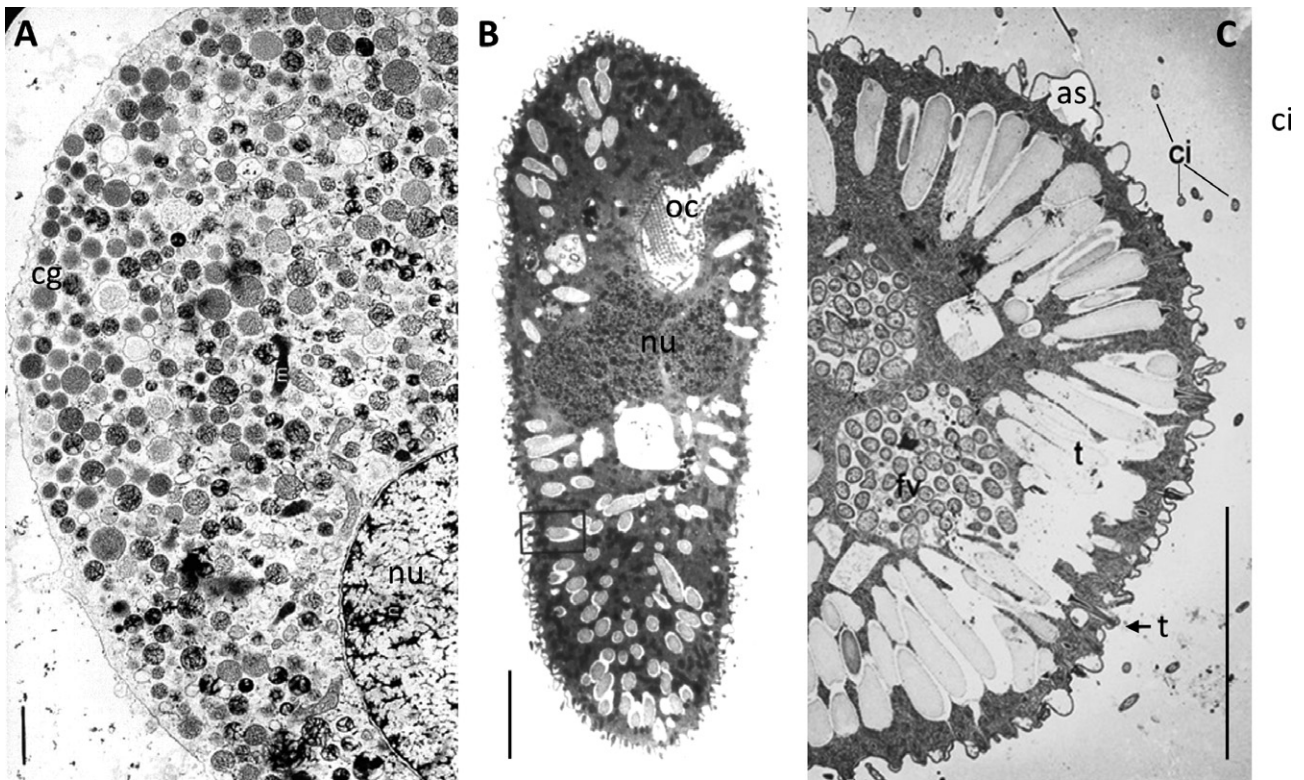


Figure 4 Mammalian and protozoan dense core-secretory systems in comparison. **(A)** Bovine chromaffin cell in primary culture, unstimulated, containing ~21,500 chromaffin granules (cg) of which only ~440, that is, ~2% of the whole vesicle population, are attached to the cell membrane in a position potentially available for immediate release upon stimulation (Plattner et al. 1997a). **(B, C)** *P. tetraurelia* cells showing abundance of trichocysts (t). More than 95% of the whole population of up to ~1,230 trichocysts are docked at the cell membrane, as can be recognized only in appropriate section planes (square in [A], arrow in [B]); of these ~95 can be immediately released upon stimulation (Plattner et al. 1985). as = alveolar sacs; ci = cilia; fv = food vacuole; nu = nucleus; oc = oral cavity. (A) is from Plattner et al. (1997a), (B) from Plattner and Verkhatsky (2016), (C) H. Plattner, unpublished. Scale bars—1 μm (A), 10 μm (B, C).

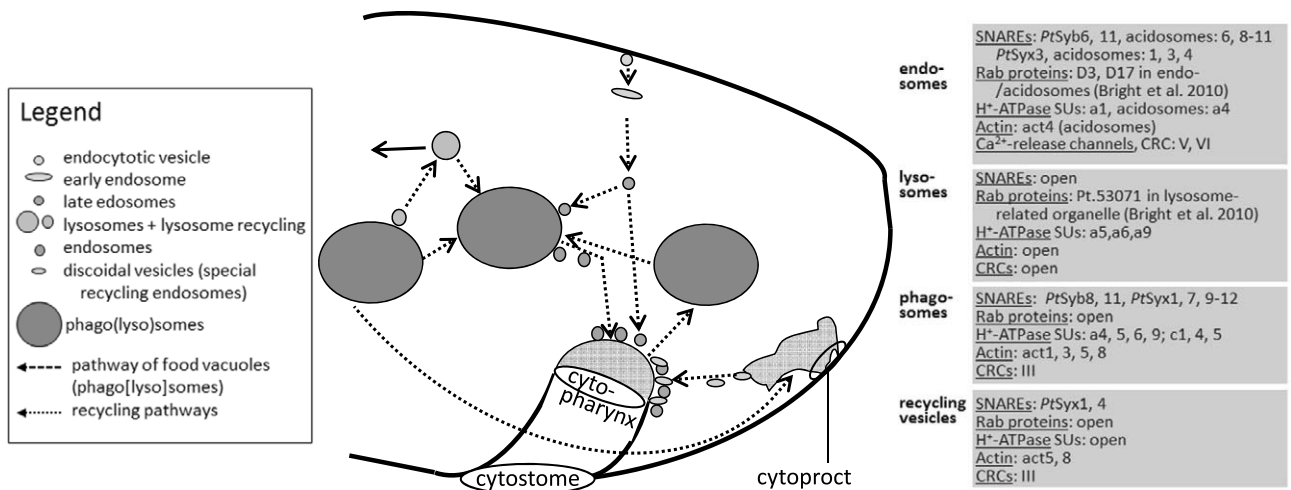


Figure 5 Trafficking of endo-/phago-/lysosomal compartments in *Paramecium tetraurelia*, showing multiple membrane/vesicle interactions (dotted arrows) on the way through the cell (dashed arrows). The boxes at the right side indicate the involvement of some of the numerous proteins, such as SNAREs, GTPases, H⁺-ATPase, actin, and Ca²⁺-release channels at different stages of the cycle. This complexity includes the exchange of variable isoforms on the way through the cell and, thus, looks as intriguing as comparable pathways in mammalian cells. For details, see Plattner (2017a,b).

still yield ~40 functionally different SNAREs (Plattner 2010a). Thus, the number of functional different types of SNAREs in *Paramecium* is about the same as in man and far beyond values extrapolated for early eukaryotic and early metazoan cells, which is estimated as ~20 and ~30, respectively (Kloepper et al. 2008).

Expansion of subunit SU-a of H⁺-ATPase

The multimeric H⁺-ATPase/pump has precursors in bacteria (Stewart et al. 2014) and is universal to eukaryotic cells where it stands out by its multi-tasking capabilities (Maxson and Grinstein 2014). This complex molecule consists of a multimeric membrane-integrated base piece (V0), a stalk (SU-a) for connecting the V0 and the V1 part. V1 is the multimeric catalytic head part that sticks out into the cytosol. In mammals, the number of genes for each of the subunit monomers is ≤ 3 , only four are for encoding SU-a (Forgac 2007). The situation is quite similar with most plants, from mosses up to flowering plants, but with a similar number of genes for SU-a as well as for each of the monomers composing the V0 and the V1 part, respectively (Schumacher and Krebs 2010).

Usually the numbers of proteins interacting in protein assemblies have to be fine-tuned to produce a functional complex. This is generally observed in the respective expression rates also in *P. tetraurelia* (Aury et al. 2006) and—as one would expect—this should also be valid for the different SUs of multimeric proteins. However, a striking exception is SU-a of the H⁺-ATPase of *P. tetraurelia*. While the number of SUs of the catalytic V1-part and of the V0-basepiece is limited as usual (Wassmer et al. 2005), these are by far outnumbered by SU-a copies, which are represented by an unprecedented number of 17 genes (Wassmer et al. 2006). In situ, the SU-a emerges from the V0 part and can be reversibly connected to variable headpiece complexes, thus forming different holoenzyme variations (Forgac 2007). In *Paramecium*, SU-a is represented by eight doublets each of them with > 82% identity and one singlet, that is, by a total of 17 genes and 5 essentially different variants (Wassmer et al. 2006). In contrast, the number of genes for the individual V1 and V0 SUs is between 1 and 4 and between 0 and 6, respectively. This is a clear exception to the general rule of stoichiometric expression of SUs in cooperating molecule complexes (Aury et al. 2006). This exception allows for a large number of functional H⁺-ATPase complexes alone on the basis of SU-a variation. What may the functional implications of an increased number of SU-a, and consequently of V1/V0 complexes in *Paramecium* be?

In mammalian cells, tuning of luminal acidification during intracellular trafficking can cause a conformational change of the cytosolic part of the H⁺-complex, followed by selective binding of ARNO (ADP-ribosylation factor nucleotide site opener). ARNO is the GDP/GTP exchange factor (GEF) for GTPase Arf6, and, thus, relevant for binding to a specific SU-a of the H⁺-ATPase at the cytosolic side; this in turn results in GTPase-specific targeting (Hurtado-Lorenzo et al. 2006). The H⁺-ATPase can,

thus, specify vesicle interaction partners by conformational coupling. Such a principle may be extended to different trafficking events in *Paramecium* where different SU-a subfamily members are distributed over specific organelles (Wassmer et al. 2006), although this still expects scrutiny.

Chlamydomonas reinhardtii possesses three genes encoding SU-a, but only one for encoding each of the monomers of the V0 and V1 complexes (Schumacher and Krebs 2010). In *Entamoeba histolytica*, where most H⁺-ATPase subunit encoding genes are singlets—with high resemblance to *D. discoideum*—two genes encode SU-a (Mélendez-Hernández et al. 2008). In plants, isoforms of V0/V1 coupling SU-a are differentially localized (Schumacher and Krebs 2010), just as one would expect for *Paramecium*. Since trafficking is also influenced by binding of actin to some of the H⁺-ATPase subunits (Beyenbach and Wieczorek 2006; Holliday 2014), this may entail additional variations in localization and function in *Paramecium* because of the large number of actin paralogs/ohnologs.

All this strongly suggests that H⁺-ATPase/SU-a isoforms may be important for differential targeting in all eukaryotes. In this context, *Paramecium* is an impressive example of selective gene expansion (Wassmer et al. 2009). This may account for the rather expansive trafficking pathways in these cells and for the involvement of a multitude of paralogs/ohnologs of SNAREs, GTPases, H⁺-ATPase SUs, and Ca²⁺-release subunits during cyclosis (Fig. 5).

Punctual changes in protein molecules and drug (in)sensitivity

When compared with mammalian orthologs, the sensitivity of ciliate proteins to different drugs may be quite aberrant (Plattner et al. 2009). This has provoked quite a few errors in cell biology of protists, claiming, for instance, the alleged absence of actin from the cleavage furrow in some protists (Shimizu et al. 2013). Also sensitivity of plant microtubules differs from that of ciliates (Pape et al. 1991) and of mammalian systems. But why altogether should the interaction of a protein with specific drugs be maintained during evolution, when there is no selection pressure by a drug?

Considering drugs relevant for Ca²⁺ and membrane dynamics examples in *Paramecium* include SERCA inhibitors, such as thapsigargin, SNARE-cleaving endo-metalloproteases type *Clostridium* toxins, and cytoskeletal elements (Plattner et al. 2009). Some drugs reactive in mammalian cells simply do not find appropriate sites for binding to, or cleavage of target proteins.

The situation is particularly precarious with RyR-LPs in *Paramecium* since these CRCs do not react to the name-giving drug, ryanodine, a diterpenoide from the South American plant species *Ryania speciosa* (family Salicaceae). This is feasible considering the diffuse character of ryanodine-binding sites, the concentration dependence of ryanodine effects, and the irrelevance of interaction in nature. Yet, *Paramecium*'s RyR-LPs clearly respond to

established activators of RyRs (Ladenburger and Plattner 2011). In contrast, IP₃Rs of *Paramecium* are fully responsive to the metabolic activator, IP₃ (Ladenburger et al. 2006).

There are little individual traits of *Paramecium* CaM which could entail a change of its main functions and drug sensitivity (Kink et al. 1990). Accordingly drug sensitivity is exceptionally well preserved for anti-CaM drugs (Ehrlich et al. 1988; Erxleben and Plattner 1994), in contrast to many other drugs (Plattner et al. 2009).

Actin filaments

However, universally distributed, microfilaments possess rather heterogenous properties. From myxamoebae to animals, from ciliates to angiosperms, the actin filament system contributes to cell organization and in the unikont branch, to motility. Work is abundant with *Dictyostelium* (and *Physarum*) as well as with angiosperms (Henty-Ridilla et al. 2013; Noegel and Schleicher 2000) and animals up to mammals (Gunning et al. 2015). In this sequence, the number of genes encoding different paralogs for cytoplasmic actin is 8 (*D. discoideum*), 10–17 (angiosperms), and 1–5 (mammals; 4 in man) (Gunning et al. 2015).

Plants use actin filaments for cytoplasmic streaming (which probably is also the case with cyclosis in ciliates), accompanied by organelle traveling, and for signaling (Henty-Ridilla et al. 2013; Shimmen 2007; Staiger et al. 2009). Just like animal cells (Bubb et al. 1994; Cooper 1987), plant actin is sensitive to the different polymerizing and depolymerizing drugs (Shimmen 2007). This is also true of *Dictyostelium* (see, e.g. [Parent et al. 1998]) and *Chlamydomonas* (Avasthi et al. 2014). In other algae, microfilaments appear incapable of binding phalloidin and, thus, to visualize the cell cleavage plane by fluorescent derivatives (Busch and Hess 2017).

The basic structure of actin appears rather conservative, but sites important for diagnostic binding of fluorescent phalloidin/jasplakinolide and cytochalasin/latrunculin, respectively, are usually well preserved. Several defined aminoacids are critical for drug binding. These are quite variable in the large number of isoforms described in *P. tetraurelia*, together with the actin-LPs, whereas they are consistently preserved in the much smaller number of mammalian actins (Sehring et al. 2007a). Concomitantly aminoacid residues responsible for binding microfilament-stabilizing and microfilament-destabilizing drugs, shifting monomer → polymer and polymer → monomer equilibrium, that is, phalloidin or jasplakinolide and cytochalasin B or latrunculin A, respectively, are quite variable between actin isoforms of *Paramecium* (Sehring et al. 2007a). This is a striking example of the largely aberrant pharmacology of many established experimental and diagnostic drugs (Plattner et al. 2009).

The fact that ciliates tolerate reduction of toxin-binding sites to different degrees may again mirror the fact that drug binding is of no direct evolutionary consequence for these molecules. Importantly, localization differs for the different actin paralogs of *Paramecium* (Sehring et al.

2007b, 2010). It may also indicate different binding to other proteins and different polymerization kinetics—predictions to be scrutinized. Remarkably, there are no consistent differences between mono- and bikonts.

Microtubules

Also microtubules are functionally and pharmacologically rather different. Several ciliate-specific epitopes of metazoan tubulins are conserved from *P. tetraurelia* to mammals (Adoutte et al. 1985, 1991). Throughout animal evolution up to man, specific covalent modifications of microtubular tubulin can take place, such as acetylation, glycylation, and glutamylation (Song and Brady 2015) and thus determine interaction with different tubulin-binding proteins (Rosenbaum 2000). In the *Paramecium* cell, just as in other taxons including plants (Parrotta et al. 2014), any covalent modifications entail selective localization in specific microtubule subpopulations, located at different sites of the *Paramecium* cell (Adoutte et al. 1991) and apparently also different drug sensitivity.

When we tested cell division activity of *P. tetraurelia* cells in the presence of drugs with established depolymerizing effects in mammalian cells, colchicine, for example, exerted no effect in concentrations ~1,000 times above those normally applied to mammalian cells (Pape et al. 1991). In this work, we have made similar observations with drugs directed against plant microtubules (Hashimoto 2015). Thus, sensitivities do not follow a strict scheme in mono- and bikonts.

Loss of a subunit

Calcineurin (CaN) is a dimer of the catalytic CaN-A and the regulatory CaN-B subunit, with widely different functions from protozoa to man (Aramburu et al. 2000, 2004; Rusnak and Mertz 2000). The occurrence of CaN SU-A, together with SU-B (Fraga et al. 2010), in ciliates would suggest its occurrence also in other bikonts, such as plants. However, in plants only SU-B is maintained as CBLs (CaN-B-like [proteins]), with an increasing number or paralogs during evolution, from Bryophyta (mosses) to *A. thaliana* (Edel and Kudla 2015). Here, CBL is widely diversified and mainly modulates ion channel activity and Ca²⁺ signaling by CIPKs (CBL-interacting protein kinases) (Edel and Kudla 2015; Sanyal et al. 2015), but altogether CIPKs regulate different signaling pathways in angiosperms (Yu et al. 2014). CBLs are also known from *C. reinhardtii* (Mohanta et al. 2015).

In summary, the selective maintenance of only one CaN-SU is specific for plant evolution, whereas both SUs are present in both of the most intensely studied unicellular representatives of the unikont and bikont lineages, that is, *Dictyostelium* (Aichem and Mutzel 2001; Hellstern et al. 1997) as well as *Paramecium* (Fraga et al. 2010). This advocates for the occurrence of both SUs also in early times of bikont evolution and secondary loss of SU-A in plants. In parallel, CBLs have arisen during evolution of the multicellular “green representatives” of bikonts, thus making arid areas accessible.

Proteins with variable covalently integrated extra-domains

The three salient examples to be discussed are PMCA, CDPKs, and R-SNAREs.

Among PMCAs, PAT1 of *Dictyostelium* is exceptional insofar as it has been described as a Ca^{2+} -ATPase most closely related with PMCAs, although it is devoid of a CaM-binding domain (Gross 2009; Moniakakis et al. 1995). Plant PMCA is also aberrant insofar as its autoinhibitory Ca^{2+} /CaM-binding domain can be found at the amino-terminus (Tidow et al. 2012). Otherwise, the molecular structure is probably the same from protozoans to mammals.

Remarkably, a CaM-like sequence is attached to the carboxy-terminal end of some protein kinases, called “ Ca^{2+} -dependent protein kinases” (CDPK), in ciliates (Kim et al. 1998), *Chlamydomonas* (Edel and Kudla 2015), and plants (Dixit and Jayabaskaran 2015). In contrast, the myxamoeba *Physarum polycephalum* is reported to possess also a genuine CaM-kinase (Nakamura et al. 2005). Altogether, CDPKs are typical of the bikont lineage.

In ciliates, R-SNAREs (“synaptobrevins”) have a longin domain integrated on the amino-terminal side (Schilde et al. 2006, 2010). This recalls the situation in plants (Sanderfoot 2007; Uemura et al. 2005; Vedovato et al. 2009). In plants, this domain contributes to vesicle targeting via interaction with other trafficking proteins. Occasionally, a similar effect has been reported for some mammalian R-SNAREs, for example, VAMP7, as analyzed in HeLa cells (Martinez-Arca et al. 2003). No such topology-determining effect has been scrutinized in ciliates as yet. In conclusion, a longin fold is an evolutionary criterion occurring much more frequently in bikonts than in unikonts.

Proteins undergoing considerable differentiation or replacement during evolution

Plasmalemmal transporters

This concerns a multitude of plasmalemmal transporters in plants. In plants, ion transport at the cell membrane is highly diversified—much more than in metazoan (and presumably in protozoan) cells (Edel and Kudla 2015). Together with exchangers, rather different ATPases are abundant in the cell membrane, that is, not only P-type Ca^{2+} -ATPases/pumps but also H^{+} -ATPases/pumps (V-type ATPases) (Duby and Boutry 2009). Not only the usual kind of V-type ATPases, composed of multimeric V0/V1 subunits and a connecting SU-a, are important as part of this inventory, but many of the transport functions can be performed by other pumps (Ratajczak 2000). Lineage-specific regulation of ion channels by CIPKs has been presented above.

A second look upon ciliary Ca^{2+} channels and pumps

Ciliary Ca^{2+} channels and $[\text{Ca}^{2+}]$ downregulation are connected with considerable relocalization of key proteins during evolution. As noted above, the subcellular localization of voltage-dependent Ca^{2+} -influx channels varies during evolution: In ciliates, they are localized in cilia of

Paramecium (Dunlap 1977; Macheimer and Ogura 1979; Yano et al. 2013) and of *Tetrahymena* (Dentler 1988) as well as in the green flagellate *Chlamydomonas* to the flagellar membrane (Mitchell 2007). This also holds for the flagellum of choanoflagellates (Cai et al. 2015). However, during metazoan evolution, other channels emerge in ciliary membranes.

TRP-type channels are not known in any detail from ciliates (Plattner and Verkhatsky 2015; yet see Plattner [2017a,b] for a possible equivalent), whereas they are known from unikonts, such as choanoflagellates (Cai et al. 2015). At first sight, the difference in voltage-dependent and nonvoltage-dependent channels would appear as a clear-cut difference between unikonts and bikonts. However, in contrast to what one would expect, *Dictyostelium* is reported to be devoid of voltage-dependent Ca^{2+} channels (Wilczynska et al. 2005). In *Chlamydomonas*, deflagellation induces upregulation of TRP1 (Fujiu et al. 2011) and electrophysiology clearly shows TRP channel characteristics (Arias-Darraz et al. 2015). While angiosperms are devoid of cilia or flagella (Moran et al. 2014), some gymnosperms, for example, *Cycas* palms and *Ginkgo* trees, still produce sperm cells with cilia; this still would have to be analyzed.

Why in evolution may TRP channels have been preferred by most systems over voltage-gated Ca^{2+} channels? TRP channels are grouped in seven subfamilies (Venkatachalam and Montell 2007), including those relevant for ciliary signaling: (i) a classical subfamily, TRPC; (ii) a vanilloid receptor subfamily, TRPV; (iii) a melastatin subfamily, TRPM; and (iv) a polycystin subfamily, TRPP (Pablo et al. 2017). TRP channels occur in, or near, motile (epithelial) as well as in immotile (primary) cilia (Kleene and Van Houten 2014). Here, in conjunction with basal body-associated F-actin, they serve as mechanosensors when cilia are bent (Battle et al. 2015; Venkatachalam and Montell 2007). TRP channels can form homo- or heteromeric channel complexes with variable channel properties including variable Ca^{2+} selectivity (Pablo et al. 2017). In the primary cilium, they can encompass, for example, TRPPs type PKD2, PKD2-1, TRPM4, and TRPV4 which are localized in the ciliary membrane (Pablo et al. 2017; Phua et al. 2015). In epithelial cells, TRPCs seem to occur, in part, in the ciliary and nonciliary cell membrane (Pablo et al. 2017). In cilia of the trachea, TRPV4 occurs, and PKD1 and PDK2 are also components of these motile cilia (Doerner et al. 2015).

So, why did this polymorphy emerge? Signal perception and also signal transmission by TRP-type channels are poly-modal depending on the stimulus and the TRP channel type activated. Altogether this allows for receptor functions in response to widely different stimuli, each with a fine-tuned reaction (Bloodgood 2010; Phua et al. 2015). Functionally, the rationale of voltage-dependent Ca^{2+} channels is the rapid, depolarization-based ciliary reversal response for immediate avoidance of mechanical obstacles and to ward off predator attacks, for example, by paramecia, as summarized recently (Plattner 2017a,b). Closure of these channels by a forming Ca^{2+} /CaM complex avoids Ca^{2+} spillover into

the cell body and, thus, avoids the additional triggering of unwanted reactions beyond cilia.

To recall, ciliary membranes in both evolutionary branches, mono- and bikonts, contain a PMCA (Fig. 1–3), as shown with *Tetrahymena* (Dentler 1988), *Paramecium* (Yano et al. 2013), *Chlamydomonas* (Pazour et al. 2005), and cilia of differentiated mammalian cells (Castillo et al. 2007). However, Ca^{2+} downregulation by PMCA activity—if present at all, for example, in the olfactory ciliary membrane—would be too sluggish (Kleene 2009). This is improved, as demonstrated with *Paramecium* cilia (Brehm and Eckert 1978) as well as with neuronal cell bodies of mammals (Levitan 1999), by rapid inactivation by CaM binding in a negative feedback loop. In summary, in widely different systems of different evolutionary levels, PMCA has only a minor, secondary effect on $[\text{Ca}^{2+}]$ downregulation after stimulation, also in cilia.

Intracellular Ca^{2+} channels

To allow for locally restricted intracellular signaling by Ca^{2+} , eukaryotic cells had to “invent” Ca^{2+} -release channels (CRCs) (Plattner and Verkhatsky 2016). Their evolution is currently in the focus of research with different protozoan groups, for example, in ciliates (Ladenburger and Plattner 2011; Ladenburger et al. 2006, 2009) and, by preliminary genomic data mining, in choanoflagellates (Cai et al. 2015).

There are evolutionary differences in the occurrence of IP_3Rs . According to informatics analysis *Dictyostelium* encodes a putative IP_3R (Traynor et al. 2000), just of the type analyzed in some detail in *Paramecium* (Ladenburger et al. 2006). An IP_3R is absent from *Chlamydomonas* (Pazour et al. 2005) and also from plant cells (Edel and Kudla 2015; Krinke et al. 2007).

The discovery of RyR-like proteins (RyR-LPs) in *P. tetraurelia* has a provocative effect. On one hand, the molecule has features typical of the superfamily of IP_3Rs and classical RyRs of mammals. This includes sequence similarity, occurrence of six transmembrane domains with a characteristic selectivity filter, and the absence of an IP_3 -binding domain in *Paramecium* (Ladenburger and Plattner 2011; Ladenburger et al. 2009; Plattner 2017a,b), just like in mammals (Efremov et al. 2015). It has to be stressed that the six transmembrane domains described for *Paramecium* RyR-LP (Ladenburger and Plattner 2011) are now established also for mammalian RyR (Efremov et al. 2015; Zalk et al. 2015). On the other hand, it may be designated merely as a RyR-LP, rather than as a conventional RyR, because of its unusually small molecular size. On one hand, this channel can be activated by classical activators of RyRs, such as caffeine and 4-chloro-meta-cresol, an effect inhibited by gene silencing (Ladenburger and Plattner 2011; Plattner 2017a,b). On the other hand, the large amino-terminal part found in mammalian RyRs, where it mediates functional feedback to the pore containing transmembrane segments (Efremov et al. 2015), is missing in *Paramecium* RyR-LPs (Ladenburger and Plattner 2011).

Remarkably, there is evidence from molecular data mining that this channel may also occur in the

choanoflagellate, *Salpingoeca rosetta* (Cai et al. 2015). Functional substitution of $\text{IP}_3\text{R}/\text{RyR-LPs}$ and of TRP channels by other channels in plants has recently been broadly discussed (Edel and Kudla 2015).

All this can be summarized as follows. Concerning intracellular CRCs, there are some similarities between uni- and bikonts in lower eukaryote representatives; for instance, IP_3R is present in *Dictyostelium* and in *Paramecium*. Again there are differences between the “green” and the other representatives of bikonts, considering that *Chlamydomonas* and angiosperms are devoid of such channels. Also the presence/absence of ciliary/flagellar channels fluctuates between representatives of the unikont and bikont branches. Altogether, loss of IP_3R channels can be considered a characteristic of plants.

Refunctionalization of proteins

Neofunctionalization and refunctionalization mean establishment of an essentially new function for a newly evolved protein and reassignment of a new function to an already existing type of protein, respectively. What previously had been considered as pre-adaptation is now called “exaptation,” that is, a shift in function of a trait/molecule during evolution (Sardar et al. 2014). Exaptation is now considered an important mechanism in evolution. Thereby, redirecting the localization of proteins (e.g. by altered targeting signals) is a mechanism of introducing new functions (Bauer et al. 2015).

A rare example of neofunctionalization is η -tubulin in *Paramecium*. It is an ohnolog emerging from a most recent whole genome duplication (Ruiz et al. 2000) serving for basal body biogenesis (Ruiz et al. 2000).

Rerefunctionalization can be exemplified as follows. The contractile vacuole complex mediates osmoregulation in some freshwater and in some parasitic protozoa. The de novo biogenesis of the organelle, as analyzed in *Paramecium*, precedes cell division and is regulated by proteins that are known as regulators of cytokinesis in any eukaryotic cells. Such proteins include calmodulin, centrin, γ -tubulin, and NIMA (never in mitosis A) kinase (Plattner 2015a). Formation of a centrin-based infraciliary lattice, intermingled with F-actin, as an immobile subplasmalemmal Ca^{2+} buffer is another example. An example of considerable interest is the dual role of some proteins, such as importin/transportin and the GTPase Ran; they regulate transport of proteins through nuclear pores as well as access to cilia (Gruss 2010). Quite recently, Shi et al. (2017) have demonstrated such dual function for IFT57A protein in *Paramecium*, where it serves for nuclear as well as for import of ciliary proteins. Despite the inability to produce cilia or flagella in higher plants, some axonemal proteins have been retained and used for alternative, cytoskeletal functions (Moran et al. 2014).

GPI-anchored variant surface antigens (vsAGs)

Other examples of acquirement of new functions are GPI-anchored proteins, or rather of their GPI anchors. In Fig. 6,

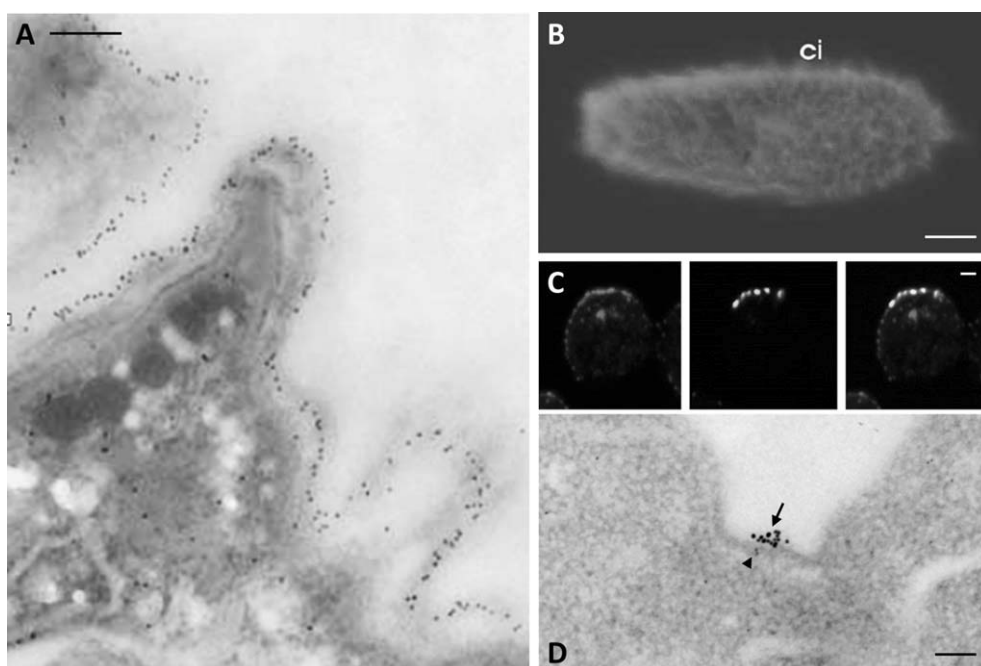


Figure 6 GPI-anchored proteins are diffusely distributed in *Paramecium tetraurelia* (A, B), but concentrated in microdomains in mammalian cells (C, D). (A, B) vsAGs in *P. tetraurelia*, (C, D) reggie/flotillin and prion protein (PrP^{Sc}) in lymphocytes. The localization of vsAG subtype G (A) by electron microscopy using immune-gold and (B) by fluorescent antibodies is diffusely distributed over the cell surface (including cilia, ci), in the absence of any recognizable microdomain formation. (C, D) The situation is different in lymphocytes where microdomain formation is shown to involve scaffolding reggie-1/flotillin-2 proteins (red, left) and co-clustering PrP^{Sc} (green, middle) shown by superposition (yellow color mix, right). Comparable co-clustering is seen in electron microscopic immunogold labeling: reggie-1/flotillin-2 labeled with 5 nm gold on the inner side (arrow head) and PrP^{Sc} detected by 10 nm gold (arrow) on the outer side of the cell membrane. (A, B) is from Momayezi et al. (2004), (C, D) from Stuermer et al. (2004). Scale bars: 0.1 μ m (A), 10 μ m (B), 1 μ m (C), and 0.1 μ m (D).

the localization of GPI-anchored proteins in ciliates is compared with that in mammalian cells, taking vsAGs and prion (PrP^{Sc}) proteins as examples. Apicomplexa and trypanosomatids as well as free-living protists, such as ciliates, are covered by a thick layer of GPI-anchored proteins; for *Paramecium*, see Preer (1986), Capdeville et al. (1987), Ferguson (1999) and Simon and Schmidt (2007). These include variant surface antigens (vsAGs) which possess a GPI anchor at their carboxy-terminal part (Ferguson 1999), and which, in *Paramecium*, represent about two-thirds of ciliary membrane proteins by mass (Yano et al. 2013). They are called vsAGs or immobilization antigens because they can be mutually exclusively expressed and exchanged and because of the effect of antibody binding, for example, in *Paramecium* (Simon and Schmidt 2007). These proteins are expressed under epigenetic control ("antigenic variation") which allows for rapid exchange of isoforms that determine the different serotypes. This is enabled by RNA-mediated reprogramming of gene expression patterns (Cheaib et al. 2015; Garnier et al. 2004) and by releasing vsAGs from the cell surface by GPI-specific phospholipases (Staudt et al. 2016).

In contrast, any functional role of vsAGs in ciliates is not well established although depleting cells of vsAGs is lethal to cells. Functions discussed range from intercellular and intracellular communication (Simon and Kusch 2013) to

receptors for chemoresponse (Yano et al. 2003). Could it be that vsAGs provide a kind of ionic microenvironment for the cells, for instance via Ca²⁺ binding, or may simply surface charges play a role? This is supported for interaction of the ciliate *Pseudomicrothorax dubius* with food cells (Kiersnowska et al. 1988) and for homologous interaction during conjugation in *Paramecium caudatum* (Kitamura and Hiwatashi 1984). Beyond this, it has not been possible so far to assign them a clear-cut function in ciliates, in contrast to the apicomplexan parasites.

The vsAGs are a rather heterogenic extended group in animals up to man where a large number of GPI-anchored proteins fulfill widely different activities. This ranges from ecto-enzymes (e.g. acetylcholine esterase, 5'-nucleotidase) to cell adhesion molecules, also in neurons and immune cells, while some serve for signal transduction (Ferguson 1999) and vesicle trafficking. Thy-1 is traditionally traded as a cell adhesion molecule, but now it is also an example of a GPI-anchored surface protein integrated into vesicle trafficking and membrane surface turnover (Stuermer 2012). Comparison of Fig. 6A/B and C/D reveals the rather homogenous distribution of the GPI-anchored proteins analyzed in *Paramecium* and the rather strict confinement of such proteins to microdomains in mammalian cells.

GPI-anchored proteins also occur in the smallest free-living nonsymbiotic alga, *Ostreococcus* (Mani et al. 2011),

in *Dictyostelium* (Haynes et al. 1993), and in plants (Eisenhaber et al. 2003), but—in the absence of an ER—not in bacteria (Mani et al. 2011). In eukaryotes, the GPI anchor is synthesized and attached to the carboxy terminus of selected proteins after truncation in the ER.

The GPI anchor of proteins can be released by specific phospholipase C (PL-C) forms. From bacterial precursors and from a small number of PL-C paralogs (Leondaritis et al. 2011), only two appear GPI-specific—a situation which is even less clear in mammals (Müller et al. 2012; Staudt et al. 2016). GPI proteins accompany evolution of Mono- and Bikonta from low levels on. Clearly, the principle of cutting a singlepass transmembrane protein type 1 at the carboxy-terminal side for transfer onto a GPI anchor (Ferguson 1999), followed by delivery to the cell surface via vesicle trafficking, is a principle maintained from protists up to angiosperms and mammals. Thereby, the protein moiety can be widely different.

Proteins newly “invented” during evolution

Filamentous structures

Intermediate filaments are most variable when different evolutionary lineages are compared. Intracellular keratin is paradigmatic for mammals and extracellular cellulose fiber formation at the cell membrane by cellulose synthase is typical of plants. No genes for the prominent mammalian intermediate filament constituents have been detected in the *T. thermophila* genome (Eisen et al. 2006). Cellulose synthesis in tunicates (phylum Chordata) has probably been enabled by horizontal gene transfer (Sagane et al. 2010). In contrast, alveolin proteins are a true characteristic of Alveolata, specifically of ciliates and Apicomplexa, where they form subplasmalemmal filaments (Gould et al. 2008). This is different from the centrin-based “infraciliary lattice” in ciliates which is composed mainly of centrin (Beisson et al. 2001), intermingled with actin (Kissmehl et al. 2004).

Cell adhesion

“Invention” of cell adhesion molecules is another achievement. It starts with cadherins (King et al. 2003, 2008; Shalchian-Tabrizi et al. 2008), specifically with a cadherin/ β -catenin complex in choanoflagellates (Nichols et al. 2012). Cell adhesion molecules from *Dictyostelium discoideum*, designated DdCAD-1, display a high degree of sequence similarity with cadherin family members, including Ca^{2+} binding (Sesaki et al. 1997; Wong et al. 1996), yet it has some unexpected functions as it binds CaM for delivery into the contractile vacuole complex (Sriskanthadevan et al. 2013). Also in *Dictyostelium*, TgrB and TgrC surface proteins, dedicated to allo-recognition during transition from uni- to multicellularity (Hirose et al. 2015), are unrelated to established cell adhesion proteins. In sum, Amoebozoa are evidently devoid of cadherins (Abedin and King 2008). In the bikont lineage, the situation may be similar with fenestriins—proteins required for cell conjugation in ciliates (Cole et al. 2008).

Further evolution of intracellular signaling

GPCRs are represented by 1,000 (or more) members in mammals where they are of paramount medical importance (Tautermann 2014). No such GPCRs are known from plants, despite the presence of trimeric G-proteins whose function, thus, differs from that in animal cells (Colaneri and Jones 2014). In protists, GPCRs are under considerable debate, except *Dictyostelium*. Only a few examples may be given. Of GPCRs, for instance, we know that they occur in *Dictyostelium* (Elzie et al. 2009), where they are coupled to folic acid-induced chemotaxis and phagocytosis (Pan et al. 2016). In contrast, we have only fragmentary information for *Paramecium* (as reviewed in Plattner 2017a,b).

Similarly, in protozoans, genuine Tyr phosphorylation for signal transduction (outside mitotic activity) is known only from choanoflagellates (Manning et al. 2008; Segawa et al. 2006), but not from ciliates (Plattner 2017a,b). More detailed information would be of paramount importance for a full understanding of signal transfer in these cells. Choanoflagellates are also unique in possessing receptor-type Tyr-kinase molecules (King et al. 2003; Miller 2012; Shalchian-Tabrizi et al. 2008); *Monosiga brevicollis* and related filasterean *Capsaspora owczarzaki* contain even more such Tyr-kinases than man and they also possess adequate protein phosphatases (Hunter 2014). In a strict sense, receptor-type Tyr-kinases are absent from ciliates and from myxamoebae (Mayer 2008) whose genome does not encode any modern tyrosine-specific protein kinases (Lim and Pawson 2010). Ciliates possess only Tyr-kinases for nuclear signaling, as summarized recently (Plattner 2017a,b). Thus, the Tyr-kinase types responsible for a large part of intracellular signal transduction, from the cell membrane on, emerge only in metazoans.

Clearly, evolution of the highest taxons entailed the development of new sensorial categories without any known structural counterpart in unicellular organisms. In vertebrates and specifically in mammals, the protein repertoire is complemented by cyclic peptide neurohormones, such as vasopressin (Baribeau and Anagnostou 2015) and oxytocin, for complex central nervous and behavioral functions (Crespi 2016; Goodson 2005). Oxytocin produces emotional effects (“love hormone”) whereby vasopressin can interact, while its main function is the regulation of water balance. The latter is handled in widely different manner in lower animals, protists, and plants. In sweet water protists, it is effectuated by the mechanistic system of an osmoregulatory organelle, the contractile vacuole complex, as reviewed recently (Plattner 2015a). Both peptide neurohormones trigger intracellular signal transduction via trimeric GTP-binding proteins (“G-proteins”) and GPCRs (Kobilka 2013). Whether plants dispose of GPCRs is the subject of intense discussions (Taddese et al. 2014). They use alternative mechanisms for osmoregulation, that is, by different ion transporters, as discussed recently by Edel and Kudla (2015). In summary, water balance is regulated by widely different mechanisms in protists, animals, and plants and the proteins involved are accordingly different during evolution.

Proteins lost during evolution

There is wide variation between retention and abolition of selective proteins during evolution. Even within animals, from fish to man, only 53% of genes are shared by different species. Similarly angiosperms retain only 51% from their precursors (Gimpel et al. 2013). As mentioned, some proteins, or subunits thereof, are completely eliminated from plants, such as SU-A of CaN and RyR-LPs.

Alveolins and epiplasmins are proteins lost during evolution. Alveolins are proteins with charged repeats which are characteristic of Alveolata, such as ciliates and Apicomplexa (*Plasmodium*, *Toxoplasma*) (El-Haddad et al. 2013). In ciliates, they are essential for the formation of the regular cell surface pattern. This is paralleled by the regular arrangement of alveolar sacs which, on the side facing the cell interior, are backed up by epiplasmins (Aubusson-Fleury et al. 2013).

While the secretory machinery is largely retained during evolution, contents are quite variable. Figure 4 compares dense core-vesicle systems in mammals and in ciliates. Three facts are striking. (i) Both use a SNARE/GTPase system. (ii) Ciliates can synchronously release their contents, in contrast to mammalian systems. (iii) Importantly in the present context, there is a complete exchange of secretory proteins during evolution. This is largely the case with mucocyst and trichocyst contents in *Tetrahymena* and *Paramecium*, respectively, aside from some proteins with similar motifs and targeting mechanisms (Guerrier et al. 2017). Reports on chromogranin A and pancreastatin in trichocysts could not be verified, as discussed previously (Plattner 2017b). This difference can be appreciated if one considers the engagement, for example, of some neurosecretory peptides in emotional conditioning and other systemic regulatory phenomena, whereas some other ones have lytic functions, to mention just two extremes.

Ion channels are another group of proteins exchanged or lost during evolution. Recent reviews—not to be repeated here—inform about appearance and disappearance, respectively, of different ion channels from protists on, up to mammals and flowering plants (Edel and Kudla 2015; Plattner and Verkhatsky 2015). Examples include bacteria (Domínguez et al. 2015), ciliates (Plattner 2015b), chlorophyceae (Edel and Kudla 2015), myxamoebae (Traynor et al. 2000), trypanosomatid flagellates (Docampo et al. 2014), other pathogenic protozoa (Prole and Taylor 2011), and, finally, choanoflagellates (Cai et al. 2015). Currently, data are frequently retrieved solely by data mining, whereas only in few protozoa, some channels have been experimentally verified and, thus, made comparable to animals and plants. Many channels emerge early on in evolution; some, like IP₃Rs, are maintained from ciliates, flagellates, and myxamoebae up to man (Plattner and Verkhatsky 2015), but up to now remained untraceable in plants (Edel and Kudla 2015) and apicomplexan parasites (Garcia et al. 2017). Remarkably *Chlamydomonas* has voltage-dependent Ca²⁺ channels as well as TRP channels, but apparently no IP₃Rs (Edel and Kudla 2015). Thus, bikonts probably have lost

IP₃Rs, voltage-dependent Ca²⁺ channels and TRP channels as soon as they became “green” (Wheeler and Brownlee 2008).

Two or more peptides/proteins from one gene and two genes for one protein

The “two or more proteins/peptides from one gene” is familiar from neuropeptides, but considerably less from protists.

In mammals, long known examples are a variety of neuropeptides released from the pituitary gland (hypophysis). One example is the cleavage of proopiomelanocortin, produced by corticotropic cells, into one dozen bioactive peptides (Dores and Baron 2011). A common prohormone is also formed for adiuretin/(Arg)vasopressin and neurophysin II (Baribeau and Anagnostou 2015). Similarly, the precursor of oxytocin is formed from a larger precursor, together with neurophysin I (Gimpl and Fahrenholz 2001). Both prohormone genes are coupled in mammals and transcribed in opposite directions; moreover, both vasopressin and oxytocin are nonapeptides requiring cyclization (Baribeau and Anagnostou 2015). Otherwise genes encoding two or more peptides are not frequent (aside from immunoglobulins) and probably have different origin. For instance, in several holometabolic insects, a translation initiation factor can be linked to a heterochromatin-associated protein, required for chromatin condensation, by removal of a stop codon and in-frame gene fusion (Krauss and Reuter 2000).

In ciliates, an adenylyl cyclase and a K⁺-channel are co-expressed as a fusion protein (Schultz et al. 1992; Weber et al. 2004). This accounts for the fact that a K⁺ conductivity stimulates cAMP formation which regulates increased ciliary beat activity (Bonini et al. 1991). In contrast, for Ca²⁺- and cGMP-mediated enhancement of ciliary reversal (Yang et al. 1997) no such connection to Ca²⁺ channels exists.

A “two genes for one protein” situation is seen with one of the CaN SUs in *P. tetraurelia*. Here, the translation products of the two CaN-B ohnolog genes—quite surprisingly—are identical (Fraga et al. 2010). Clearly this reflects permanent trial and error on the nucleotide level which results in identical proteins because of constraints. Such constraint is also observed with the highly conserved binding site of CaN-B on the CaN-A SU. Genes encoding identical isoforms are observed more extensively with actin in *E. histolytica* and *Dictyostelium* (Gunning et al. 2015). In *E. histolytica*, there exists one cytoplasmic actin encoded by 7 genes, and in *D. discoideum*, eight actins are encoded by 24 genes. In such cases, one may consider a function by translational amplification effects. The numerous ohnologs of different genes/proteins in *P. tetraurelia* may be considered additional examples, considering that many of them, for example, SNAREs (Plattner 2010a,b), display only very little difference between the ohnologs (Plattner 2010a).

The requirement of only one gene for producing, by posttranslational cleavage, several proteins with different

functions is overcompensated by the requirement of additional genes for receptors. In summary, it seems that such tandem genes do not remarkably affect the total balance of genes in different organisms. Occurrence of many ohnologs can serve an amplification effect and may also be the basis for further differentiation during evolution.

Proteins under discussion

Surprisingly, IP₃Rs have been identified by mass spectroscopic analysis in the membranes of carefully purified cilia of *Chlamydomonas* (Pazour et al. 2005) and of *Paramecium* (Yano et al. 2013). However, in *P. tetraurelia*, molecular analyses and immuno-localization of a selection of the IP₃Rs revealed restriction to vesicles participating in trafficking, whereas IP₃Rs remained undetected in cilia (Ladenburger et al. 2006). The discrepancy may be explained by different methodical sensitivities.

Also trimeric G-proteins, GPCRs, luminal CaBPs in Ca²⁺ stores, and annexins have been only tentatively identified in ciliates (Plattner 2010b). Other proteins identified insufficiently in ciliates are those involved in nuclear death, an apoptosis-related process involved in elimination of the old macronucleus after conjugation (Guerrier et al. 2017). There would be many more examples.

SYNOPSIS OF CHANGES FROM PROTISTS TO HUMANS AND PLANTS

Altogether, this review considers the evolutionary “career” of molecules from protists on, asking “what has become of prominent proteins during evolution?” To mediate and govern increasing complexity, evolution of eukaryotic cells has required an increasing inventory of proteins, some found already in bacteria, and many more in unicellular organisms. Essentially, this includes cytoskeletal elements, motor proteins, signaling molecules, components enabling vesicle trafficking, scaffolding proteins, and etc. Representatives of all these have been identified in different protists, although with variations. This is summarized in Tables 1, 2.

How is increasing complexity achieved? Is there an ancient core inventory transmitted during evolution? Consider that green plants are made of ~70 cell types and that our body contains ~240 cell types, to give some typical pilot values reported in the Internet and based on methodology reported by Bard et al. (2005), with nearly 100,000 proteins species, despite the only moderate increase of protein-coding genes in man (below). In principle, evolutionary differentiation of proteins can include further use of old genes, their modification by the mechanisms discussed above, by formation of new genes (and deletion of some old genes, as exemplified), formation of splice variants, posttranslational modifications, increasing numbers of transcription factors, non-coding regulatory DNA, and etc. All these mechanisms can contribute to evolution of the eukaryotic cell from unicellular to multicellular forms. The question arises

whether precise numbers currently available can be accommodated as essential “core” genes/proteins of protists in metazoans and in green plants or, vice versa, which percentage this heritage may occupy in multicellular organisms.

Can protistan genes and proteins potentially be accommodated in higher eukaryotes?

The number of (protein-encoding) genes during evolution increases much less than previously assumed—quite in contrast to the enormous increase in DNA per nucleus (“C-value paradox,” [Fedoroff 2012]). Numbers of predicted (protein-coding) genes are reported in the following ranges: 27,424 in *T. thermophila* (Eisen et al. 2006); 39,642 in *P. tetraurelia* (Aury et al. 2006); 8,657 in choanoflagellates (Suga et al. 2013); 14,516 in *Chlamydomonas* (Fritz-Laylin et al. 2010); ~10,300 (Williams 2010) to 13,574 in *Dictyostelium* (Fritz-Laylin et al. 2010); 26,541 in *A. thaliana* (Fritz-Laylin et al. 2010; The Arabidopsis Genome Initiative 2000); finally an estimated number of between 20,344 (Uhlén et al. 2015), ~20,700 (The ENCODE project consortium 2012) and 23,328 (Fritz-Laylin et al. 2010) are reportedly expressed in humans. The Charophyta species *Klebsormidium flaccidum* disposes of 16,215 (nuclear and organellar) protein-encoding genes (Hori et al. 2014).

The wide variations call for interpretation. The general line is an increase of the number of genes with increasing organizational level. But there are exceptions to this rule. The extraordinary number of genes in *P. tetraurelia*, in contrast to only 18,509 in *P. caudatum* (McGrath et al. 2014) reflects at least two whole genome duplications with little diversification of genes from the last two, out of probably three duplication rounds in *P. tetraurelia* (Aury et al. 2006; McGrath et al. 2014) which produced very similar paralogs called “ohnologs” (Ohno 1970). Thus, the number of subfamilies, rather of individual genes, may eventually be considered as a criterion for the real extent of differentiation, as discussed for SNAREs by Plattner (2010a). Although the number of genes published for some of the species mentioned is not precisely settled, it is clear that the number of protein-encoding genes has increased by only less than twofold in higher levels of multicellular organisms. Aside from gene transfer from other species, new genes arise from divergence of duplicated genes and by de novo formation from a non-(protein-)coding gene (Andersson et al. 2015). Nevertheless, there is ample space for maintaining quite a few genes/proteins during evolution, and “modern genomes and proteins are also fossils in their own way, littered with evidence of genes that were useful to ancestral organism” (Hunter 2013). Among them are functionally most important genes/proteins, as mainly discussed in this review.

There is an enormous contrast between the number of genes and of nucleotides per nucleus which, during evolution, accumulates an enormous amount of “non-(protein-) coding DNA.” Thus, the coding density decreases dramatically during evolution (Fritz-Laylin et al. 2010; Suga et al.

2013). As we now know, during evolution this reflects an increasing contribution of regulatory factors, such as transcription factors and RNAs regulating transcription of protein-encoding genes, for example, during tissue differentiation.

There are still other mechanisms which have contributed to diversification of proteins. Some diversification of the protein inventory is enabled by alternative splicing of mRNA. This is considered an important aspect already for the latest eukaryotic common ancestor (Irimia and Roy 2014). The number of introns per gene in the protozoa under consideration is between 1.3 (*D. discoideum*), 3.6 (*T. thermophila*), and 2.3 (*P. tetraurelia*), whereas the value indicated for *A. thaliana* is 4.8 (Atambaeva et al. 2008) and that for *H. sapiens* is 12.4 (Xiong et al. 2012). The percentage of genes with introns is 80–84% in *P. tetraurelia* (Aury et al. 2006; Zagulski et al. 2004), 68% in *D. discoideum* (Zagulski et al. 2004), 79% in *A. thaliana* (The Arabidopsis Genome Initiative 2000), and 85% in *H. sapiens* (Aury et al. 2006). However, this does not reflect the low number of splice variants detected in ciliates (Jaillon et al. 2008). In *P. tetraurelia*, less than 0.9% of the introns are alternatively spliced (Jaillon et al. 2008); in *T. thermophila* ~5% of alternative splicing has been reported (Xiong et al. 2012). Also in angiosperms, the rate of splicing is low (The Arabidopsis Genome Initiative 2000). Proteomics analysis available so far indicates 86,771 proteins in *H. sapiens* (Wilhelm et al. 2014). When compared with the number of genes (above) this is compatible with an estimated number of splice forms per proteins which reportedly is between 4.5 (Chen et al. 2014) and 6.3 (The ENCODE Project Consortium 2012). Only among vertebrates, the number of splice variants increases with evolutionary level (Chen et al. 2014). According to RNA analysis, in *A. thaliana*, 23,840 transcripts (> 29,000 with splice variants included) have been assigned to 22,076 genes (Liang et al. 2016). The low rate of splicing in *P. tetraurelia* explains that only ~30,000 proteins are to be expected (Zagulski et al. 2004). Here, in *P. tetraurelia*, some of the numerous ohnologs, for example, of Ca²⁺-release channels (Ladenburger and Plattner 2011; Ladenburger et al. 2009) and of SNAREs (Plattner 2010a), may either serve for amplification effects or they may provide similar effects as splice variants do in vertebrate cells.

Additional variation comes from covalent posttranslational modifications such as aminoacylation, for example, of tubulins from ciliates (Adoutte et al. 1991) to mammals (Wloga and Gaertig 2010), fatty acylation, isoprenylation of GTPases, and ubiquitylation (Duan and Walther 2015)—phenomena, in part, known also from protozoans where this is only insufficiently analyzed so far.

The predominant mass of DNA per nucleus in mammals and higher plants is nonprotein coding; it is rather engaged in regulatory processes via regulatory RNAs (Morris and Mattick 2014) and formation of an ever increasing number of transcription factors, from ~1,400 to 1,900 (Vaquerizas et al. 2009) to several thousand estimated in man. In fact, the number of transcription factor genes steadily

increases, 1,558 being reported by Wingender et al. (2013), with an additional increase to > 2,900 when isoforms generated by alternative splicing or protein processing events are included. In a single-cell system, such as protozoa, their number will necessarily be considerably smaller. This follows from a comparative genomic analysis where the content of transcription factor-encoding genes relative to that in man was 10% in the choanoflagellate *M. brevicollis*, 9% in *D. discoideum*, 7% in *T. thermophila*, and 12% in *C. reinhardtii* (De Mendoza et al. 2013). The value was higher only in *A. thaliana* (135%); the unexpectedly high value in *P. tetraurelia* (42%) is probably due to the severalfold whole-genome duplications. In summary, a crucial aspect pertinent to the topic of the present review is the fact that (protein) coding density in humans is only ~1.5% (International Human Genome Sequencing Consortium 2001) or 2.8% in a selected sample (Alexander et al. 2010) and ~4.5 in *Arabidopsis* (Arabidopsis Genome Initiative 2000), in contrast to ~85% in *Paramecium* (Aury et al. 2006), to give an example.

In humans, widely varying expression in different tissues is required for differentiation which is enabled by epigenetically controlled transcription factors and their splice variants (Kim et al. 2014). In humans, only 44% of the protein-encoding genes are expressed in all major tissues (Uhlén et al. 2015) which, therefore, may represent some basic equipment for basic functions. As mentioned, bacterial genes contribute by ~37% to mammalian gene sequences and 28% are all-eukaryotic (McFall-Ngai et al. 2013), whereas over 50% are retained from low metazoans up to man and from low plants up to angiosperms (Gimpel et al. 2013). The percentage of protistan genes retained during evolution remains to be established. Nevertheless, there appears to be enough storage capacity to accommodate much of the basic inventory of lower eukaryotes in their highest multicellular descendants. Concomitantly, as far as analyzed so far, orthologs of functionally important genes are found throughout eukaryote evolution, although with some variations.

Essential changes and innovations during evolution

From bottom to top of organismic evolution, some proteins may display high conservation of structure and localization. Examples are calmodulin, P-type Ca²⁺ pumps, GTPases, and inositol 1,4,5-trisphosphate receptors in ciliates and metazoans. Most impressive changes of protein structure, function, and/or localization are as follows.

- (i) The exchange of voltage-dependent Ca²⁺-influx channels in cilia of protozoa for transient receptor potential (TRP)-type channels in epithelia and primary cilia allows to achieve more flexibility, although with slower responses. Fast response is required, for example, to avoid obstacles and predators by swimming *Paramecium* cells via ciliary reversal, as summarized recently (Plattner 2017a,b). Analogously, in mammals, voltage-gated Ca²⁺ channels are engaged in rapid neuronal communication.

- (ii) Some proteins are endowed with specific, but evolutionarily variable domains (e.g. R-SNAREs, type synaptobrevins, in animals vs. longins in ciliates and plants, synaptotagmin and enhanced synaptotagmins in *Paramecium* vs. mammals). With R-SNAREs, targeting effects are obvious for plants.
- (iii) Expansion of some protein subunit family members (ohnologs) can eventually change intracellular localization of holoenzyme combinations. In *Paramecium*, increased gene numbers encoding SU-a of the V-type H⁺-ATPase allow to connect different V0 and V1 parts and this may potentially mediate binding of different partner proteins and overall variable targeting.
- (iv) Proteins with occasional incorporation of regulating sequences (e.g. a calmodulin-like domain in “Ca²⁺-dependent protein kinases,” CDPK, of ciliates and plants). This entails strict co-localization of effector (kinase) and regulator (CaM).
- (v) Loss of a subunit (e.g. of calcineurin A in plants) is connected with regulation of ion channels by CBL kinase.
- (vi) Loss of entire proteins, alveolin, for example, can be explained by absence of alveoli outside Alveolata, although alveoli resemble structurally and functionally rather closely cortical ER sacs of some metazoan cells (Plattner 2014).
- (vii) Proteins differentiating from atypical precursors during evolution, for example, from ryanodine-receptor-like Ca²⁺-release channels in ciliates, may be the starting point for RyRs which now requires extensive scrutiny in different taxons.
- (viii) There are proteins with pharmacological characteristics varying between subfamily members (e.g. varying drug binding by actin isoforms in protists).
- (ix) In some other proteins, we observe an inconsistent expression of subfamily traits, for example, flotillin/reggie vs. stomatin characters in ciliates.
- (x) Neofunctionalization (e.g. η -tubulin in *Paramecium*) and refunctionalization of proteins (e.g. calcineurin B in plants; centrin in centrosomes vs. plasmodesmata in plant). Such phenomena are considered a driving force in evolution.
- (xi) Endowment of proteins with a GPI anchor (e.g. glycosylphosphatidylinositol-anchored “variant surface antigens” in ciliates and Apicomplexa vs. numerous GPI-anchored glycolyx proteins in metazoans) can mediate flexibility of such proteins with regard to trafficking and signaling (Stuermer 2010, 2012).

- (xii) Some proteins are newly “invented” during evolution (e.g. neuronal secretory peptides; keratin-type intermediate filaments in animal cells; plant-specific proteins, such as cellulose synthase).

In this review, frequently similarities between monokonts and bikonts are mentioned, but we also see many more variability and dissimilarity when molecular structure and subcellular localization are compared (Table 2). This is compatible with the emergence of the two lineages from common ancestors, possibly already during early eukaryote evolution. Diversification can have been accompanied by loss, modification, or new formation of specific molecules. However, it should not be denied that some authors have questioned the mono-/bikont subdivision of organisms altogether, based on the analysis of a broad range of proteins (Baldauf et al. 2000) or on rare genomic changes (Rogozin et al. 2009). Similarities would be compatible with the preservation of early protein components before organisms have split into the two lineages. Beyond this, we have seen examples that “green” multicellular bikonts (plants) differ, in part, from flagellated algae and even more from ciliates, for example, with regard to centrin localization, CBL activity, and absence of IP₃Rs and TPRs.

Some components occurring already in protozoans (SNAREs, GTPases, CaM, some CRCs) are retained up to highest evolutionary levels. Some become essential in man for long-term potentiation, that is learning, and for immune defense (CaN). Except CaN and CRCs, similar proteins are found in selected (archae) bacteria. This includes protein sequences related to SNAREs, GTPases, and their modulators. (However, occurrence of such protein equivalents in some pathogenic bacteria could also indicate top-to-bottom gene transfer.) Also CaM and PMCA are known from some eubacteria, and recent analysis of Lokiarchaeota-type archaeobacteria was very much enlightening since it also revealed the presence of some precursors of some additional molecules under consideration here (Spang et al. 2015). In total, many proteins dedicated to most basic and complex functions in our body have been available already early on in evolution. Eventually proteins have changed intracellular position during eukaryotic evolution. A good example are voltage-dependent (depolarization-sensitive) Ca²⁺-influx channels which, in ciliates, are restricted to cilia, but in mammals to neurons.

In summary, from the analysis of a limited set of proteins probed so far in protists for molecular structure, function, and subcellular localization, it appears that early eukaryotes had collected most components essential for establishing a complex, dynamic intracellular structure from different types of bacteria where many putative precursors still can be found. One driving force was leakage of Ca²⁺ from the outside which required counter-regulation already in bacteria. Early eukaryotic cells have reverted this disadvantage to an advantage, that is, spatially and temporally restricted signaling. Thus, with increasing cell size, this principle was overriding mere

Ca²⁺ downregulation. This recalls the detoxification and energetic exploitation of increasing atmospheric oxygen concentration during evolution—another innovation critical for prokaryote-to-eukaryote transformation.

Many of the basic regulatory and functional mechanisms related to Ca²⁺ are essentially maintained during evolution, from protozoa to man. Many details had to follow, for example, a dramatic increase of the number of effector molecules for Ca²⁺, that is, CaBPs, during evolution. Essential molecular features observed in protozoa and specifically in ciliates are operating in the most complicated functional capabilities of man. As outlined at the beginning, just considering size, the nuclear genome of protists could potentially have been accommodated in the genome of the most highly evolved metazoans. As mentioned, bacterial genes contribute by ~37% to mammalian gene sequences (McFall-Ngai et al. 2013) and the percentage of protozoan precursors, although not known in detail, will probably be even higher. Metazoans have steadily increased their genome which is about 50% larger than that of unicellular organisms. In addition, posttranslational processing and modification have increased the number of protein species. Considerable changes are also observed during evolution of higher plants. On several occasions, whole-genome duplications provided play material for diversification (Van de Peer et al. 2009).

In retrospect, work with different protists gives excellent examples of the evolutionary phenomena described. The kind of “evolutionary cell biology” (Lynch et al. 2014) addressed here will advance as the number of protein molecules investigated in protists will steadily increase.

ACKNOWLEDGMENTS

The author’s work cited herein has been supported up to 2014 by the German Research Council (Deutsche Forschungsgemeinschaft, DFG). The author also wants to thank the many coworkers who contributed over the years, as cited, to the analysis of trafficking and signaling in *Paramecium* cells.

ABBREVIATIONS

[Ca²⁺]_i: intracellular free Ca²⁺ concentration
 CaBP: Ca²⁺-binding protein
 CaM: calmodulin
 CaM-kinase: Ca²⁺/CaM-activated protein kinase
 CaN: calcineurin (protein phosphatase 2B, PP2B)
 CBL: CaN-B-like
 CDPK: Ca²⁺-dependent protein kinase with integrated CaM-motif
 CIPKs: CBL-interacting protein kinases
 COP: coatomer protein
 CRC: Ca²⁺-release channel

GPI-: glycosylphosphatidylinositol
 G-protein: trimeric GTP-binding protein
 GTPase: monomeric low-molecular-weight GTP-binding/ GTP hydrolyzing proteins
 IP₃: inositol 1,4,5-trisphosphate
 IP₃R: IP₃ receptor
 LP: like protein
 NSF: N-ethylmaleimide sensitive factor
 PL-C: phospholipase C
 PMCA: plasmamembrane Ca²⁺-ATPase/pump
 RyR: ryanodine receptor
 SERCA: sarcoplasmic/endoplasmic reticulum Ca²⁺-ATPase/pump
 SNAP-25: soluble NSF attachment protein-25
 SNARE: soluble N-ethylmaleimide-sensitive factor attachment protein receptors
 SPFH: stomatin/prohibitin/flotillin (reggie)/Hflk protein family
 SU: subunit
 TRP channel: transient receptor potential channel
 vsAG: variant surface antigen

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