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Unicellular Eukaryotes as Models in Cell and Molecular Biology: Critical Appraisal of Their Past and Future Value

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Contents

Abstract

Unicellular eukaryotes have been appreciated as model systems for the analysis of crucial questions in cell and molecular biology. This includes *Dictyostelium* (chemotaxis, amoeboid movement, phagocytosis), *Tetrahymena* (telomere structure, telomerase function), *Paramecium* (variant surface antigens, exocytosis, phagocytosis cycle) or both ciliates (ciliary beat regulation, surface pattern formation), *Chlamydomonas* (flagellar biogenesis and beat), and yeast (*S. cerevisiae*) for innumerable aspects. Nowadays many problems may be tackled with "higher" eukaryotic/metazoan cells for which full genomic information as well as domain databases, etc., were available long before protozoa. Established molecular tools, commercial antibodies, and established pharmacology are additional advantages available for higher eukaryotic cells. Moreover, an increasing number of inherited genetic disturbances in humans have become elucidated and can serve as new models. Among lower eukaryotes, yeast will remain a standard model because of its peculiarities, including its reduced genome and availability in the haploid form. But do protists still have a future as models? This touches not only the basic understanding of biology but also practical aspects of research, such as fund raising. As we try to scrutinize, due to specific advantages some protozoa should and will remain favorable models for analyzing novel genes or specific aspects of cell structure and function. Outstanding examples are epigenetic phenomena a field of rising interest.

1. INTRODUCTION

Eukaryotic cells are complex four dimensional systems, like a 4D puz zle, with molecular components varying in space and with time, for exam ple, after stimulation or during development. Furthermore, elements have to be routinely exchanged, for instance for repair work, as it occurs during molecular and organellar autophagy. All parts have to fit together at all times, not only in the individual organelles but in the cell as a whole and, in mul ticellular organisms, in the context of tissues. This makes it difficult to dissect individual aspects of cell structure, biogenesis, and function. Therefore, yeast and protists (protozoa and some algae), both endowed with some simple traits, have been appreciated as model systems over decades. In Section 2, we explore the possibility of using unicellular eukaryotes as models in the past and possibly also in the future.

Mitochondria, Golgi apparatus, secretory organelles, cilia, and flagella look essentially the same throughout eukaryotes, from protists to mammals. Even plant cells follow essentially the same construction principle, disre garding the fact that, on the one hand, they harbor additional elements

(chloroplasts and their derivatives, glyoxysomes, etc.) and, on the other hand, they lack some components. Differences concern the lack of a centri ole, for instance in Angiosperms (flowering plants) and some Gymnosperms (Gnetales, Coniferae) (Hodges et al., 2012). Also some of the unicellular model organisms can live without a centriole (Bettencourt Dias, 2013; see Section 3), although centriole equivalents, the ciliary basal bodies, are present in unsurpassed numbers. This is an example of how unicellular organisms with particular traits can be favorable objects for the analysis of specific questions. In this context, the essential question we may ask a model system is which components are essential for centriole and which ones for basal body function.

Taking advantage of specific traits of unicellular organisms has intensely facilitated the elaboration of new concepts in cell biology, as we will testify in this review. This view is not shared by all. For instance, Munro stated in a recent "highly accessed" article: "*It is unlikely that the planet's tax payers will be willing to pay for enough cell biologists to investigate every last intriguing invertebrate or bizarre bikont, and thus future work is likely to focus on particular key cell types, especially those found in tax payers themselves*" (Munro, 2013). Does that mean the end of work with model cells, with any model? We here try to inves tigate the potential advantages and disadvantages of unicellular models with out talking up or down their advantages and their disadvantages. We are encouraged by a recent comment on "the genome's rising stars," where the perspectives of epigenetic research are appreciated (Maxmen, 2013): "*Some experiments also call for experience with model systems. . .*" As we outline in Section 4, epigenetics is one of the topics which promises a future par ticularly for protistan model systems. In Section 5, we try to give nonprotist models a fair chance. Finally, in Section 6, we try to reach an objective judg ment about the future of unicellular models.

2. WHAT IS SPECIAL ABOUT UNICELLULAR MODELS

Nature provides a plethora of cells with special traits that appear aber rant when compared with "normal" cells, particularly when these are inte grated in a tissue such as liver. Consider, for instance, the example of the centriole in Section 1. However, deviations from what we consider "nor mal" from the mammalian point of view can not only fulfill special require ments in a "lower" eukaryote growing in the wild but may also offer to the

experimentalist a handle on specific problems which otherwise would be difficult to address.

2.1. Unicellular models

To serve as a model, easy cultivation and established genomics are important prerequisites. In this section, we present lower eukaryotes preferably used as unicellular models, together with their systematic/evolutionary positions, and the type of problems for which they were or are still considered as models.

2.1.1 Dictyostelium *(Amoebozoa, Mycetozoa)*

Dictyostelium (Amoebozoa, Mycetozoa) has served as a model for many aspects (Müller Taubenberger et al., 2013) including amoeboid movement and phagocytosis (Cosson and Soldati, 2008) as well as for cell adhesion (Annesley and Fisher, 2009; Bagorda and Parent, 2008; Harloff et al., 1989). Note that cell adhesion molecules were detected in *Dictyostelium* (Beug et al., 1973; Müller and Gerisch, 1978). They are of particular impor tance for this organism whose amoeba stage is capable of developmentally controlled cell aggregation (King et al., 2003; Williams et al., 2005). Haploid *Dictyostelium* cells greatly facilitate genetics studies.

2.1.2 Paramecium *and* Tetrahymena *(Alveolata, Ciliata)*

Paramecium and *Tetrahymena* (Alveolata, Ciliata), mainly *P. tetraurelia* and *T. thermophila*, have served as models for the function (Sleigh, 1969) and bio genesis (Smith et al., 2005) of cilia. In ciliates, metachronal waves of ciliary beat (Fig. 3.1) are easy to study. Because of the surface mucus layer on mam malian ciliated epithelia it was impossible to analyze their metachronal beat which in ciliates is clearly imposed by hydrodynamic coupling. Only recently has this phenomenon been formally described on a more general basis (Elgeti and Gompper, 2013). The regular construction of the cortex of *Tetrahymena* and *Paramecium* (Fig. 3.2) was helpful in analyzing pattern formation, with epigenetic effects involved (Aufderheide et al., 1980; Frankel, 1973). This regularity also greatly facilitated the analysis of exocy tosis and endocytosis (Section 3.4). Study of phagocytosis under precisely timed conditions was another aspect (Allen and Fok, 2000). In *Paramecium*, synchronous exocytosis of a large number of dense core secretory organ elles, the trichocysts, can be achieved (Plattner et al., 1984), because a cell contains up to ~1000 trichocysts ready for immediate release upon

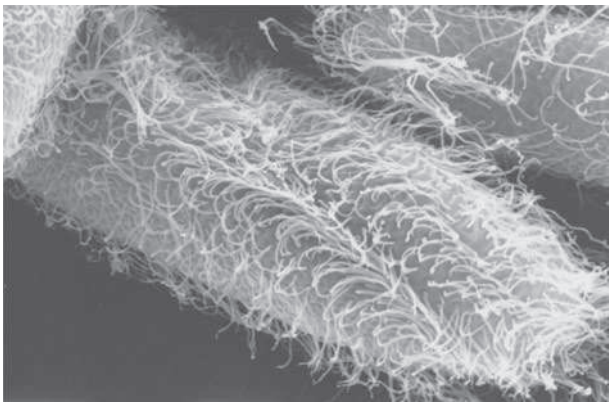

Figure 3.1 Scanning electron micrograph of the surface of a *Paramecium* cell. Cilia are arranged in waves due to metachronal beat activity. Already early on, the great number of cilia and easy accessibility of *Paramecium* to electrophysiology have made this cell an invaluable model. Magnification 1100×. *Unpublished micrograph by J. Hentschel (University of Konstanz).*

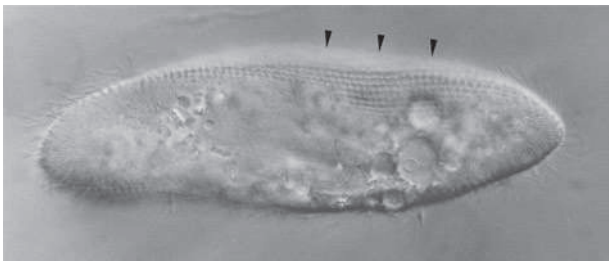

Figure 3.2 Phase contrast image of a *Paramecium* cell revealing the regular structure of its surface (arrowheads in top part). Note the occurrence of rows (kineties) of unit fields (kinetids), delineated by longitudinal and perpendicular ridges on the egg case-like cell surface. Magnification 700×.

stimulation (Figs. 3.3 and 3.4). Specifically this allowed the analysis of rapid processes, such as membrane fusion during exocytosis and exo /endocytosis coupling (Plattner and Hentschel, 2006). Recently *Paramecium* has served for studying the consequences of whole genome duplications, gene differenti ation, pseudogene formation, and epigenetic regulation phenomena. Work with *Tetrahymena* does not have the problem of multiple paralogs—in many regards an advantage of this system.

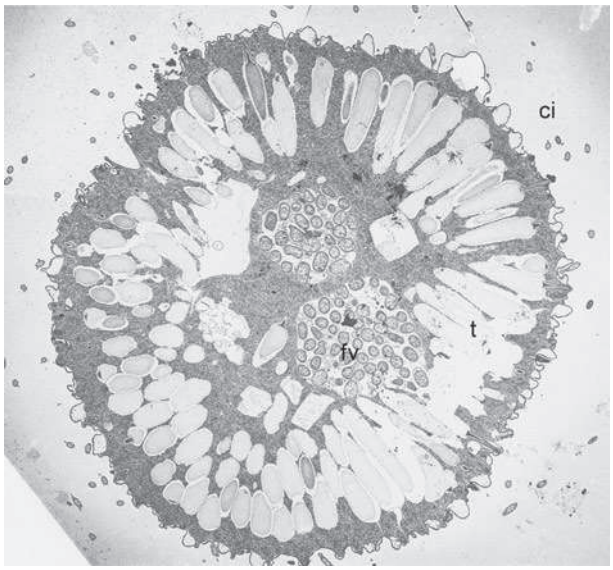

Figure 3.3 Low power EM micrograph of a cross-section through a *P. tetraurelia* cell, surrounded by many cross-sectioned cilia (ci). The cell contains numerous dense-core secretory organelles, called trichocysts (t), of which mainly the carrot-shaped, electron-translucent main "body" is seen and much less frequently the rod-shaped, electron-dense tip. By this tip, trichocysts are attached at the cell membrane, ready for discharge by exocytosis upon stimulation. Also note the occurrence of two food vacuoles (fv), that is, phagosomes, containing many bacteria for digestion. Magnification 2700×.

2.1.3 Chlamydomonas *(Chlorophyta, Volvocales)*

Chlamydomonas (Chlorophyta, Volvocales) is a model for microtubule assembly as well as for biogenesis and activity of flagella—a starting point for the analysis of human syndromes related to ciliary or flagellar deficiencies (Snell et al., 2004).

2.1.4 Yeast (Fungi, Ascomycota)

Yeast, mostly *Saccharomyces cerevisiae* (Fungi, Ascomycota), represents the most important model for many aspects of cell biology. Its dominating role as a unicellular model makes it unnecessary, even impossible, to elaborate here on all aspects. Particular advantages are a greatly reduced genome and the ease of identifying mutations in haploid cultures (Section 5.3). Earlier, yeast was the dominating model for the biogenesis of mitochondria (Section 3.1). In the past two decades, it became significant for detecting basic aspects of intra cellular trafficking, cell growth and polarization (Pruyne and Bretscher, 2000), cell division, signaling, and aging (Klipp, 2007). Notably the targeting

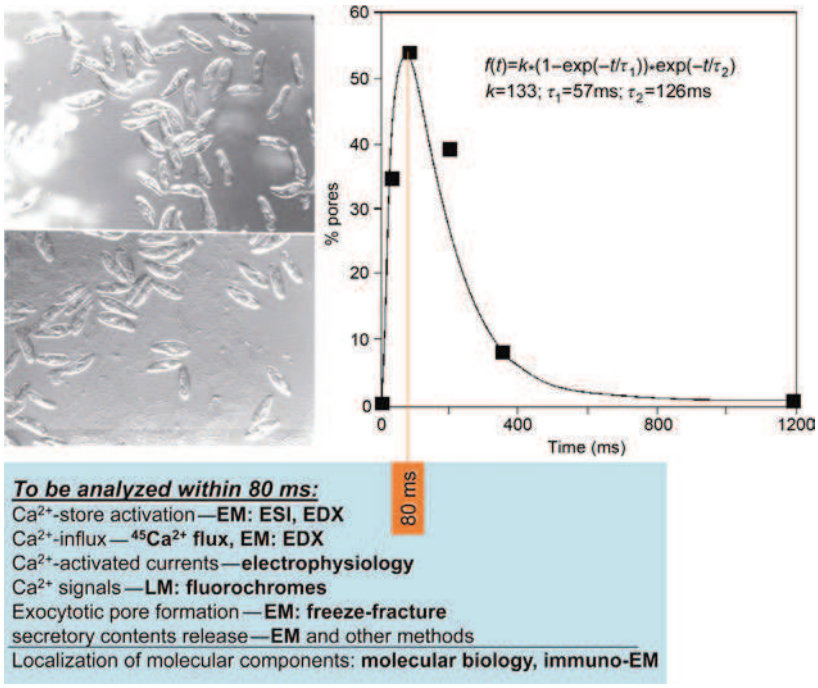

Figure 3.4 Live paramecia before (top left) and after (middle left) induction of synchronous exocytosis of trichocysts. Due to decondensation (stretching) of the trichocyst body contents they become visible as needles in the medium. Right: Time-resolved analysis of exo-/endocytosis coupling, analyzed by evaluation of freeze-fracture replicas after fast freezing at different time points during synchronous exocytosis stimulation, using a quenched-flow apparatus. The number of exocytotic openings (pores) increases up to 80 ms and then decreases due to exocytosis-coupled endocytosis (membrane resealing). The apparent time constants (τ) for exo- and endocytosis, respectively, are calculated for all events in all cells thus analyzed, whereas individual events take much less time. The bottom part summarizes methods combined for a timed analysis (example: within 80 ms exocytosis only), for example, of Ca^{2+} signaling by ESI (electron spectroscopic imaging) and EDX (energy-dispersive X-ray microanalysis), complemented by Ca^{2+}-flux measurements and fast Ca^{2+} imaging, also within the short time during which synchronous exocytosis takes place. Light micrographs are from Plattner et al. (1984), the time course, based on experiments by Knoll et al. (1991), has been calculated by Plattner et al. (1992).

function of low molecular mass/monomeric G proteins (GTP binding proteins, small GTPases), though detected in mammalian CHO cells (Melançon et al., 1987), has been scrutinized with *S. cerevisiae*, for example, in the work of Novick and associates (Mizuno Yamasaki et al., 2012). They

also analyzed the relevance of GTPase modulating proteins (GAP, GEF) (Grosshans et al., 2006; Mizuno Yamasaki et al., 2012) and of exocyst components for membrane tethering (TerBush et al., 1996) occurring before membrane fusion can take place. A decisive advantage of yeast is its small genome and easy to handle mutational work. *S. cerevisiae* as a model has brought about much important work. This includes *Schizosaccharomyces pombe* for seminal discoveries pertinent to the regulation of epigenetic phenomena (Section 4.2.2).

Any work with model cells also calls for comparison with "normal" cells up to humans. So, why not start with human cells right away? At the end of this article, we shall try to give an answer to this question that currently becomes increasingly important, not only directly for science per se but also indirectly for fund raising (see Section 1).

In the pregenomic era, mutants, spontaneous or induced, substantially contributed to the usefulness of different protozoan models. Such efforts with *Paramecium* and *Dictyostelium* were summarized by Vayssié et al. (2000) and Noegel and Schleicher (2000), respectively. Basic features of eukaryotic cells have thus been unraveled. In subsequent steps, mutants were repaired by injection of the gene (or occasionally of the protein) under consideration. In practice, in such complementation cloning experiments, sets of genes available from indexed libraries were microinjected. This eventually resulted in the identification of genes relevant for defined functions, for example, in *Paramecium* (Keller and Cohen, 2000).

2.2. Unicellular models: Examples, pitfals, and perspectives

Surprisingly lower eukaryotes, such as choanoflagellates and slime molds, possess precursors of cell adhesion molecules (King et al., 2003, 2008; Shalchian Tabrizi et al., 2008). With choanoflagellates this also includes signal transmission by changing the phosphorylation state of tyrosine residues—a crucial aspect of cell integration into tissues (Mayer, 2008; Miller, 2012; Shalchian Tabrizi et al., 2008) up to humans, with dis regulation phenomena in cancer. In a recent congress report, Carpenter (2012) summarized work by King showing that some bacteria drive the formation of multicellular aggregates of choanoflagellates—a new paradigm for an important evolutionary step. Already several generations ago some biologists propagated choanoflagellates as precursors of metazoans. This is one of these fascinating old hypotheses which now become increasingly supported by molecular biology (other examples are the symbiotic bacterial origin of

chloroplasts and mitochondria). In *Dictyostelium*, a small molecular com pound (cyclic di [3′:5′] guanosine monophosphate) was identified as the agent that causes amoebae to aggregate to a multicellular "stalk" (Chen and Schaap, 2012). All this shows old models in new light and their ongoing validity as model systems.

There are many other examples of the elucidation of signal transduction in unicellular models. As generally known, Ca^{2+} is a universal second mes senger and mediator of membrane–membrane interactions. Ca^{2+} may come from the outside medium or from internal stores (Berridge et al., 2003; Laude and Simpson, 2009; Petersen et al., 2005). In the field of Ca^{2+} sig naling, there exist many examples supporting the value of such models. This is true specifically for ciliates, particularly because of the easy applicability of conventional electrophysiology. Originally the low capacity/high affinity type of cytoplasmic Ca^{2+} binding protein, calmodulin, was found in mam malian brain and testes (Cheung, 1980), but when it was detected in proto zoa some crucial observations were made: Calmodulin in a complex with Ca^{2+} activates a variety of plasmalemmal cation channels (Ehrlich et al., 1988; Saimi and Kung, 2002), but it shuts down conductivity of voltage dependent Ca^{2+} influx channels in the ciliary membrane (Brehm and Eckert, 1978; Saimi and Kung, 2002). Such negative feedback of sub plasmalemmal Ca^{2+} had been searched for in brain cells. Only after the dis covery of this phenomenon in *Paramecium*, the same inhibitory mechanism, based on a Ca^{2+}/calmodulin complex, was found also in neuronal cells (Levitan, 1999; Xia et al., 1998). Recently an additional regulator for the inactivation of such channels has been found in brain neurons, that is, Ca^{2+} binding protein 1, which acts in competition with calmodulin (Oz et al., 2013).

Other molecules crucial for the brain function and immune defense, such as calcineurin (protein phosphatase 2B, PP2B; Klee et al., 1998), surprisingly occur in protozoa. Interestingly plants (Angiosperms) only express the B subunit as part of the stress tolerance machinery (Gu et al., 2008). This exemplifies why plant cells are less suitable as general models in cell biology. The occurrence of both, the catalytic and regulatory subunits was well established for protozoa, including parasitic Apicomplexa and free living cil iates (Fraga et al., 2010). Experiments with *Paramecium* taught us its involve ment in exocytosis (Momayezi et al., 1987) and this aspect has been followed up subsequently up to man. However, this may encompass widely different aspects up to fusion pore expansion (Samasilp et al., 2012) and ensuing exocytosis coupled endocytosis of empty "ghosts" (Lai et al., 1999).

Evidently such basic mechanisms are conserved from protozoa to man where the activity of calcineurin culminates in long term potentiation, that is, learning (Mulkey et al., 1994), and immune defense via activation of tran scription factor NFAT in T cells (Bueno et al., 2002). However, as the func tion of calcineurin is not yet fully understood in any system, further experiments with protists may yield important clues.

Considering the large number of genes, a cell is a complicated puzzle indeed. With ongoing evolution, the increase in the number of protein encoding genes is surprisingly moderate. Which advantages can protozoa offer along these lines? In higher eukaryotes, alternative splicing can generate many more protein forms which can also help to fit building blocks more precisely and flexibly into the 4D puzzle. Moreover, different posttransla tional modifications can increase the complexity of a cell. Estimates of the average number of splice variants in mammalian cells range from three to seven per transcript. In contrast, alternative splicing is almost absent in ciliates, for example, *Paramecium* (Jaillon et al., 2008). Rare examples are certain types of intracellular Ca^{2+} release channels (CRCs) such as *Pt*CRC VI 3, but here this may indicate a pathway to pseudogenization (Ladenburger and Plattner, 2011). In principle, the general absence of alter native splicing in ciliates can facilitate analyses of function and intracellular localization. This advantage may be canceled whenever there occurred whole genome duplications; these can result in a number of similar paralogs (also called ohnologs) as described below. In this regard, *Tetrahymena* is more favorable than *Paramecium*. Posttranslational modifications can be manifold also in protozoan cells. An example is glycination and acetylation of tubulin. This results in topologic diversification, that is specific microtubule subpop ulations achieve specific subcellular localization and stability in *Paramecium* as well as in *Tetrahymena* (Adoutte et al., 1991; Libusová and Dráber, 2006; Wloga and Gaertig, 2010).

In *Paramecium*, the plethora of extensive gene families encoding many protein isoforms complicates the situation. These cells, in contrast to *Tetra-hymena*, have numerous paralogs because of several rounds of whole genome duplications (Aury et al., 2006). Such paralogs/ohnologs are known from many *Paramecium* gene families. Examples are not only tubulin (Dutcher, 2001) but also actin, with differential positioning of isoforms in the cell (Sehring et al., 2007a,b). This special situation makes the analysis of some aspects with this model rather intriguing. However, in these cells, closely related paralogs from whole genome duplications can also provide access to the analysis of further specification of protein molecules. This can then

become an alternative to alternative splicing. An example is the neo functionalization of η tubulin which may contribute to epigenetically con trolled surface structuring (Ruiz et al., 2000). Thus, some models may entail specific problems but also open up new perspectives.

While this situation shows specific aspects of, and problems with some models, this can be a chance for evolutionary studies. Moreover, it is possible to switch from *Paramecium* to a related model with less paralogs, such as *Tetrahymena* although this cell is less easily amenable to microinjection and electrophysiology. The same is true of yeast, with the advantage of still fewer genes. Thus, one has to find the proper balance between advantages and disadvantages to select the proper model for a specific problem. A model should have a special feature that makes it suitable for the analysis of a specific aspect, thus following the postulate of the founding father of genetics, Wil liam Bateson, about 100 years ago: "*Foster your exceptions*." Toward the end of Bateson's life, Morgan established *Drosophila* as the most successful model in classical genetics. So, there is no one model for everything and different models should be available for different problems.

Let us now illustrate the potential of unicellular organisms as models by some of the most recent and dramatic discoveries in cell biology. Due to the special trait of chromosome fragmentation, *Tetrahymena* has allowed for the discovery of ribozymes, that is, catalytic self splicing RNA (Kruger et al., 1982). This resulted in a Nobel Prize in 1989. Similarly it stimulated the recognition of telomeres and telomerase (Blackburn, 2010; Blackburn and Gall, 1978; Greider and Blackburn, 1985) honored with the 2009 Nobel Prize. All this was based on the knowledge of extensive chromo some fragmentation which greatly increases the number of telomeres in cil iates such as *Tetrahymena* (Katzen et al., 1981). Recently this advantage has facilitated the elucidation of the molecular structure of the telomerase holoenzyme (Jiang et al., 2013). Fragmentation of chromosomes is now known to be even much more abundant in the ciliate *Oxytricha* (Swart et al., 2013) which, thus, would be an even better model. Currently reg ulation of epigenetic inheritance in ciliates is an example of rising interest (Section 4).

3. UNICELLULAR MODELS FOR ORGANELLE BIOGENESIS

Unicellular model cells have substantially contributed to our under standing of the biogenesis of different organelles.

3.1. Biogenesis of mitochondria in yeast

Mitochondria are not formed de novo, but this has not always been so clear. In *S. cerevisiae* cells, under anaerobic growth or glucose repression, mito chondria are no more seen in the electron microscope. On this basis some groups advocated for de novo biogenesis. Remarkably mtDNA persists whereas a number of protein components disappear. Since disappearance of these structures can be reversed by aeration the dispute arose about de novo biogenesis versus de /redifferentiation of mitochondria—the first encounter of the senior author with a model system. With appropriate prep aration conditions for EM analysis and using radioactive label transfer exper iments it became clear that mitochondria persisted as structural entities (called promitochondria by Schatz and coworkers), ready for redifferentiation. Although there were claims of similar de / redifferentiation also in liver cells, this was an inappropriate extension of the model, yeast. The exciting analyses using yeast as a model were recently summarized by Schatz (2012).

3.2. Biogenesis of secretory organelles, cilia, and flagella

An average mammalian cell expresses about 10,000 or more different pro teins, generally between several hundred to over 1000 in one organelle. This is true, for instance, for cilia and flagella. In other organelles the number may be smaller, as is the case with dense core secretory organelles. In any case, any analyses are greatly facilitated in cells that possess large numbers of the respective organelle. Figures 3.1 and 3.3 give examples relevant for cilia and trichocysts in *Paramecium*. Moreover, stimulation of synchronous exocytosis (which is possible, for instance, with ciliates) induces increased transcription of a much larger number of genes of which only some are in overt connec tion with secretory function (Haddad and Turkewitz, 1997). The demon strated link between stimulated secretion and increased transcriptional activity, though not fully elucidated up to our days, was well worth an edi torial in PNAS (Hutton, 1997). In a detailed analysis, a link between syn chronous secretion and transcription, as well as induction of autogamy and reciliation after experimental deciliation was also found in *Paramecium* (Arnaiz et al., 2010). Since only some of the genes induced can be seen in line with organelle biogenesis and function, all of the other genes with unknown functional connection or even of unknown function altogether deserve even more interest for innovative research in the future. The advan tage of such models for biogenetic studies is evidently founded on the high numbers of organelles (cilia) and/or synchrony of events (exocytosis).

Concomitantly, colleagues interested in the biogenesis of cilia and flagella in mammals assign a valuable role to ciliates and to *Chlamydomonas*, respec tively, for studying the molecular background of organelle biogenesis (Vincensini et al., 2011). Despite their stereotypical construction principle, cilia and flagella from different organisms, including protists, show some var iations in ultrastructural details (Fisch and Dupuis Williams, 2011). Their molecular background, though not always known, may make them an inter esting resource for molecular analysis up to mammals.

Considering the conceptual problems concerning the biogenesis of the centriole, a model with many such organelles was looked for. At the end of the nineteenth century, practically at the same time, both, Henneguy and Lenhossek formulated independently the hypothesis that centrioles are equivalents of ciliary/flagellar basal bodies and that one can be transformed into the other (Caullery, 1941). Margulis (1993) fostered the idea of a sym biotic origin also of this organelle. Then, there was some back and forth considering organellar DNA mediated (Hall et al., 1989) and de novo for mation, respectively. After alledged proof of the presence or DNA in basal bodies, the presence of organellar DNA was experimentally disproved shortly afterward (Johnson and Rosenbaum, 1990). The arguments were conducted mainly with *Chlamydomonas* (Chapman, 1998).

Even recent reviews assign to flagellates and ciliates a key role with great potential for the exploration of centrioles (Gönczy, 2012) and basal bodies (Pearson and Winey, 2009). Recently the Henneguy/Lenhossek hypothesis received further support by the demonstration that the conserved centrosomal protein FOR20 is required for the assembly of the transition zone, for basal body docking at the cell membrane and for ciliogenesis in *Paramecium* (Aubusson Fleury et al., 2012), as it is during primary cilium assembly in man (Sedjaï et al., 2010). Clearly, due to sheer numbers, details can be analyzed more easily in a ciliated protozoan. Evidently research on centrioles/centrosomes is important also for the understanding of cancer cells because of their ongoing involvement in mitotic activity. On the other hand, many organisms, including protists such as *Paramecium* (Sections 1 and 4.3), can live without a centrosome at the polar spindle (Bettencourt Dias, 2013), thus offering a promising basis for scrutiny of molecular details pertinent to its function.

3.3. Phagocytotic pathway

More than 100 years ago, phagocytosis and intracellular digestion were detected in phagocytes of starfish larvae by Élie Metchnikoff who pointed

out the similarity with neutrophil granulocytes. A model frequently used over decades, even before its genome analysis had been completed, was *Dictyostelium*, as summarized by Gerisch et al. (1999), Chisholm and Firtel (2004), and Williams et al. (2005). Here, the involvement of actin with all its facets was explored. This was not easy with the other frequently used models, *Paramecium* (Allen and Fok, 2000) and *Tetrahymena*. It became evi dent only recently that in *Paramecium* actin is represented by a complicated collection of paralogs/ohnologs with partially aberrant binding of diagnostic drugs such as phalloidin (Plattner et al., 2009; Sehring et al., 2007a). This may require reinvestigation of some aspects, not only of phagocytosis. Remarkably, in *Paramecium* phagosomes (food vacuoles) change their coat of actin isoforms during cyclosis (Sehring et al., 2007b).

In their consistent and profound work with *Paramecium*, Allen and Fok (2000) developed new seminal ideas. For instance, the nascent phagosome membrane is formed largely by vesicles recycling from maturing and spent phagosomes, whereas in mammalian cells the impression has prevailed for some time that phagocytosis involves primarily the invagination of the cell membrane (see Section 3.4 for more details).

A general drawback of lower eukaryotes was and—concerning topolog ical information—still is to a certain extent the lack of a comprehensive set of markers, such as small GTPases (Section 5.3), for identifying different sources of membrane input. This refers to different types and stages of endo somes and lysosomes. To overcome this problem, before molecular biology became available for ciliates, Fok et al. (1986) introduced monoclonal anti bodies for such identification.

In retrospect, work with protozoan models has been of paramount importance for the analysis of phagocytosis. Such work still is of particular interest for intracellular parasitic protozoa. Moreover, work with *Amoeba proteus* has allowed for tracing a most important evolutionary step, the "domestication" of phagocytosed bacteria to indispensable symbionts whereby both become mutually dependent on each other (Jeon, 1995, 2004). At this point, the question arises whether pathogenic bacteria may once decide to select a similar pathway. In this context, and knowing that free living amoebae can harbor the strong pathogen *Legionella pneumophila*, analyses with such models appear also of great practical value. As recently found with *Dictyostelium*, phagocytotic ingestion of bacteria can be selective, for example, by distinction between Gram negative and Gram positive forms (Nasser et al., 2013; Snyder, 2013).

3.4. Qualifying for model system by precise timing

With ciliates it is not only the sheer number of regularly arranged organelles but also the possibility to synchronize culture growth and biogenesis of organelles as well as synchronous performance of different processes, which qualifies them as models.

3.4.1 Cilia

Amplified reciliation after radical deciliation and secretory organelle biogen esis after massive exocytosis stimulation, respectively, as analyzed in *Paramecium* by Arnaiz et al. (2010), enable the analysis of systemic feedback between actual and setpoint values of organelle components. In fact, it is not known how a cell senses the loss of its cilia or secretory vesicles. Such cells then can be used as model systems by virtue of the particular advantage of redundancy of components and/or of synchrony effects. The possibility to synchronize cultures of *Tetrahymena* (opposite to *Paramecium*) allowed for the microarray analysis of gene expression during its life cycle (Miao et al., 2009). 1068 predicted genes are specifically expressed and 1753 are significantly upregulated during sexual activity (conjugation). This would hardly be accessible to any of the "higher" eukaryotic cells.

3.4.2 Exocytosis and exo-/endocytosis coupling

Ciliates are favorable objects to study stimulated exocytosis. When com pared with mammalian cells (Kasai, 1999) *Paramecium* shows the highest degree of synchrony (80 ms) ever observed with stimulated dense core secretory organelle exocytosis and almost 100% efficiency (Plattner and Hentschel, 2006; Plattner and Kissmehl, 2003; Plattner et al., 1984). This is an unsurpassed chance for studying the secretory cycle with diverse methods (Fig. 3.4). The presence of integral and peripheral proteins, their rearrangement during fusion, protein mediated fusion of the focal type in the millisecond time range was thus observed from about 1980 on (Plattner, 1981), as summarized by Plattner and Hentschel (2006). This was also achieved with patch clamp electrophysiology which allows for sin gle event analyses, even with higher resolution, in mammalian cells (Neher and Marty, 1982). As explained in Fig. 3.5, special structural and functional features, that is, regular design and synchronous activity profiles, have made *Paramecium* appropriate to serve as a model system following Bateson's pos tulate "*foster your exceptions*." Nevertheless it has not been sure whether

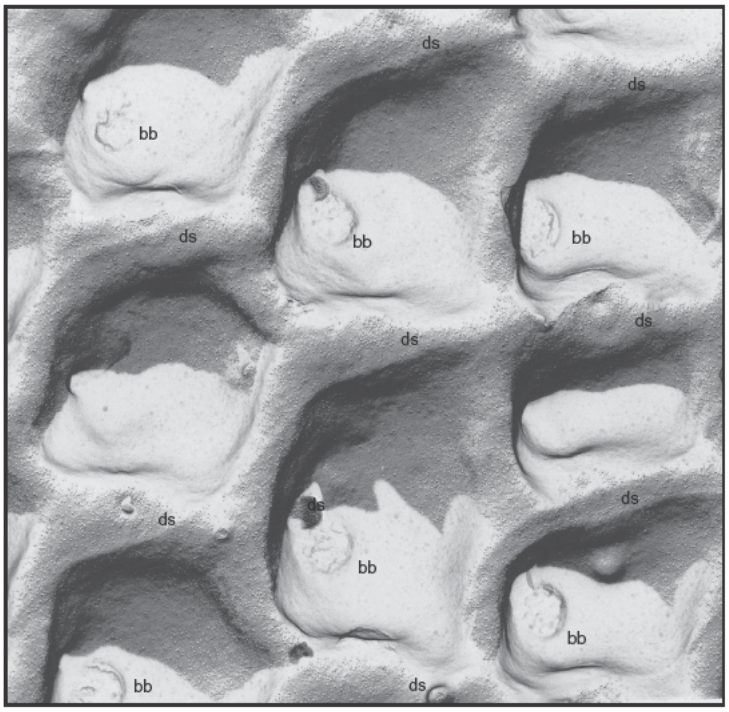

Figure 3.5 Detail from a freeze-fractured *Paramecium* cell membrane showing three kineties separated by longitudinal (vertical) ridges. Note perpendicular ridges (horizontal) between kinetids and linear arrangement of ciliary basal bodies (bb) and of trichocyst docking sites (ds), that is, preformed exocytosis sites recognizable by arrays of intramembranous particles/proteins. As described in the text, synchronous exocytosis stimulation in conjunction with quenched-flow/fast freezing greatly facilitated the timed analysis of structural changes occurring in the cell membrane during exocytosis- and exocytosis-coupled endocytosis. Magnification 20,000×.

SNARE (*s*oluble *N* ethylmaleimide *a*ttachment protein [SNAP] *re*ceptor) proteins—a general prerequisite for membrane fusion (Jahn and Scheller, 2006)—would be present at the level of protozoa (Hutton, 1997). Here, they have been characterized only with some delay (Plattner, 2010a,b).

In most cases, endocytosis is coupled to exocytosis. That this coupling works very fast, within a second time range, was shown first with *Paramecium* (Knoll et al., 1991; Plattner et al., 1992; see Fig. 3.4). Estimates using exog enous tracers, added to mammalian dense core secretory systems to follow retrieval of empty "ghost" membranes, were by several orders of magnitude

too slow, as summarized by Plattner and Hentschel (2006). At that time, patch clamp electrophysiology could not yet tackle the problem of exo / endocytosis coupling; for technical reasons this became possible several years later (Henkel and Almers, 1996; Rosenboom and Lindau, 1994).

3.4.3 Phagocytotic cycle

Another case showing the advantage of synchrony, though at a much slower time scale, is phagocytosis. *Paramecium* cells engulf bacteria for digestion in a defined 20–40 min cycle (depending on species and strain), when the phagosome cycles through the cell (cyclosis), as summarized by Allen and Fok (2000). As outlined in Section 3.3, the invagination of the cell membrane at the cytopharynx is mediated by membrane material recycling from different phagosomal stages. Subsequently the nascent phagosome is detached from the cytopharynx and this entails, after a delay of ~1 min, fusion of acidosomes. These represent late endosomes (Fok and Allen, 1993) and provide the phagosome with H^+ ATPase. This in turn is required to inactivate food bacteria and to activate a great part of the lysosomal enzymes which have an acidic pH optimum of different degree. During cyclosis, pH rises and parts of the membrane and of the lysosomal enzymes are retrieved for reuse. At that period of research, variable membrane components were identified by monoclonal antibodies and localized at the light and electron microscope levels (LM, EM).

In *Tetrahymena*, Vosskühler and Tiedtke (1993) elaborated a method for the isolation of phagosomes with precise timing by feeding magnetobeads. A thorough mass spectroscopic identification of organellar proteins was achieved with phagosomes, first from macrophages (Garin et al., 2001) and later from *Tetrahymena* (Jacobs et al., 2006). In either case, roughly 100 putative organellar proteins were identified. A time resolved assignment of stage and input specific small GTPases or other markers would greatly facilitate further analysis of details of the trafficking pathways which cooperate during phagosome formation and maturation. In this regard, ciliates still lag behind other cells.

3.5. Free-living forms as models for pathogenic forms

The cell membrane of Alveolata is covered with densely packed variant surface antigens (vsAG). This holds for free living and pathogenic ciliates (the fish pathogen *Ichthyophthirius*), Apicomplexa as well as for pathogenic flagellates. Apicomplexa encompass severe pathogens, such as *Toxoplasma*

(potentially teratogenic) and *Plasmodium* (the malaria causing agent). *Leishmania* and *Trypanosoma* are examples of pathogenic flagellate genera causing different types of leishmaniosis and of trypanosomiasis, respectively, such as African sleeping disease (*T. brucei*) and Chagas disease (*T. cruzi*). All these protozoa can spontaneously and rapidly shed their vsAGs so that, in the case of parasites, the host's immune system cannot produce antibodies rapidly enough to cope with this molecular camouflage. The vsAG systems of nearly all pathogens and also of free living ciliates and of fungi have in common that only one vsAG is expressed at a time (called the serotype) whereas other members of the multigene family are silent (Deitsch et al., 2009). Since Apicomplexa are close relatives of ciliates, their vsAG proteins and their expression mechanisms reveal several similarities which legitimate a comparison of the antigenic systems between free living and pathogenic forms (Simon and Schmidt, 2007).

In many cases, surface proteins are attached to the cell surface by a glycosylphosphatidyl inositol (GPI) anchor, from protozoa and yeast up to flowering plants and man (Eisenhaber et al., 2003; Ferguson, 1999; Orlean and Menon, 2007). In *Paramecium*, the switch to a newly expressed vsAG requires removal of the old one which is cleaved off by a GPI specific phospholipase C (PLC) and subsequently released into the medium, as demonstrated in Fig. 3.6 (Klöppel et al., 2009; Müller et al., 2012). Therefore, expression of a pure new serotype specific vsAG requires not only a complex mechanism of exclusive gene expression, as discussed below in Section 4.2.3, but also an active release mechanisms and, to start with, a complex mechanism for surface attachment. Such release mechanisms by cleavage of GPI anchors are not understood in depth in any of the different organisms. This is surprising especially since similar mechanisms have to be postulated even for mammals (Simon and Kusch, 2013).

It now appears of paramount importance to scrutinize the molecular background of all regulation steps involved in vsAG expression in free living unicellular model systems. Here, its analysis is much easier to achieve than in parasites or in multicellular organisms.

4. MODELS FOR EPIGENETIC PHENOMENA

Here, Darwinism meets Lamarckism. There is growing knowledge that phenotypes of cells and organisms can change epigenetically. Epigenetic inheritance means the transgenerational transfer of information which is not encoded by DNA (nonchromosomal inheritance). Examples of such

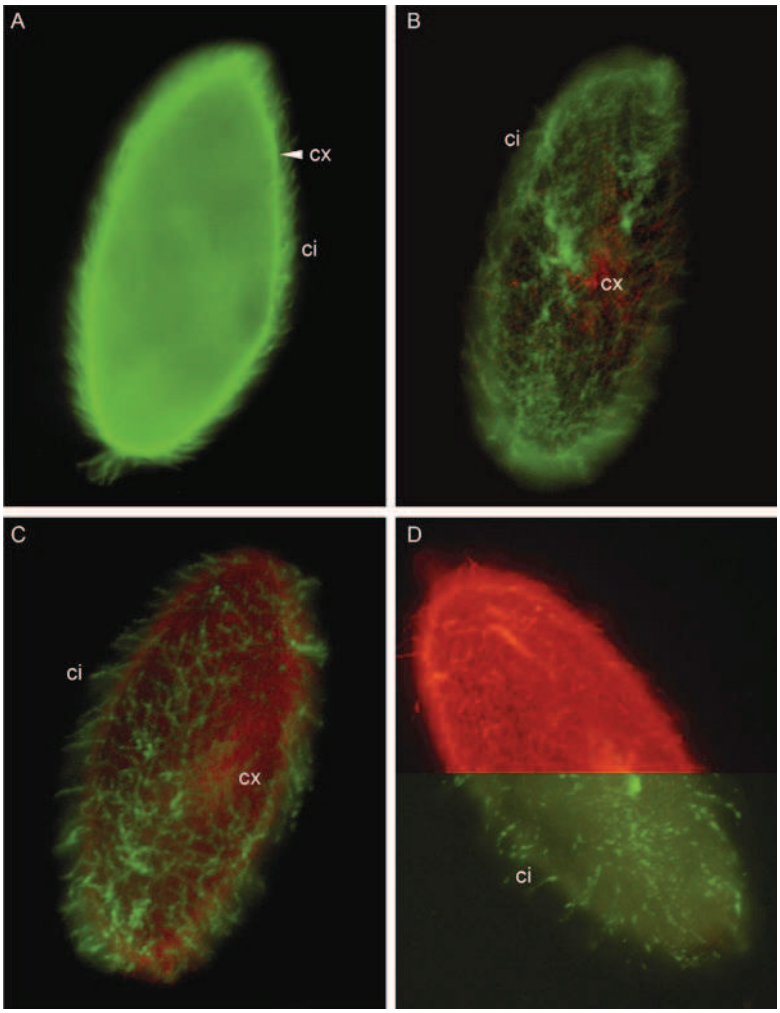

Figure 3.6 Immunolocalization of variable surface antigens on *P. tetraurelia* cells. (A) Shows a cell expressing pure serotype 51A. Specific antibodies indicate the presence of the antigen on cilia (ci) and on the cortex (cx), that is, the nonciliary cell membrane. (B–D) A switch from serotype 51A (green fluorescence) to 51D (red fluorescence) has been induced via RNAi by experimental suppression of the old antigen, 51A. The new surface antigen can first be detected on the cortex membrane whereas the "old" antigen remains on the cilia, as shown in (B and C). In a late stage of serotype switching (D), some residual 51A antigen is detectable only on the tips of some cilia (lower half of the cell), whereas the upper part displays the new serotype (red) on cilia and the nonciliary cell membrane (cortex). Magnification 800 ×. *Figures (B–D) are from Simon et al. (2006).*

non Mendelian inheritance have been reported first from unicellular organ isms since it is rather easy to phenotypically characterize genetically identical cells when they have short generation times. As we begin to understand these mechanisms at the molecular level, we try to give an overview of current achievements with unicellular eukaryotes and their implications for our current understanding of inheritance, adaptation, and evolution.

Ciliates have a nuclear dimorphism. During evolution, they decided to split germline and soma into different nuclei, in spite of being unicellular organisms. As a result, germline (micronuclei) and somatic line (macronu cleus) exist in the same cytoplasm in one single cell (Fig. 3.7). The

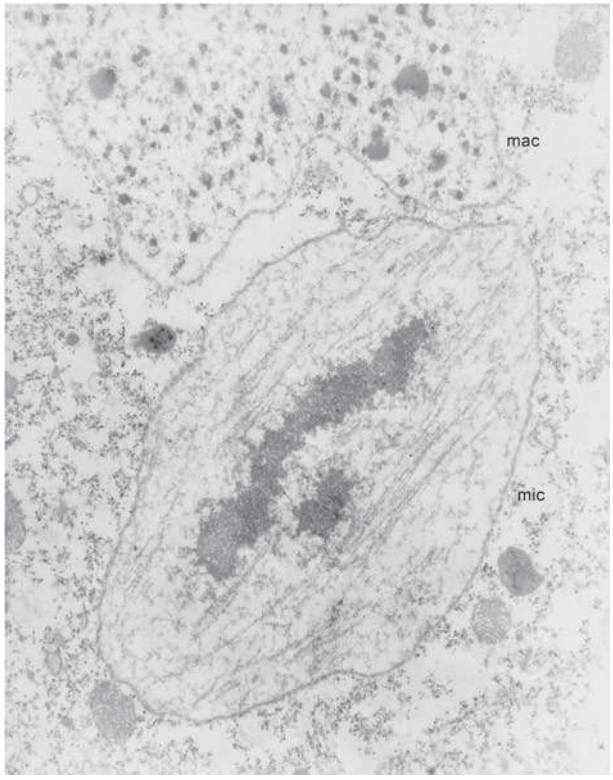

Figure 3.7 Portrait of a micronucleus (mic) side-by-side with a macronucleus (mac) of which only a small part is visible. Note the numerous small heterochromatic patches in the transcriptionally active macronucleus. Also note the condensed chromosomes, flanked by spindle microtubules, in the micronucleus undergoing division in the presence of an intact nuclear envelope (closed mitosis) and in the absence of a centriole. Magnification 14,000 ×.

micronucleus is diploid and transcriptionally inactive; the macronucleus (~800 fold polyploid in *Paramecium* and ~45 fold polyploid in *Tetrahymena*) is transcriptionally active, thus providing plenty of transcripts for vegetative functions and growth.

4.1. Epigenetic phenomena from molecules to ultrastructure

An exciting example is the so called "structural inheritance" when proteins are assembled or folded in a new conformation according to the scaffolding effect of another protein, for example, of a misfolded form of the same trans lation product.

4.1.1 Prion proteins

The most popular form of epigenetically determined structures is represen ted by prion proteins. The normal cellular form, PrP^{c}, can be folded according to the misfolded form, PrP^{sc}, whenever present. This causes the scrapie disease in sheep. However, Prions have been detected in fungi long before this novel concept of nongenetic inheritance became practically important for veterinary and human pathology.

In her early work, Beisson detected extrachromosomal inheritance in the filamentous fungus *Podospora anserina* (Beisson, 2008; Beisson Schecroun, 1962). The discovery and definition of the novel concept of a prion protein, called [Het s] in *Podospora*, was put into the limelight in a monograph by Prusiner (2004). He was honored by a Nobel Prize in 1997 for unraveling mammalian prion protein in its misfolded conforma tion, PrP^{sc}, as a source of transmissible spongiform encephalopathia causing the "mad cow disease" syndrome (Prusiner, 1982) or, in man, a syndrome similar to the Creutzfeldt Jakob disease. These pathogenic effects are now attributed not to genetically, but rather to epigenetically encoded phenom ena. Prusiner (2004) cites Beisson's pioneering work: "*The thorough physiological and genetic investigation on* [*Het c*] *performed by George Rizet and Jeanine Beisson in the 1950s and 1960s indicated that* [*Het c*] *is a cytoplasmic infectious element.*" Thus, the discovery of prions in a unicellular model laid the basis for medical aspects which became fully appreciated, however, only several decades later.

In the past few years, *Saccharomyces* has become attractive for the study of phenotypic inheritance of prion proteins (Halfmann et al., 2012) and of underlying mechanisms (Liebman and Chernoff, 2012).

4.1.2 Cell-surface structure

Apart from the practical importance of the prion concept, until recently epi genetic effects have been underestimated. Currently the appreciation of work with lower eukaryotes, including ciliates, greatly increases (Sperling, 2011).

Beisson pursued this novel line of research with *Paramecium*, whose cor tex structure is epigenetically determined, first with Sonneborn (Beisson and Sonneborn, 1965) and later with members of her group and with Jerka Dziadosz (Beisson and Jerka Dziadosz, 1999). In ciliates, surface structures are arranged with extreme regularity. In *P. tetraurelia*, about 4000 unit fields, kinetids, are arranged in longitudinal rows (kineties), as shown in Figs. 3.2 and 3.5. The unit fields, several μm^2 in size, are visible in the light micro scope where kinetids are delineated by longitudinal and perpendicular ridges. From the slightly depressed center of each kinetid, one cilium emerges, rarely two. Alternating with ciliary bases trichocysts are docked, with membrane specializations recognizable in freeze fracture replicas (Plattner et al., 1973).

How is this regular design determined? Originally epigenetic regulation of surface pattern was demonstrated in *Euplotes* by the following observation (Frankel, 1973). When conjugants prematurely divide they may carry along kinetids from the prospective partner exconjugant and then retain and trans mit them in the partner's (wrong) orientation. Such nonsurgically produced transplants have their counterparts in *Paramecium* where kinetids transplanted in the wrong orientation are also transmitted in the artificially produced ori entation. Work along these lines is summarized by Beisson (2008) and in a book entitled "Protein based inheritance" (Chernoff, 2007). Though less evident, epigenetic cell surface determination appears relevant up to mam malian cells and tissues (Marshall, 2011) and may play a role in evolutionary adaptation (Soto, 2012). This concept has been anticipated by the analysis of unicellular organisms.

Which epigenetic determinants stay behind such incomparable regular ity? Several gene silencing studies resulted in aberrant cell shape and surface pattern and, thus, pointed out several potential key players. In ciliates, epigenetic determinants of cortex structure may act by the cooperative effect of several cortical proteins. In *Paramecium*, this includes specific centrin isoforms (Gogendeau et al., 2008). Centrin is a widely distributed Ca^{2+} binding protein endowed with several low capacity/high affinity Ca^{2+} binding sites of the EF hand loop type, and in addition with less orga nized acidic residues serving for high capacity/low affinity Ca^{2+} binding.

This is also true of ciliates, as shown with *Tetrahymena* where distinct domains exert distinct functions in basal bodies (Vonderfecht et al., 2011). In *Paramecium*, the manipulation of centrin (Ruiz et al., 2005) or of η tubulin (Ruiz et al., 2000) causes structural changes in the cell cortex. The shape of a *Paramecium* cell also changes in consequence of silencing cor tical actin4 (Sehring et al., 2010) or of one of the SNARE proteins, Syb6 (Schilde et al., 2006). Surely, the cortex of ciliates is a highly specialized example—but just therefore it may be a Rosetta stone for analyzing the epi genetic control of self assembly processes.

In *Xenopus* embryos, centrin is now recognized as a regulator of basal body assembly, together with cortical actin (Werner et al., 2011). The latter was difficult to ascertain for ciliates because the usual LM visualization with fluorescent phalloidin does not work due to aberrant drug binding (Sehring et al., 2007a). This situation may serve as another example of the interactive progress achieved with "model" and "normal" cells, that is, lower and higher eukaryotes. As to ciliates, the case of actin also exemplifies specific experimental difficulties which may arise with lower eukaryotes. In sum mary, it looks from work with protozoa that several proteins may cooper atively regulate surface structure formation in an epigenetic process.

4.2. Models for RNA-mediated epigenetic phenomena

The structural inheritance discussed above represents an example where a preexisting protein structure controls the *de novo* assembly of new structures. Clearly, the information about the arrangement of proteins and organelles is not encoded directly by DNA and, therefore, the information about the assembled structure is derived from an epigenetic signal. After detection of this kind of protein based inheritance, the field of epigenetics advanced within the past decade to one of the most important areas of life sciences.

During the past 15 years, another substance, RNA has been recognized as an additional class of epigenetic transfer molecule and this discovery was again heavily supported by work with ciliates. By the characterization of different classes of previously unknown RNA species, we are able to answer questions related to epigenetic phenomena which were asked decades before even the term "gene" was defined. But what is the advantage of ciliates in epigenetic research? To answer this question, we summarize the molecular mechanisms behind transgenerational RNA in the following sections. Figure 3.8 outlines the mechanisms behind the problems to be sub sequently discussed.

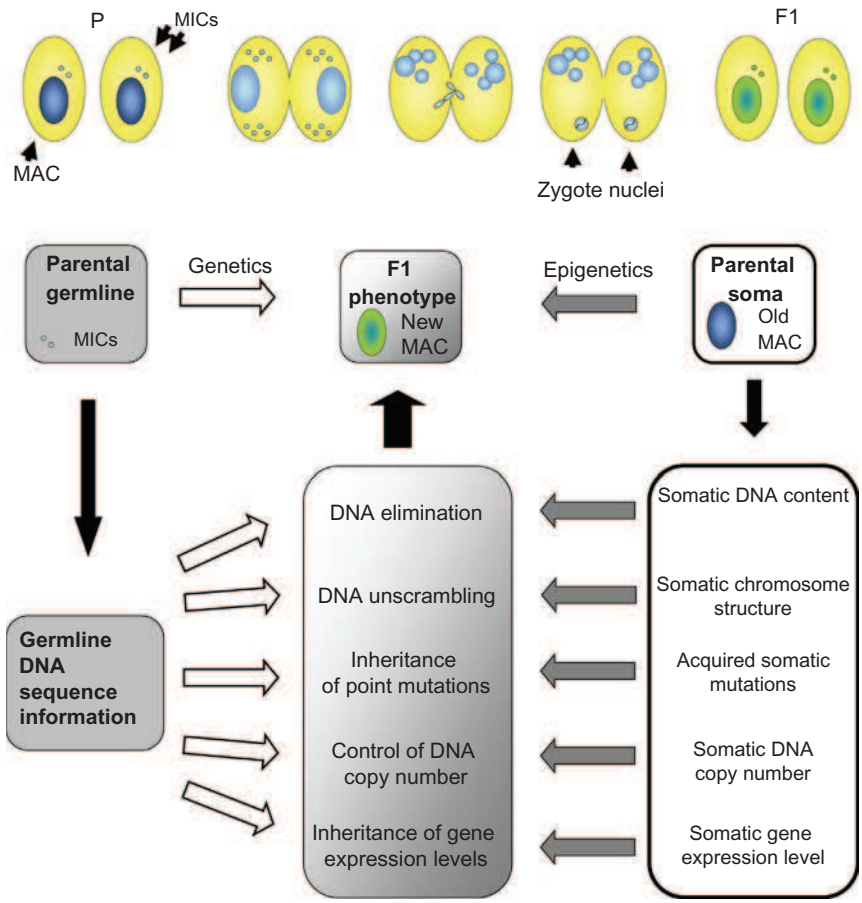

Figure 3.8 Current knowledge of non-Mendelian inheritance in ciliates. *Top row*: Simplified scheme of conjugation in the *Paramecium aurelia* complex. Two vegetative cells contain two generative diploid micronuclei (MIC, green) in addition to one somatic polyploid macronucleus (MAC, blue). The MICs undergo two meiotic divisions into eight haploid nuclei per cell when conjugation is initiated. Then, mating pairs attach themselves to each other at the oral cavity, thus forming a cytoplasmic bridge between the cells. The old macronucleus starts to fragment, but macronuclear fragments are still responsible for cellular metabolism. Only one of the haploid nuclei persists per cell and migrates to the oral cone formed near the oral cavity where each of the micronuclei divides mitotically once more. After exchange of one of the micronuclear division products, these fuse to form a diploid zygote nucleus. When the conjugant cells separate, the diploid zygote nucleus mitotically divides twice into four diploid nuclei. From these, two form the anlagen of the new macronuclei which segregate into one of the cells at the following karyonidal cell division. The remaining two nuclei represent new micronuclei and undergo mitosis with the karyonidal division (not included in the scheme). In sum, four karyonidal cells result (only two are drawn in the scheme) and start growing and

4.2.1 *Seminal insight from* Paramecium

In the early 1930s, Sonneborn discovered two of the most exciting examples of epigenetic inheritance in *Paramecium*. First, Sonneborn and LeSuer (1948) published a precise analysis of non Mendelian inheritance of serotypes. Paramecia can express and maintain individual serotypes resulting from mutually exclusive expression of the respective vsAG encoded by a multigene family. As outlined in Section 3.5, this is analogous to what we know about parasitic variable surface antigen systems (Simon and Kusch, 2013; Simon and Schmidt, 2007). By inducing conjugation (mating) between cells expressing different serotypes (vsAGs), Sonneborn detected that, although being genetically identical, both exconjugants proceed with the expression of the serotypes of their "cytoplasm parents." This finding was contrary to the doctrines of classical genetics.

A second example of non Mendelian inheritance reported by Sonneborn appears similar at first glance: The *Paramecium aurelia* complex exhibits binary mating systems, meaning that cells of individual species are capable of expressing two complementary mating types (Sawka, 2012). Right after the discovery and description of these mating types in 1938, Sonneborn and colleagues realized that only *P. tredecaurelia* showed Mendelian mating type inheritance (Sonneborn, 1966). All other species of the *aurelia* complex exhibited karyonidal inheritance. After conjugation, newly formed macronuclei derived from the zygotic nucleus segregated into separate cells in the very first division. These were called "karyonides" and Sonneborn realized that they determined their mating type independently of the cytoplasmic parent or the synclone (four karyonides derived from a conjugation pair). However, one group of species including *P. tetraurelia* showed mating type determination according to the cytoplasmic lineage. Later experiments by Sonneborn and Nanney provided evidence that this kind of cytoplasmic factor itself was under the control of parental macronuclei (Nanney, 1957; Sonneborn, 1954).

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dividing under normal vegetative (nonsexual) conditions. The *lower part* of the figure shows the contributions to the F1 phenotype by genetic inheritance of DNA sequence information from the parental micronucleus (left) and the epigenetic contributions from the parental macronucleus (right). According to actual knowledge from *Paramecium*, *Tetrahymena*, *Oxytricha*, and *Stylonychia* epigenetic phenomena can be classified as DNA elimination (e.g., IES excision), DNA unscrambling, inheritance of point mutations, control of DNA copy number, and inheritance of gene expression levels.

It was Sonneborn's achievement, not only based on these two examples (serotype and mating type inheritance) but also on several additional facts, to conclude that inheritance of phenotypes is controlled not only by DNA. In his work "Beyond the gene," Sonneborn (1949) defined his "plasmagene" hypothesis by giving a precise model of the mechanisms which we can clarify at the molecular level only today, as outlined below.

In spite of this pioneer work in the first half of the twentieth century, ciliate genetics experienced drastic repression. The situation became so drastic that Preer (1997) complained in his article "*Whatever happened to Paramecium genetics*?" that the classical textbooks in genetics had lost almost all their chapters about ciliates. In between, from the 1950s on, DNA sequencing technologies allowed for the identification of gene sequences, for example, of serotype proteins. Subsequent experiments identified homology dependency of serotype regulation (Leeck and Forney, 1994, 1996) or, in other examples, of homology dependent non Mendelian mutations (Epstein and Forney, 1984; Garnier et al., 2004). Therefore, from the current point of view, the reason of this depression was not a decreasing number of excellent publications, but probably rather the fact that the great theories were formulated so early. Although scientists had described the epigenetic phenomena around the plasmagene hypothesis in considerable detail, the breakthrough allowing for a description of the plasmagene theory on the molecular level was still missing up to the 1990s.

4.2.2 Molecules and molecular modifications behind epigenetics

Fire et al. (1998) pointed toward a new class of regulatory molecules: They applied long double stranded RNA (dsRNA) to *Caenorhabditis elegans* and realized the power of these molecules to interfere with gene expression in a homology dependent manner. Although the initial observations about RNA interference (RNAi) were made in plants much earlier (Ecker and Davis, 1986; Napoli et al., 1990) it was the Fire group that started a new field in life sciences. Knowledge about the regulatory pathways of RNAi and later precise analysis of the short interfering RNAs (siRNAs) in almost all eukary otic model organisms became of paramount importance. The Nobel Prize committee considered this to have made an "*immense impact on biomedical research*" and awarded the 2006 Nobel Prize in Medicine to the researchers. Knowledge about the regulatory pathways of RNAi as well as subsequent analysis of the siRNA pathways in general and of the regulatory function of siRNAs in particular was a breakthrough for all fields in the life sciences.

Scientists realized that the newly detected RNA species in eukaryotic cells represented a highly complex homology dependent control of gene expression of overwhelming importance.

However, the relation of RNAi to non Mendelian inheritance still had to be elaborated. Its identification, as described below, is an example of how work with a unicellular model, yeast, led to the combination of two fields of research which enabled one of the most important breakthrough events. In contrast to most other model organisms, *S. pombe* was recognized as a perfect model for studying RNAi effects because it contains no redundancy in key components of RNAi, such as RNA dependent RNA polymerase, Dicer and Argonaute proteins. Thus, in fission yeast these exist only as a single homolog, as reviewed by Volpe and Martienssen (2011). The early observations in fission yeast indicated that mutants of RNAi components had defects in silencing of reporter genes when these were located near centromeres, while the heterochromatin at the centromeres themselves showed a loss of methylated Lysine 9 in histone H3 (Volpe et al., 2002). These findings suggested a surprising fusion of two issues: RNAi and heterochromatin formation. This indicated for the first time that small RNAs not only guide posttranscriptional events but also mediate the formation of heterochromatin by homology dependent recruitment of chromatin modifying components. This was seminal to the understanding of homology dependent and RNA mediated heterochromatin formation.

In the following years, hundreds of individual histone modifications were described and characterized for their effects. Chromatin associated enzymes can be classified into "writers" (setting covalent histone modifications) and "readers" (recognizing modifications). However, knowing that chromatin writers can be attracted to individual regions in the nucleus in a homology dependent manner, allowed one to understand how the "epigenome" restricts writer's activity to individual regions of the genome without creating identical chromatin modifications all over the nucleus. The "histone code" hypothesis initiated detailed characterization of individual histone modifications and their subsequent effects on heterochromatin formation and, as a consequence, on transcriptional activity (Jenuwein and Allis, 2001). These hypotheses were based on studies with *S. cerevisiae* although this cell shows drastic differences to other eukaryotes as it lacks nearly all enzymes of the RNAi pathway. Thus, there are considerable differences between the yeast species, *S. cerevisiae* and *S. pombe*, since the latter exhibits RNAi pathways and, therefore, is more similar to higher

eukaryotes. A comparison of both yeast species made this tandem an ideal model system for epigenetic research.

4.2.3 Non-Mendelian transgenerational inheritance of epigenetic information

Later, epigenetic modifications across the genome were found to play a key role during the differentiation of stem cells into differentiated adult tissues in metazoans. This way of regulating gene expression can create different phe notypes from genetically identical cells of a body. This is called "mitotic stability," meaning that the epigenetic information becomes "*replicated and transmitted upon cellular proliferation through the mitotic process*" (Skinner, 2011). Otherwise, epigenetic information would be lost during cell division which would make multicellular life impossible.

In contrast to "mitotic stability" (passing on epigenetic information to daughter cells during mitosis), the term "epigenetic inheritance" defines the information transfer between generations of an organism (Skinner, 2011). Transgenerational transfer of epigenetic information appears aston ishing at first glance because it requires molecular communication between soma and germline. Consequently, the question of the underlying molecular mechanisms turned one's attention again to ciliates. They exhibit RNAi pathways and possess one additional advantage which other model organ isms do not have: During ciliate conjugation, the newly formed zygote develops within the parental cytoplasm, thus allowing for elegant experi ments in search of small RNAs which can transfer epigenetic information across generations (Nowacki and Landweber, 2009); this also enabled the molecular identification of Sonneborn's plasmagenes.

During the past 10 years, analysis and characterization of small RNAs during vegetative growth and sexual activity in ciliates changed our point of view not only about epigenetic inheritance in ciliates, but also about RNA as a transgenerational carrier of epigenetic information. For instance, serotype expression, one of the above mentioned classical examples of plasmagene regulated phenomena (Section 4.2.1), has been shown to be controlled by RNAi. This means that antigen encoding genes (all but the single one expressed) become silenced by small RNAs which are likely involved in the formation of heterochromatin (Marker et al., 2010). Details of the mechanism still have to be elaborated. For example, how can one of the serotype genes escape silencing? It would be logical that the heterochromatin associated siRNAs are responsible for the transfer of the individual state of gene expression to sexual progeny. But then, is there

any difference between the vegetative siRNAs (controlling gene expression) and the conjugation associated siRNAs (passing on gene expression)?

This example of epigenetically controlled mutually exclusive gene expression and antigenic variation on the basis of transacting mechanisms is important also for pathogenic protozoa. It explains the evasion from the host's immune system; alternating surface antigen coats clearly represent a crucial component of the survival strategy of such pathogens. The general system of mutually exclusive gene expression is more or less conserved in unicellular fungi and protists, not only in parasites but also in free living species (Deitsch et al., 2009; Merrick and Duraisingh, 2006; Simon and Kusch, 2013).

Similar transacting mechanisms also exist in multicellular organisms up to man. Examples are small RNA mediated epigenetically controlled paramutation in plants, an epigenetic interaction between two alleles in the form of a transacting inheritable epigenetic silencing (Arteaga Vazquez and Chandler, 2010) and genetic imprinting in mammalian heterozygotes where RNA controls exclusive expression of either the paternal or the maternal allele (Royo and Cavaillé, 2008).

The perspectives of model systems are as follows. We need further examples of such mechanisms to understand their genetic requirements. In detail, one aspect (i) emerges in the context of siRNA synthesis and transport. (ii) Another aspect concerns the requirements of individual genes to become epigenetically controlled. This has to consider the importance of chromosomal location, homology, and occurrence of repeated sequences. Finally, (iii) in ciliates, we ought to understand possibilities to analyze inheritable, RNA mediated chromatin patterns. All this will greatly contribute to answer some of the essential questions about epigenetic control mechanisms.

However, these examples of control of gene expression have to be distinguished from other RNA mediated phenomena in ciliates. These can include DNA rearrangements which do not occur in the examples mentioned above. Moreover, as described in the following section, several lines of evidence show that small RNAs are also able to transfer DNA sequence information to sexual progeny, either indirectly by altering chromosomal architecture or directly by altering the DNA sequence. This astonishing diversity of RNA mediated epigenetic inheritance is right now described only in ciliates, thus underlining their model function in epigenetic research. The details of these RNA mediated genome rearrangements in ciliates are summarized below. There is no reason to suspect that such pioneer work would not yield interesting hints for metazoans.

4.3. Excision of IESs during macronuclear development: scnRNA model

To recall, ciliates have a nuclear dimorphism (Figs. 3.7 and 3.8), that is, a diploid, transcriptionally inactive micronucleus and a polyploid, transcrip tionally active macronucleus. Figure 3.7 shows a micronucleus in a stage of "closed mitosis" without spindle pole centrioles, as first described by Eichenlaub Ritter and Tucker (1984). An intriguing aspect of epigenetic control in ciliates is the formation of the macronuclear genome as a modified copy of the micronuclear genome. During conjugation, the micronucleus and macronucleus are newly formed by sexual activity after disintegration of the old macronucleus (see Fig. 3.8). During the formation of macronu clear genome, many thousands of "internal eliminated sequences" (IES) have to be excised from the micronuclear DNA to create functional genes. In contrast to *Paramecium*, *Tetrahymena* reveals a more or less imprecise elimination of IESs. This difference makes sense because in *Paramecium* IESs are often found in the coding region of genes whereas in *Tetrahymena* IESs are mostly located in intergenic regions or introns (Coyne et al., 2012). Within the ciliate clade IES sequences share similarities to each other and, moreover, to transposons in nematodes and other metazoans (Klobutcher and Herrick, 1995). From this, the following questions emerged: (i) What are the origin and function IES? (ii) Which mechanism controls their precise elimination?

Elimination of IES was shown to occur with a large fraction of IESs under epigenetic control of the parental macronucleus by previously unknown epi genetic mechanisms (Duharcourt et al., 1995, 1998). During macronuclear development from the zygote, that is, the diploid synkaryon produced by the fusion of two haploid micronuclei, several DNA rearrangement processes take place (Fig. 3.8). These are controlled by transgenerational RNA. The meiotic micronucleus produces so called scnRNAs (scan RNA, 25–30 nucle otides in length) derived from the entire germline genome (Mochizuki et al., 2002). These are transported into the parental macronucleus where they pair with long noncoding RNAs produced by the old parental genome. Those scnRNAs that do not pair with sequences in the old macronucleus become transported into the new mature macronucleus where they are targeted to homologous sequences to initiate their degradation (Meyer and Chalker, 2006). In *Paramecium* (Lepère et al., 2008, 2009) and *Tetrahymena* (Mochizuki et al., 2002; Yao et al., 2003), several studies characterized this kind of homology dependent mechanism, demonstrating that these scnRNAs promote the elimination of IES regions; this involves targeting by chromatin

modifications, as shown in *Tetrahymena*. In the end, this leads to a most efficient transcriptional silencing by DNA elimination. One can summarize these aspects as follows: The micronuclear derived scnRNAs and macronuclear (maternal) transcripts are hybridized and the subtraction of the two sequences determines sequences destined for the new macronucleus (Duharcourt et al., 2009; Schoeberl and Mochizuki, 2011).

As mentioned above, IESs were suspected to be derived from transposons (Klobutcher and Herrick, 1995). This view was supported by the fact that their excision mechanism involves a domesticated *PiggyBac* transposase (PiggyMac), as shown for *Paramecium* and *Tetrahymena* (Baudry et al., 2009; Cheng et al., 2010). A recent global analysis of IESs in the *Paramecium* germline confirms the homology to transposable elements at least for a fraction of IESs (Arnaiz et al., 2012; Dubois et al., 2012) and indicates that selective pressure counterselects IESs in highly transcribed genes. This makes sense as IESs excision is not 100% efficient and their efficient and precise excision seems crucial for the new macronucleus to work properly.

But why did ciliates tolerate in evolution the massive spreading of transposons in their germline, thus requiring this enormous apparatus for their elimination with every sexual recombination? The first example that the scnRNA pathway can be recruited to control endogenous gene expression was recently reported for the mating types in *Paramecium*. An IES—obviously not derived from transposable elements—was shown to contain the promoter region of the gene encoding a mating type protein (cf. Section 4.2.1). The presence of promoter/IES and the expression of mating type proteins are, therefore, under maternal control by the scnRNA machinery (Singh et al., in revision). These authors concluded that the scnRNA pathway, whose original function is the elimination of transposon derived sequences from the germline, can be recruited to control the expression of endogenous genes and to allow for its maternal inheritance. In contrast to the above mentioned example of serotypes, mating type inheritance involves DNA rearrangements. Both examples represent two independent mechanisms of inheritance of gene expression mediated by small RNAs. Some transposons can contribute to the remodeling of other transposon derivatives, e.g. by IES excision (Vogt et al., 2013).

Therefore, the massive DNA rearrangements in ciliates allow fast adaption of the macronuclear genome, not only by variability due to spreading of transposon like IESs, but also by recruitment of this mechanism to the endogenous gene expression machinery. If one single event in a single cell leads to an altered excision of an IES with possible advantages for this

individual, positive selection can occur. Thus the scnRNA pathway can allow passage of an advantageous feature to sexual progeny.

Recent studies indicate a similarity between ciliate scnRNAs and Piwi associated RNAs (piRNAs, name derived from *P* element *i*nduced *wi*mpy testis) in higher eukaryotes (metazoans). These are involved in the suppres sion of transposon derived sequences and self renewal of germline stem cells (Bamezai et al., 2012). Especially the analysis of piRNAs in germline and somatic stem cells will benefit from basic research into maternal inheritance mediated by scnRNAs in ciliates. From this, one may also gain new insight, for example, in cancer development. In fact, currently cancer is increasingly recognized as a genetic disturbance with considerable epigenetic feedback (Shen and Laird, 2013).

4.4. Maternal RNA controlling DNA copy number

In *Oxytricha trifallax* and *Stylonychia lemnae*, most of the polyploid somatic chromosomes consist of single unlinked genes as a consequence of "DNA scrambling" (Prescott, 1994). Two studies have identified maternal RNA transcripts to control the degree of polyploidy. It has been demonstrated that the DNA copy numbers of sexual progeny can be altered positively or neg atively in *Oxytricha* and *Stylonychia* by interference with the RNA content during conjugation. Higher gene copy numbers can be achieved by injec tion of additional RNA templates during conjugation, whereas a decrease in the number of gene copies can be initiated by RNAi which causes a decrease of RNA templates (Heyse et al., 2010; Nowacki et al., 2010). A similar mechanism occurs in *Paramecium* (though in a more complex manner because of the large macronuclear chromosomes): Prezygotic silencing by transgenes or by dsRNA feeding was shown to induce deletions of genes from larger macronuclear chromosomes (Garnier et al., 2004).

4.5. Maternal RNA matrices providing template for DNA unscrambling in *Oxytricha*

Genome rearrangements in *Paramecium* and *Tetrahymena* differ at least in two essential regards from those in *Oxytricha trifallax* (Bracht et al., 2013; Fang and Landweber, 2013; Fang et al., 2012). First, scnRNAs in *Oxytricha* do not target DNA sequences to elimination but, in contrast to *Paramecium* and *Tetrahymena*, to retention in the developing somatic genome. Second, the assembly of a functional vegetative macronucleus requires unscrambling of the remaining sequences by permutation or inversion. This is due to the

fact that segments destined for coding sequences in the vegetative macronu cleus are fragmented into a great number of tiny pieces in the micronuclear genome. If so, where is the information for the correct order to create intact open reading frames during micronuclear fragmentation in an early step and unscrambling in a later step? Nowacki and collaborators (Nowacki et al., 2008) identified parental RNA transcripts serving as templates for the new somatic genome. In their model, the entire parental macronucleus is transcribed into an RNA reference sequence which orchestrates the assem bly of the scrambled genetic information in the nascent macronucleus. Sur prisingly, they observed that somatic point mutations are transferred to the sexual progeny. The evidence came from the injection of alternative DNA or RNA templates. This did not only alter the assembly of sequence seg ments, but it was also found that single nucleotide substitutions were trans ferred from the artificial template to the progenies, thus indicating that RNA directed DNA proof reading occurs during assembly of the new macronucleus (Nowacki and Landweber, 2009; Nowacki et al., 2008). This model, therefore, opens up a regulatory pathway without precedent—so far no comparable mechanism is known from any other organism.

4.6. Impact of epigenetic studies with unicellular models

In summary, current research in ciliate epigenetics provides the characteri zation of several mechanisms of non Mendelian inheritance. Clearly such discoveries of rather complicated mechanisms depend on detailed molecular analysis of suitable models, that is, cells with short generation time which are appropriate for easy mass culturing. Among them are not only yeasts, *S. pombe* and *S. cerevisiae*, but also ciliates. These, furthermore, enable easy analysis of nuclear transfer of molecules to solve the mechanisms underlying their nuclear dimorphism. These include maternal control of IES excision, gene unscrambling, gene expression, and somatic mutations (cf. Fig. 3.8). Ongoing projects aim at a precise description of the role of RNA as a trans generational carrier of maternal epigenetic information.

From the evolutionary point of view, the epigenetic inheritance of alter nating somatic (macronuclear) epigenomes provides a powerful tool for short time adaptation to rapidly changing environmental conditions, with out waiting for random germline mutations and subsequent Darwinian selection. Therefore, our growing knowledge about RNA as a substance which is able to transfer epigenetic information across generations supports in principle—though not in all of its facets—the Lamarckian theory.

Rejected over a long time, the postulated inheritance of acquired characters currently finds an equivalent on a molecular level in the epigenetic phenomena described in ciliates. In fact, research with ciliates has considerably contributed to this recent appreciation of Lamarckism. It recently culminated in the establishment of a European Research Network "Ciliates as model systems to study genome evolution, mechanisms of non Mendelian inheritance, and their roles in environmental adaptation" coordinated by Miceli and Meyer.

The question remains whether ciliates simply exhibit more cases of non Mendelian inheritance because of the fact that the germline and soma equivalents, that is, micro and macronuclei, respectively, exist in the same cytoplasm or whether examples of non Mendelian inheritance in metazoa are more difficult to observe. On the one hand the respective influence of sperm and oocyte RNA content in the mammalian zygote will be more difficult to analyze than in ciliates. On the other hand medical science cannot ignore the fundamental advances in RNA biology; therefore, investigation on transgenerational RNA in the end needs to become extended to the metazoan zygote.

In the foreword to his textbook on *Paramecium* genetics, Beale mentioned: "*One may even get the impression in my opinion false - that paramecia differ fundamentally from other organisms. . .. Because geneticists limited their attention to one kind of determination unit the gene they have brought about an unnatural divorce between their subject and the rest of biology*" (Beale, 1954). In fact, he was still right in 1990 when the Human Genome Project was started with something like a medial hype—with the expectation of complete decoding of human life. However, knowledge about the "blueprint" of the human genome did not tell us how regulation occurs and why genetically identical cells in different parts of our body differentiate into different phenotypes—the tissues.

At this point, it was consequent that the upcoming epigenetics community started epigenome projects as the next step, including the characterization and mapping of histone modifications and patterns of DNA methylation to gain insight into the epigenetic differences of an untouchable genome sequence (Beck et al., 1999). In light of what we know from basic research on ciliates and other microorganisms, this will also result "only" in a description of the actual epigenetic situation, very likely with limited insight in its regulation. Quite late, but finally in agreement with the conclusions drawn by Sonneborn, Preer, and Beale, the "regulome projects" become or will become initiated by combining the epigenome information with the

locus specific information about regulatory RNA. Thus, the whole coding and noncoding RNA content finally can provide insight into phenotype expression, regulation, and inheritance.

At last, we now investigate what was suggested more than 60 years ago by implication. This now appears as some kind of final proof of the indispens ability of unicellular models for basic research in cell and molecular biology and genetics. Looking back, nobody can deny the importance of impulses from ciliates, the highly honored detection of ribozyme and telomerase function (see Sections 2.2 and 5.4) being just highlights of a broad spectrum of seminal discoveries. Thus, lower eukaryotes undoubtedly have been, are, and will be important models, for example, for the forthcoming elucidation of the many facets of epigenetic inheritance.

5. EXPLORING POTENTIAL OF NEW MODEL SYSTEMS

A considerable handicap of work with protozoa as models is their largely aberrant pharmacology, as summarized with many examples from ciliates (Plattner et al., 2009). However, eons of evolution have also pro duced legions of unicellular systems with special traits that make them attrac tive as model systems even if they also have some disadvantages. Nevertheless it would be unfair not to disclose the specific advantages of some mammalian cells where spontaneous or induced mutations can pro duce pathogenic effects and consequently unique features which render them interesting as models. The perspective of curing diseases would be an even more convincing argument. Nevertheless, one should not disregard the considerable potential of unicellular eukaryotes which have allowed for important discoveries, such as Prions and Prion based diseases (Section 4.1.1). An interplay between the two systems appears most feasible.

5.1. Human diseases as new models

In humans, different inheritable disturbances in one molecule, for example, point mutations in different regions of a gene, can cause different syndromes and a variety of similar syndromes can be caused by different genetic distur bances. With an ever expanding list of mutated gene loci, cells derived from patients with different syndromes more and more entered the scene of research. Many thousands of genetic probes would currently be available for human diagnostics (though in practice the number is restricted and selec tion is made by reasons of anamnetic background). Such cells evolved to

new model systems, with the emerging prospect of cure by somatic gene therapy, as in examples of the following type.

One of the first cases was the elucidation of lysosomal storage diseases, such as I cell disease. The pioneer was a pediatrician, Sly. He found mannose 6 phosphate as the tag responsible for the targeting of acid hydro lases to lysosomes (Sly and Fischer, 1982). A mutation in a transferase mol ecule can inactivate this tag. Meanwhile the list of lysosomal storage diseases is long and the concept of protein targeting is well known. Another example concerns amoeboid movement. The Wiscott–Aldrich syndrome, a failure in the immune response to bacterial infection, enabled the identification of the name giving Wiscott–Aldrich syndrome protein (WASP). This is required for amoeboid movement of neutrophil granulocytes. In detail, it mediates the progression of polymerized F actin to branched filaments at the anterior end of the cell, that is, the leading edge.

Why then still care for model systems such as unicellular eukaryotes (although admittedly important details of amoeboid movement had been detected mainly with *Dictyostelium*)? The counterargument is that, in reality, it is difficult to anticipate whether there are any more details to be detected in organisms such as *Dictyostelium*. For example, this organism has allowed the detection of cross reactive effects between WASP and SCAR proteins (Section 5.2). Another recent example is that it has provided hints to an unknown source of mitochondria based disease, that is, the assembly factor Ndufaf5 which is required for complex I assembly (Carilla Latorre et al., 2013). In summary, there are good arguments for mutual stimulation of work with lower and higher eukaryotes.

The cell biology of cilia and flagella is another example. Vincensini et al. (2011) acknowledged that "*pioneering studies carried out in the green alga Chlamydomonas established the link between cilia and several genetic diseases. . .*" and that "*protists such as Paramecium, Tetrahymena and Trypanosoma or Leishmania each bring specific advantages to the study of cilium biology. For example, the function of genes involved in primary ciliary dyskinesia (due to defects in ciliary motility) can be efficiently assessed in trypanosomes.*"

Earlier, the Kartagener syndrome gave important clues to the molecular basis of ciliary and flagellar beat (Afzelius, 1976). Patients suffer from inflam mation of the upper respiratory tract because cilia are immobile and male patients are infertile since their spermatozoa are also immobile due to the lack of dynein arms (motor ATPase). Since this also entails a *situs inversus* of abdominal organs (heart wrongly on the right side, etc.) this syndrome was also appropriate to give important clues to left right symmetry

determination during development. Many more details about ciliopathies are accumulating. Mostly they are oligogenic, but with pleiotropic effects (Davis and Katsanis, 2012). To give just two additional examples: In man, polycystic kidney disease is caused by a mutated *Tg*737 gene (Pazour et al., 2000), the homologue of *IFT*88 in *Chlamydomonas*; here it is known to participate in intraflagellar (nonvesicular) transport. Like other distur bances, those underlying the Bardet–Biedl syndrome (BBS) were discovered in patients, rather than in lower eukaryotic models. However, considering their complexity, analysis of many molecular disturbances is more difficult in mammalian cells than in protists. As to be demonstrated, these have the potential to shed light on important molecular details. For instance, it is now argued that in *Chlamydomonas* the BBSome (protein aggregate) is sub stoichiometric to the intraflagellar transport (IFT) particle and, thus, may not, or not always be part of the latter (Wei et al., 2012). In retrospect, stud ies with models, such as *Chlamydomonas*, and human ciliary deficiencies resulted in mutual stimulation (Snell et al., 2004).

As mentioned, in ciliates—mainly *Paramecium* being analyzed—voltage dependent Ca^{2+} influx channels are localized to the ciliary membrane (Brehm and Eckert, 1978; Saimi and Kung, 2002). They are activated by membrane depolarization via mechanosensitive Ca^{2+} influx channels enriched in the anterior somatic cell membrane (Machemer, 1989; Machemer and Ogura, 1979). The spatial separation of the sensor and the response channel, therefore, is different from the situation in the primary cilium, for example, of kidney derived MDCK cells (Praetorius and Spring, 2003). The arrangement in *Paramecium* cells helps them to avoid, by inducing ciliary beat reversal, hitting too vigorously an obstacle and, thus, to avoid useless struggling with forward swimming. It remains to be scruti nized why nonciliates up to man do not spatially separate the two functions of Ca^{2+} signaling. One advantage for ciliates can be seen in the avoidance of spill over phenomena by a local restriction of Ca^{2+} signals (Plattner et al., 2006). Signals for ciliary and secretory activity can thus be clearly separated. On the one hand centrin located inside cilia forms part of the Ca^{2+} signal transducing machinery (Gonda et al., 2007) and on other hand, centrin assembled in the infraciliary lattice (Beisson et al., 2001) around ciliary bases serves as a sink for Ca^{2+} after massive exocytosis (Sehring et al., 2009) and possibly in the course of ciliary reversal.

In summary, analysis of lower eukaryotic models and human cells, including those with pathological disturbances, can mutually complement each other and provide important suggestions for ongoing research.

Of course, such dual approach will not be possible with all aspects of cell function and their disturbances. For instance, white Lipizzan horses from famous Spanish Riding School at the Emperial Court in Vienna, Austria, are a longstanding and unique model for cell biology of melanoma cells (Curik et al., 2013). Generally mass cultivation of stable cell lines, from protists to mammals, is a clear advantage for serving as a model system.

5.2. Protozoan models: Once highly qualified—Now disqualified?

In practice, it has to be feared that "lower" eukaryotes may be increasingly considered of lower interest (as stated, yeast is an exception—some even say they believe novel discoveries only if backed by data from yeast). This chal lenging question should now be dissected with one of the most intriguing phenomena, that is, amoeboid movement and chemotaxis. As mentioned, this field has greatly profited from work with *Dictyostelium* (Bagorda and Parent, 2008), notably by Gerisch (Bretschneider et al., 2009; Gerisch, 2009) and Noegel (Noegel and Schleicher, 2000). *Dictyostelium* amoebae follow by chemotaxis bacteria which they ingest by phagocytosis and digest as a food supply. They also follow an extracellular cAMP gradient to form a multicellular complex of *Dictyostelium* cells. As this aggregation can be induced by a point source of cAMP, the system became very attractive for analyzing amoeboid movement. Again, the availability of mutants was of big advantage (Noegel and Schleicher, 2000).

Extensive study of the actin cytoskeleton was prerequisite to the eluci dation of amoeboid movement and phagocytosis. Currently, as the genome has been annotated and extensively analyzed, more detailed questions can be asked with these cells (Williams et al., 2005) and *Dictyostelium* still stays in competition with mammalian cells. Immunologists have learned a lot from diseases, such as the Wiscott–Aldrich syndrome (Section 5.1). Another key player in amoeboid movement, SCAR, is conserved from *Dictyostelium*, where it was detected (Bear et al., 1998), up to man. Using *Dictyostelium* knockout cells it was found that SCAR can override the effect of WASP (Veltman et al., 2012). Is there a similar situation to be expected in mam malian cells—as suggested by the model? Alone to pose this question qual ifies *Dictyostelium* as a model up to our time.

Clearly, with mammalian cells one has the advantage of the availability of the whole repertoire of tools, from molecular biology to ever advancing pharmacology (e.g., to inhibit or stimulate specific functions) and to com mercial antibodies. Many of these instruments are not yet available for

unicellular models. In reality, however, unicellular models catch up in several regards. Complete genome analysis of *Dictyostelium*, *Paramecium,* and *Tetrahymena* is in the advanced stage. The comparison with sequences from largely characterized genes from other species, from protists to mammals, now allows considerable progress. This includes annotations, first fragmentary and provisional, but then with increasing precision and certainty. A combination of methods from the large repertoire of molecular and cellular biology, including localization and functional studies as well as gene silencing, greatly accelerates further work with protozoa and, thus, can bring about a turning point for unicellular models. Then, identification of new genes of unknown function calls for elucidation and such genes may become important also for mammalians where their analysis would be more intriguing.

Nevertheless, it must be admitted that many questions previously accessible only to protozoan models, with their eventual spontaneous or randomly induced mutations, can now be scrutinized with mammalian cells. Here, deliberate mutations can be set to analyze function, targeting, turnover, etc. Is it, therefore, nostalgic to adhere to lower eukaryotes as model systems? In fact, in part it may appear so, considering the unlimited accessibility of genomic information and the availability of even more extensive and well established tools for selective overexpression (and localization as GFP fusion proteins), down regulation, site directed mutational analyses, knock out and knock in, interactomes and other " omes"... This makes it possible to analyze specific questions with specific cells, including those of medical or other practical interest. Alternatively, all these methodologies become increasingly available also for protozoan cells which offer some additional advantages, for example, for the analysis of completely unknown genes or of novel epigenetic mechanisms (Maxmen, 2013; Section 4), to give just two important examples.

5.3. Boon and bane of genome size: Small versus large

S. cerevisiae is still highly appreciated as a unicellular model system, particularly because of its greatly reduced genome, availability of haploid cultures and of defined genetic situations, together with easy accessibility to different manipulations. The yeast two hybrid method enables the detection of protein–protein interactions in different cells. The fact that important aspects of molecular regulation of vesicle trafficking have been developed and in part even discovered in yeast may be accounted for by the small

genome that is restricted to the absolutely necessary. Here, small GTPases and their regulators have been studied most carefully (Mizuno Yamasaki et al., 2012).

Evolution of essential regulators of vesicle trafficking from yeast to man entails an increase in molecular diversity. However, an increase in the num ber of protein encoding genes hardly goes strictly proportional to an increas ing evolutionary level. Nevertheless, in principle such a relationship exists with defined gene families, for example, small GTPases. These are grouped in different subfamilies, such as Ras, Rho, Rac, and Rab, the latter being most essential for vesicular transport, together with SNAREs. Let us com pare "low" and "high" eukaryotes and have a critical look upon established unicellular models.

The number of GTPases increases from 20 in yeast and 53 in *C. elegans* to 163 in man (Rojas et al., 2012). The yeast model reflects minimal require ments for basic functions in a simple eukaryotic cell: Its number of G proteins is as low as extrapolated for the last eukaryotic common ancestor, that is, 20, as recently published (Elias et al., 2012; Stenmark, 2012). Almost all GTPase subfamilies relevant for vesicle trafficking acquire increasing numbers of subfamily members during evolution. This holds true for pro teins of the families type Ras (involved in intracellular signaling, often starting with extracellular stimuli and resulting in cell growth and differen tiation), Rho (actin regulation, cell cycle progression, gene expression, etc.), Rab (vesicular trafficking, from vesicle budding to exocytosis and dif ferent pathways of endocytosis), and Arf (for additional intracellular vesicle trafficking regulation in concert with vesicle coat protein assembly). For these families, in this sequence (Ras/Rho/Rab/Arf), the number of mem bers rises from 4, 3, 8, and 5 (yeast) to 40, 27, 67, and 29 (man), respectively (Rojas et al., 2012). Apart from the GTPases mentioned, the Rac subfamily serves for controlling the actin cytoskeleton and the Ran type for nucleo cytoplasmatic transport; Ran is kept at the lowest level of one gene per genome (Rojas et al., 2012). Clearly, by virtue of its comprehensibility due to its small genome, yeast has given seminal hints to these functions, in concert with GTPase modulating proteins, before research could be expanded to other cells, up to the human brain.

In *Paramecium*, many more GTPase sequences have been found, namely 229 (Saito Nakano et al., 2010) whereas there are only 88 in *Tetrahymena* (Bright et al., 2010), that is, more than in *C. elegans* (Rojas et al., 2012). The high number in *P. tetraurelia* reflects the several fold whole genome duplications in *Paramecium* (Aury et al., 2006), with the possibility of

amplification effects (if all genes are transcribed) and/or potential for further differentiation during evolution. It will be difficult to localize and to selec tively silence all ohnologs differentially (i.e., paralogs from whole genome duplications). Such complications do not improve the usefulness of *Parame-cium* as a model for intracellular trafficking as the great number of isoforms jeopardizes selective labeling (unless as fluorescent fusion proteins) and selec tive silencing. With *Tetrahymena*, in vivo labeling is possible (Bright et al., 2010), but with *Paramecium* this may be much more difficult.

Another example is the exorbitant number of 17 a subunits of the V0 part of the H^+ ATPase in *Paramecium* (Wassmer et al., 2005), in contrast to four in mammals. This may turn out to be very interesting as it may indi cate further differentiation of the H^+ ATPase complex in *Paramecium* by increased combination with the numerous additional subunits (which are not extended to any comparable numbers). Different combinations of subunits could allow for functional and/or topological fine tuning of H^+ transport within the manifold organelles participating in trafficking in this complex cell. *Paramecium* could serve as a model for evolution of molec ular complexity. For all, this one has to consider the age of ciliates of ~800 million years (Douzéry et al., 2004; Wright and Lynn, 1997). Parallel evo lution in lower and higher eukaryotes comes into play. How is the situation with other components involved in vesicle trafficking and signaling?

SNAREs are another example. They were discovered in mammalian cells, including brain cells (Söllner et al., 1993; Weidman et al., 1989). Anal ysis with protozoa lagged behind. From the *Paramecium* database, Fasshauer and colleagues (Kienle et al., 2009; Kloepper et al., 2007) retrieved ~70 SNARE sequences. Detailed domain analysis identified a number of ~44 in *Paramecium* (Plattner, 2010a). Taking into account the close similar ity ($\geq$85% of basepairs) of a fraction of them, one may consider some ohnologs for gene amplification, rather than for diversification. This leaves us with ~40 experimentally identified different SNAREs in *Paramecium* for differential localization and function (Plattner, 2010b), which is equivalent to the number in man (Kloepper et al., 2007). Considering a number of ~20 extrapolated for the ur eukaryote (Kloepper et al., 2008) this clearly indicates evolutionary progress at the protozoan level. Such parallel evolu tion has also been derived from the analysis of CRCs in *Paramecium*, as specified below.

The question of parallel evolution also arose in the context of the analysis of proteins involved in cell signaling, including Ca^{2+} regulated processes. In *Paramecium*, there are 14 very similar genes for the catalytic A subunit (SU)

of calcineurin (protein phosphatase 2B, PP2B) (cf. Section 2.2), but only two for the regulatory B SU (Fraga et al., 2010). The translation products of SU B are identical for the two genes. In A SU, only the binding site for SU B is highly conserved. Clearly there are gene duplications at work, together with a specific selection processes for B SU due to functional restrictions. In other words, the genome may play in a bewildering manner, but it faces its limits when function calls for maintaining "discipline." The question remains whether different A SU ohnologs account for different functions of this pleiotropic gene/enzyme. In fact, unexpected functions are detected even in mammalian cells (Samasilp et al., 2012) and such aspects have to be expected also with protozoa. Here, calcineurin has been analyzed so far only in *Dictyostelium* and in *Paramecium* and only to a small extent. More research with *Paramecium* may yield important clues even for mamma lian cells, as it did for signaling during exocytosis (Momayezi et al., 1987), as summarized by Fraga et al. (2010).

Particularly bewildering is the enormous number of genes encoding K^+ channels in *Paramecium*; it amounts to several hundred (Haynes et al., 2003), in contrast to ~80 in man. Is all that luxury or for amplification effects? Or is it to provide different channel properties and/or to allow for differential positioning? One would have to concede to *Paramecium* a value in itself to delve into such complexity. Its analysis could possibly con tribute to a basic understanding of K^+ channels, based on their intramolec ular variability. However, this is probably much easier to settle by mutational analysis in other systems.

We face a similar, though less dramatic problem with intracellular CRCs. Preliminary information indicates the presence in *P. tetraurelia* of two pore channels and of transient receptor potential type channels (Plattner et al., 2012) though they are not yet analyzed here in any detail. Much more thor oughly analyzed are inositol 1,4,5 trisphosphate (IP_3) receptor type CRCs (IP_3R), as well as ryanodine receptor (RyR) type CRCs (Ladenburger and Plattner, 2011). In *Paramecium*, genes related to IP_3Rs and RyRs are grouped in 6 subfamilies, with a total of 34 members (only few being wrongly transcribed or pseudogenized). The subfamilies are differentially localized to vesicular compartments that participate in vesicle trafficking. Does this first unambiguous identification of such CRCs in a protozoan imply a primeval aspect of Ca^{2+} signaling (Plattner and Verkhratsky, 2013), possibly with a parallel evolution to forms found in higher eukary otes, up to man? Or could it be due to vertical gene transfer from metazoans, as recently proposed (Mackrill, 2012)? The latter view is rather unlikely

considering (i) the complex domain structure of these molecules, (ii) the occurrence of intermediate domain structure combinations in both types of such CRCs, (iii) the simultaneous occurrence of a broad spectrum of other molecules engaged in Ca^{2+} signaling (Plattner et al., 2012), and (iv) because of the absence of evident link to any free living gene "donors." To appreciate this complexity and the distinct intracellular distribution of CRC isoforms in *Paramecium*, one has to bear in mind that vesicle trafficking requires locally restricted regulation mechanisms for Ca^{2+} signals. Thus, detailed signaling mechanisms must have evolved in early times. In essence, the *Paramecium* model is appropriate to widen our horizon on evolution, and there are practical consequences: Recently similar channels were identified also in some parasitic trypanosomatides (Hashimoto et al., 2013; Huang et al., 2013) where they are important for mammalian host cell infection.

In retrospect, already at an early stage a minimum set of key players evolved. One may assume some parallel evolutionary diversification of some of the proteins involved in signaling and trafficking, also including CRCs (Plattner and Verkhratsky, 2013) in lower and higher eukaryotes.

In summary, several whole genome duplications (Aury et al., 2006) have imposed some practical problems to those who work mainly with *P. tetraurelia* cells; this concerns mainly the differential localization and functional analysis of ohnologs. *Tetrahymena*, *Dictyostelium*, *Chlamydomonas,* and yeast do not share these problems. Though the small genome size of yeast clearly facilitates analyses it can give, of course, less clues to further diversification during evolution which appears most pronounced in the most complicated model cell, *Paramecium*.

5.4. Birth and death of nuclei, rather than of cells

One of the most fascinating aspects is the nuclear dimorphism of ciliates (Fig. 3.8) and it would be like the soma/germline aspect of our body. Thus, ciliates can be considered at the same time as cells and organisms, as proposed by Hausmann and Bradbury (1996). Ciliates as cells are frequently described as potentially immortal. They, as well as yeast, have a basic equipment for ongoing DNA replication and cell division, notably a constitutively active telomerase activity serving for the maintenance of telomere length (Eckert and Collins, 2012; Hug and Lingner, 2006). In contrast, "normal" somatic metazoan cells count cell divisions by proceeding telomere shortening as a kind of mitotic clock (Shay and Wright, 2010). Important components contribute to mitotic checkpoint control, though not all can be traced

in evolution down to ciliates (Vleugel et al., 2012). The elimination of parental macronuclei during conjugation has some resemblance to apoptosis. Reportedly, in ciliates this includes DNA ladder formation, involvement of an apoptosis inducing factor and of caspase (Akematsu and Endoh, 2010; Lu and Wolfe, 2001), although it has been reported that some apoptosis relevant molecules, including caspase, are metazoan specific (Srivastava et al., 2010). If so, ciliates may be considered as models from birth to death.

5.5. Special aspects

Extracellular signaling via pheromones is also a topic investigated with unicellular models. Work with yeast includes pheromone induced signal transduction via trimeric GTP binding proteins and downstream protein kinases (Klipp, 2007; Kofahl and Klipp, 2004; Zhang et al., 2006). Some studies with the ciliate *Euplotes* focused on the mitogenic pheromone receptor loop, pheromone/receptor interaction and cell–cell interaction at a molecular level, including evolutionary aspects (Ortenzi et al., 2000; Vallesi et al., 2005, 2008). The disadvantage of poorly developed proteomics of these cells can be compensated by heterologous expression of putative signal transducing elements in a mammalian system where the corresponding signaling cascades can easily be followed (Cervia et al., 2013). This may enable the identification of signaling components in the respective model protist.

In *Tetrahymena vorax* expression of alternative morphs, microstome and macrostome, can be induced by extracellular messengers called kairomones. The mechanism underlying this interesting aspect of morphogenesis has recently been studied by electrophysiology (Grønlien et al., 2013).

Some unicellular models are also suitable for studying the principles of graviperception and graviresponses. Such studies were performed with *Paramecium* (Hemmersbach and Braun, 2006; Nagel and Machemer, 2000). Specific ion channels in cooperation with cortical F actin are likely involved. Exceptionally also pathogenic protozoa have served as models. To give here just one example, the acidocalcisome was detected in *Trypansosoma*. Only with some delay counterparts of these acidic Ca^{2+} stores were found in metazoan cells up to man (Docampo et al., 2005).

Allen (1995) concluded from cryo EM studies that the tandem arrangement of mitochondrial coupling factor F0F1 in *Paramecium* transforms the inner mitochondrial membrane to its typical tubular invaginations. Dimers have been confirmed in yeast by molecular biology combined with 3D evaluation of EM cryo tomograms (Davies et al., 2012; Strauss et al., 2008). Recently, all these

observations culminated in the identification of such dimers, or components thereof, as the long searched for permeability transition pore—the mitochon drial channel for rapid Ca^{2+} uptake (Giorgio et al., 2013). These examples illustrate how unicellular models may give us an answer to the manyfold questions a cell biologist is confronted with inadvertedly. In the fungus, *Podospora anserina*, dissociation of ATP synthase dimers during aging entails loss of cristae structures in mitochondria (Daum et al., 2013).

6. EPILOGUE

Unicellular models have substantially contributed to the progress of cell and molecular biology. As we have tried to demonstrate, many func tional aspects could be more easily elucidated with cells possessing specific traits or capabilities, thus speeding up scientific progress. Important details, such as existence, structure and function of telomeres, were detected in cil iates. Currently telomeres are hotly debated in the context of stem cells (Hoffmeyer et al., 2012), organismal aging (Donate and Blasco, 2011) and cancer (Ding et al., 2012). Epigenetic regulation, including the effects of misfolded proteins, such as prion proteins, were first observed and defined in a relative of yeast. Only after detection in a lower eukaryote model this could be followed up in medically oriented work. At the end of the past cen tury, misfolded Prions became recognized as pathogenic agents. Work of this kind is frequently hypothesis driven. Data driven work conducted in parallel (e.g., large scale genomic analyses) improves the capabilities of models for serving as innovative model systems also in the future. By sub sequent or parallel work with higher eukaryotes, sometimes with some delay, a generalization of results has been frequently achieved. In retrospect, many small details of research have resulted in important concepts with important practical consequences. This is clearly in contrast to Munro's pro vocative and derogative statement (Munro, 2013) cited in Section 1.

Even if one would brush aside some of our arguments in favor of uni cellular models, the famous dictum by Theodosius Dobzhansky still should be considered: "*Nothing in biology makes sense except in the light of evolution*." In fact, well explored models are currently fully integrated in discussions about evolutionary aspects.

The inaccessible expectations from the human genome project (Section 4.6) are one of the most convincing negative examples of work focused selectively on the most highly evolved organisms; clearly this may not always be the most fruitful approach. Also strictly profit oriented research

would hardly have detected many of the treasures available in nature's invaluable treasure box with its protist model cells. Recently Botstein (2012) nicely summarized arguments in favor of such attitude in an article entitled: "*Why we need more basic biology research, not less.*" And to finish, we also agree with Bolker (2012), "*There is more to life than rats and flies.*"

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