A fundamental question in biology is how multicellular organisms can arise from their single-celled precursors. The evolution of multicellularity requires the adoption of new traits in unicellular ancestors that allows the generation of form by, for example, increasing the size and developing new cell types. But what are the genetic, cellular and biochemical bases underlying the evolution of multicellularity? Recent advances in evolutionary developmental biology suggest that the regulation of gene expression by cis-regulatory factors, gene duplication and alternative splicing contribute to phenotypic evolution. These mechanisms enable different degrees of phenotypic divergence and complexity with variation in traits from genomes with similar gene contents. In addition, signaling pathways specific to cell types are developed to guarantee the modulation of cellular and developmental processes matched to the cell types as well as the maintenance of multicellularity.

“...one of the most important discoveries in recent developmental genetics has been the context-dependent actions of regulatory genes.” (Scott F. Gilbert, 2005)1

The confusing maze behind the molecular mechanisms underlying phenotypic divergence is one of the most interesting and, at the same time, the most complicated processes in biology. This also includes the transition from unicellular ancestors to multicellular organisms and the generation of new cell types. The main challenge to understanding the molecular mechanisms of evolution is to identify the genetic basis behind developmental processes that lead to shapes. For a long time, it was largely assumed that species-specific proteins contribute, almost single-handedly, to phenotypic divergence. Although changes in protein function can generate new phenotypes, there is growing evidence indicating that many homologous proteins show highly conserved functions among closely related species. Recent advances in evolutionary developmental biology show that the regulation of gene activity is the main source of biodiversity.2 The first evidence of the importance of gene regulatory mechanisms in evolutionary changes came from studies during the 1960s and 1970s that proposed that affecting gene expression by mutations and other regulatory mechanisms probably led to the evolution of organismal diversity.3,4 Knowledge gained in recent decades confirms this assessment and shows that gene regulatory factors play a central role in the evolution of biodiversity and the generation of form (reviewed in5). Collaboration between various sophisticated gene regulatory mechanisms enables evolutionary novelties by fine-tuning gene expression and depends on location, timing and cell type, notably with respect to the developmental history of the cell. In this way, various phenotypes can form from genomes with similar gene contents. Cis-regulatory elements,6 gene duplication,7 alternative splicing8 and potentially microRNAs9,10 are the key actors in evolutionary developmental biology. Armed with these insights, I will give an overview of the potential impact of these regulatory mechanisms on the evolution of multicellular organisms from unicellular ancestors.

The members of the volvocine algae, a group of chlorophytes including unicellular Chlamydomonas reinhardtii (hereafter Chlamydomonas) and multicellular Volvox carteri (hereafter Volvox), represent a
The evolution of multicellular life from a common unicellular ancestor.11 The evolution of multicellular life from Chlamydomonas to Volvox required several developmental traits, including asymmetric cell division and embryonic morphogenesis. However, the most exiting developmental trait was the evolution of germ-soma differentiation.12 Unlike Chlamydomonas, Volvox has 2 cell types, i.e. 2000-4000 biflagellate, motile, terminally differentiated somatic cells and around 16 much larger immotile reproductive cells, with a clear division of labor (Figure 1). The cells are embedded in a transparent sphere of a glycoprotein-rich extracellular matrix (ECM).13 At the molecular level, however, both organisms possess similar gene contents. The nuclear genome of Chlamydomonas contains 118 Mbp, and that of its multicellular relative Volvox contains 138 Mbp. The larger genome of Volvox (≈17%) is attributed to its higher content of transposons and repetitive DNA, because both species have almost identical protein-coding potentials, i.e., 14,516 and 14,520 protein-coding genes in Chlamydomonas and Volvox, respectively.14,15 Only a few gene families, i.e. the pherophorin genes, the VMP genes (Volvox matrix metalloproteinases) and the cyclin-D-related genes, have more members in Volvox than in Chlamydomonas.14 The same situation can be observed in the human genome, which contains almost as many genes as that of Caenorhabditis elegans.16 This fact strongly supports the theory of evolutionary developmental biology and suggests that the transition from a unicellular Chlamydomonas-like ancestor to multicellular Volvox did not require major changes in gene content.14,17 Based on this observation together with the fact that Volvox cell types represent differential patterns of gene expression in various functional classes,18-20 the development of species-specific proteins could not account for the development of Volvox from a Chlamydomonas-like ancestor. This is also supported by experimental evidence that showed that 2 important proteins, GlsA and InvA, which are responsible for essential developmental processes behind the evolution of multicellularity in Volvox, namely asymmetric division and embryo inversion, respectively, are conserved in unicellular Chlamydomonas. Interestingly, Chlamydomonas orthologs can rescue Volvox glsA and invA mutants.21-23 Thus, rather than species-specific proteins, the functional divergence of gene regulatory elements could be the main contributor to the development of multicellularity during evolution.

We and others have recently shown that alternative splicing could contribute to the appearance of multicellularity by generating multiple transcripts from a single gene.24,25 In many cases, alternative splicing seems to be a part of the molecular mechanisms that allow organisms to decrease the expression of specific genes by generating non-functional or modified variants toward attenuation or alteration of specific cellular and physiological processes. Our analyses show that at least ~2.9% of the intron-containing genes in Volvox are alternatively spliced. Considering the number of analyzed ESTs, it is very likely that the Volvox genome possesses more favorable conditions, e.g. changes in the length and GC content of introns, for the occurrence of alternative splicing than those of the closely related Chlamydomonas.24,26 On the other hand, an analysis of the alternative-splicing status of homologous genes from the closely related alga Chlamydomonas could show that a large fraction of the genes that are alternatively spliced in Volvox are not alternatively spliced in Chlamydomonas. Concurrently with our study, Urrutia and colleagues examined how alternative splicing was related to organismal complexity by analyzing alternative splicing in 47 eukaryotic species. They found that alternative splicing has steadily increased over eukaryotic evolution and is strongly associated with organismal complexity and cell-type number.27 Therefore, it might be conceivable that alternative splicing acts as a key regulatory factor to facilitate the evolution of multicellularity in volvocine algae. However, more effort should be made to provide more insight into the evolutionary aspects of alternative splicing behind the development of multicellularity, for example by a genome-wide comparative analysis of alternative-splicing events between Chlamydomonas and Volvox (to investigate species-specific alternative-splicing events) as well as by investigating the cell-type-specific regulation of events in multicellular Volvox. In this respect, it is also worth noting that the impact of environmental factors, e.g., light cues, which have a large impact on the growth and development of photosynthetic organisms, on the regulation of alternative splicing should be taken into account. Light-regulated gene expression, mediated by photoreceptors, acts as a
multifaceted regulator to control the abundance of functional genes at different levels (reviewed in^27). Surprisingly, *Volvox* photoreceptors are mostly expressed in a cell-type-specific manner,^28 enabling the alga to use distinct light-signaling pathways to modulate the expression of genes involved in various cellular and metabolic pathways in a cell-type-specific manner. This reflects an early development of cell-type-specific signaling mechanisms during evolution to ensure the development as well as the maintenance of cellular differentiation.^30

Another important regulatory mechanism that should be moved increasingly into focus is the role of cis-regulatory elements. Cis-regulatory elements (such as promoters and enhancers) are transcription-factor binding sites and other non-coding DNA that are normally located upstream, downstream or in the introns of genes. These regulatory elements regulate gene expression in a cell-type-, tissue- or developmental-stage-specific fashion. Considering the fact that *Volvox* has almost as many genes as *Chlamydomonas*, the species-specific regulation of gene expression could be the main source of diversity across volvocine algae. To go further, the first step will be to identify the cis-regulatory elements in those genes (as has been partially done for *regA* gene)^31 that are of particular importance for the evolution of multicellularity. It has been shown that around 50% of the genes from closely related species show differences in cis-regulatory elements. However, again genome-wide comparative analyses (e.g. prediction of cis-regulatory elements)^34,35 and supporting experiments would be of great benefit for identifying the developmentally important elements—as well as their candidate sites—and for studying cis-regulatory divergence and activity during the transition from unicellular to multicellular life.

Gene duplication and microRNAs (small noncoding RNAs that regulate gene expression post-transcriptionally) could also be considered as additional sources of novel evolutionary diversity. In particular, gene duplication could contribute to the creation of species-specific transcription factors and other essential proteins for the evolution of gene regulatory networks. An example of species-specific transcription factors is *RegA*, a *Volvox*-specific transcription factor involved in cellular differentiation. Interestingly, *regA* gene seems to be absent in the closely related *Chlamydomonas*. Phylogenetic analysis suggests that *regA* gene was present in a common unicellular ancestor of *Volvox* and *Chlamydomonas*, but was later lost in *Chlamydomonas.^37* In *Volvox*, reproductive activities (and subsequently growth) are suppressed in somatic cells by the transcription factor *RegA*, which is expressed at very high level in these cells. Conversely, the dark green reproductive cells, which show a low *regA* transcript level, possess more photosynthetic activities. It is known that an inverse correlation exists between the size of a gene’s family and its use of alternatively spliced isoforms in humans, mice and worms. However, a recent study by Cooper and colleagues demonstrated that the reduction in alternative splicing was independent of the size of the gene family in zebrafish. In *Volvox*, conversely, it even seems that the more gene duplicates there are, the more alternative splicing is observed (Kianianmomeni et al., unpublished data). Thus, coordination between gene duplication and alternative splicing provides resources for functional innovation to expand protein diversity during the evolution of multicellularity. Moreover, microRNAs, which play an important role in closely related species show differences in cis-regulatory elements. However, again genome-wide comparative analyses (e.g. prediction of cis-regulatory elements) and supporting experiments would be of great benefit for identifying the developmentally important elements—as well as their candidate sites—and for studying cis-regulatory divergence and activity during the transition from unicellular to multicellular life.

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No potential conflicts of interest were disclosed.

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