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Co-option during the evolution of multicellular and developmental complexity in the volvocine green algae

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Despite its major impact on the evolution of Life on Earth, the transition to multicellularity remains poorly understood, especially in terms of its genetic basis. The volvocine algae are a group of closely related species that range in morphology from unicellular individuals (*Chlamydomonas*) to undifferentiated multicellular forms (*Gonium*) and complex organisms with distinct developmental programs and one (*Pleodorina*) or two (*Volvox*) specialized cell types. Modern genetic approaches, complemented by the recent sequencing of genomes from several key species, revealed that co-option of existing genes and pathways is the primary driving force for the evolution of multicellularity in this lineage. The initial transition to undifferentiated multicellularity, as typified by the extant *Gonium*, was driven primarily by the co-option of cell cycle regulation. Further morphological and developmental innovations in the lineage leading to *Volvox* resulted from additional co-option events involving genes important for embryonic inversion, asymmetric cell division, somatic and germ cell differentiation and the structure and function of the extracellular matrix. Because of their relatively low but variable levels of morphological and developmental complexity, simple underlying genetics and recent evolutionary history, the volvocine algae are providing significant insight into our understanding of the genetics and evolution of major developmental and morphological traits.

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Introduction

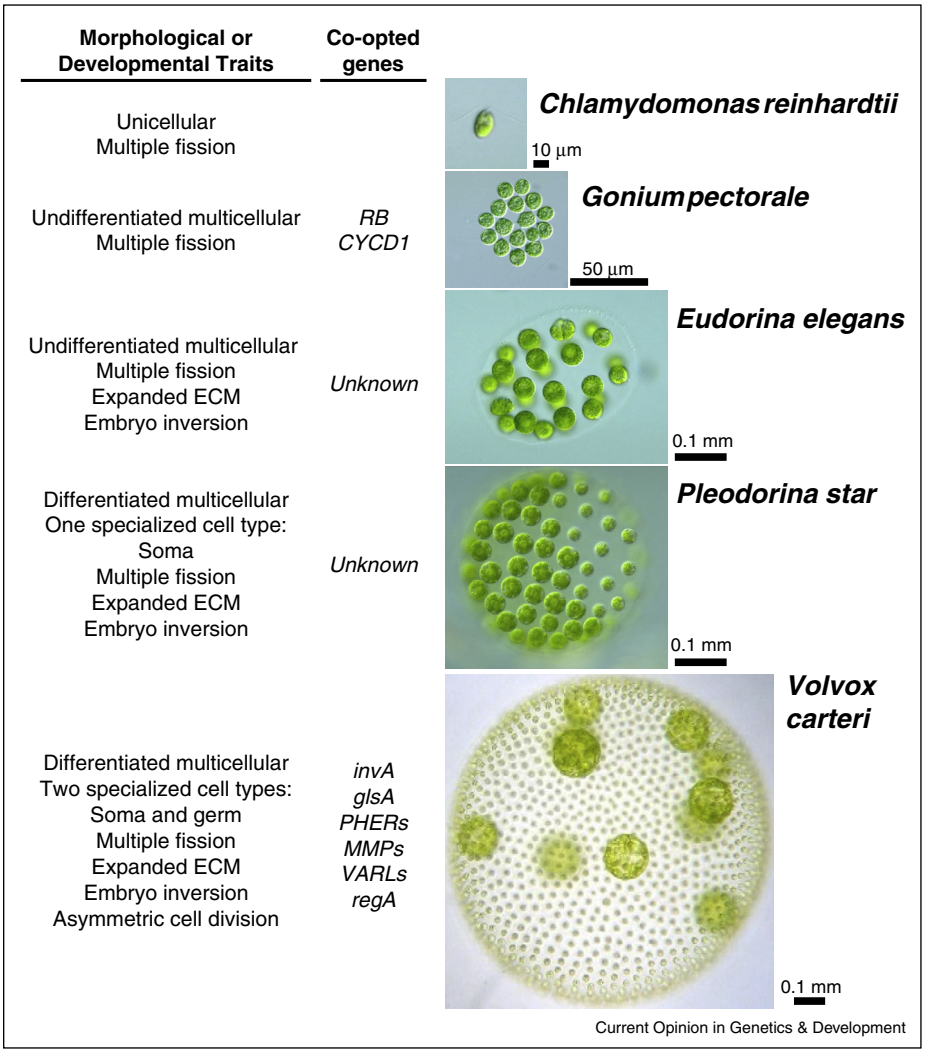
Multicellularity evolved independently at least twenty-five times, in both prokaryotic and eukaryotic clades [1,2,3••,4], suggesting it is a common adaptation in response to various ecological pressures such as predation, nutrient limitation or changing environments (reviewed in [3••,4,5]). The transition to simple multicellular life opened up unprecedented opportunities for the evolution of complex bodies with specialized cells and novel developmental plans. Most multicellular organisms, including plants and animals, develop from a single progenitor cell, a process known as clonal/unitary development (see [3••,6] for alternative developmental modes). Despite being one of the few major evolutionary transitions that shaped Life on Earth [3••,7••,8], the genetic basis for the evolution of multicellularity has been elusive, partly because extant lineages and their genomes have changed significantly since diverging from their unicellular ancestors [9•,10•,11,12].

Generally, the evolution of new traits relies on two major processes: *de novo* gene evolution and co-option of existing genes for new functions [13,14•,15]. The former is less understood, however, with more genomes being sequenced and more advanced computational approaches becoming available the role of *de novo* genes to the evolutionary process is being re-assessed [13,14•,16]. Gene co-option can involve single-copy or duplicated genes. Both structural (i.e., coding for RNAs or proteins other than regulatory factors) and regulatory genes can be co-opted via changes in either their coding or regulatory sequences, or both [13,14•,17•]. Co-option can also involve a change in the binding spectrum of an existing protein by the virtue of a fortuitous interaction with a newly evolved protein, a process referred to as 'molecular exploitation' [18]. Co-option of existing genes has been often invoked to underlie the evolution of numerous adaptive traits, including those associated with the multicellular phenotype [13,14•,17•,19•]. However, the relative significance of the postulated co-option mechanisms remains a matter of debate, with changes in gene regulation often considered the primary contributors to morphological evolution [19•,20•,21•,22]. As genomics is transforming our understanding of the genetic basis of many processes, the mechanisms and sequence of events involved in the evolution of multicellular and developmental complexity are starting to become clear in several major multicellular lineages. Here we focus on recent advances in our understanding of multicellular evolution using the volvocine green algae as a model system, and argue that co-option of both regulatory and structural genes involving changes in regulatory as well as coding sequences played a major role in the evolution of morphological and developmental complexity in this group.

The volvocine algae, in the order Volvocales, include a series of species with morphologies ranging from unicellular

forms such as in *Chlamydomonas*, to multicellular groups of undifferentiated cells (e.g. *Gonium* and *Eudorina*), and to complex multicellular individuals with one (e.g. *Pleodorina*; somatic cells), or two (*Volvox*; somatic and germ cells) specialized cell types (Figure 1). The Volvocales are an experimentally tractable model-system for understanding the mechanistic basis for the evolution of multicellular complexity for several reasons [5]. Multicellularity in the Volvocales occurred more recently, ~200 Mya [23••], than in other multicellular lineages; for instance, in the animal and land plant lineages multicellularity evolved ~0.65–1 Bya [9•,10•,24–26]. The genomes of 3 volvocine species — *Chlamydomonas reinhardtii*, *Gonium pectorale*, and *Volvox carteri* — have been sequenced [27••,28••,29], and although these species span the range of complexity from unicellular to multicellular forms with simple or complex developmental programs, their genomes appear overall similar, with some of the differences likely to have contributed to the evolution of multicellular complexity in this group. Indeed, both genomics and genetics have revealed that the genetic basis for major leaps in developmental and morphological complexity in the Volvocales is rather simple [30–40]. Genetic tools are also available to tease apart the contributions of the various postulated genetic mechanisms underlying the evolution of morphological innovations [41–45]. Furthermore, genetics screens have been a powerful tool in advancing our understanding of developmental pathways in *V. carteri*, where a number of mutants have revealed genes involved in important developmental and morphological traits [30–33,40,46,47]. Lastly, while the transition to multicellular undifferentiated forms occurred only once, several traits associated with organismal size

Figure 1



Evolution of multicellular and developmental complexity in the Volvocales. Representative volvocine species with distinct levels of complexity, from single-celled individuals (*Chlamydomonas*) to complex multicellular organisms with two specialized cell types (*Volvox*). Genes that have been co-opted for novel morphological and developmental traits in this group have been mapped according to the current understanding of their evolutionary history (see Table 1 for more information).

expansion and developmental programs have been repeatedly gained and lost in this group [12,23••,41,48,49,50•,51], opening up the possibility to address the genetic basis of morphological convergence.

Co-option of cell cycle regulation during the evolution of undifferentiated multicellularity in volvocine algae

In metazoans and plants, the regulation of the cell cycle involves a group of retinoblastoma-related proteins; these are transcriptional regulators that repress and activate cell cycle regulated genes through binding to, and directly affecting the activity of E2F-DP transcription factors [62]. When hypo-phosphorylated, RB proteins repress the cell cycle, but when hyper-phosphorylated, primarily by cyclin-CDK dimers, RB becomes inactivated, thereby derepressing and activating S phase-related transcription driving the cell into mitosis [62].

A recurring theme of multicellular evolution in the Volvocales involves modifications to their cell cycle program [5,12,41,50•] (Figure 2). Our understanding of the volvocalean cell cycle is primarily derived from work in *C. reinhardtii*. In this species, each single-celled individual follows a multiple fission cell cycle, where cells grow 2^n in size, followed by n alternating rounds of divisions. The cycle becomes highly synchronized with the diurnal light-dark cycle, resulting in a prolonged G1 period during light and a series of S-phase and mitosis (S/M) in the dark, to produce a uniform population of unicellular daughter cells (Figure 2) [52–57]. Interestingly, in *C. reinhardtii*, the number of alternating rounds of S/M phase is determined by the mother cell size, which regulates the activity of the *Chlamydomonas* retinoblastoma protein, RB (encoded by the *MAT3* gene), and determines the number of rounds of cell division after cells have reached a minimum size to divide at least once [52,53,55,56,58] (Figure 2).

Multiple fission has been co-opted for multicellularity in the Volvocales. While in unicellular *Chlamydomonas* individual daughters break apart from division clusters, in undifferentiated multicellular species such as *Gonium* failure to separate at the end of the process results in multicellular daughter colonies (Figure 2). In *V. carteri* (Figure 1) and its close relatives, clusters of undifferentiated cells ultimately undergo a series of asymmetric cell divisions resulting in the establishment of the germ and somatic cell lines [5,33,41,51,59–61]. Because RB is a key regulator of multiple fission and of the number of division cycles [52–54,56], it is likely that this gene has been important during the evolution of multicellularity in the volvocine lineage (Figure 2).

In contrast to most plants and metazoans, which have multiple isoforms of RB, E2F and DP with unique and overlapping roles [62–65], *C. reinhardtii*, *G. pectorale* and *V. carteri* have single copies of RB, E2F and DP. However, the RB cell cycle regulatory pathway differs between the unicellular and multicellular volvocine algae [27••,66•]. The RB gene itself is regulated by dimers of two proteins, a cyclin and a cyclin-dependent kinase (CDK), which act as dimers to phosphorylate RB. Cyclin D plays a critical role in regulating the transition from G1 to S phase (Figure 2). Interestingly, *C. reinhardtii* has a single cyclin D1 gene, while *G. pectorale* and *V. carteri* each have an expanded repertoire of cyclin D1 genes [27••,28••]. Similarly, the linker domain of the RB protein — where cyclin-CDK dimers phosphorylate RB [52,66•], is different between the unicellular *Chlamydomonas* and the multicellular *Gonium* and *Volvox* [27••]. Interestingly, RB genes in the Volvocales are also tightly linked to their mating type loci, likely because they have secondary roles in regulating the sex cycle [41,66•,67•,68]. Because the RB pathway regulates the transcription of cell cycle related genes, it is likely that the transcriptional output of the RB pathway has been co-opted for a role in multicellularity, and subsequently for cell differentiation, and sexual development [27••].

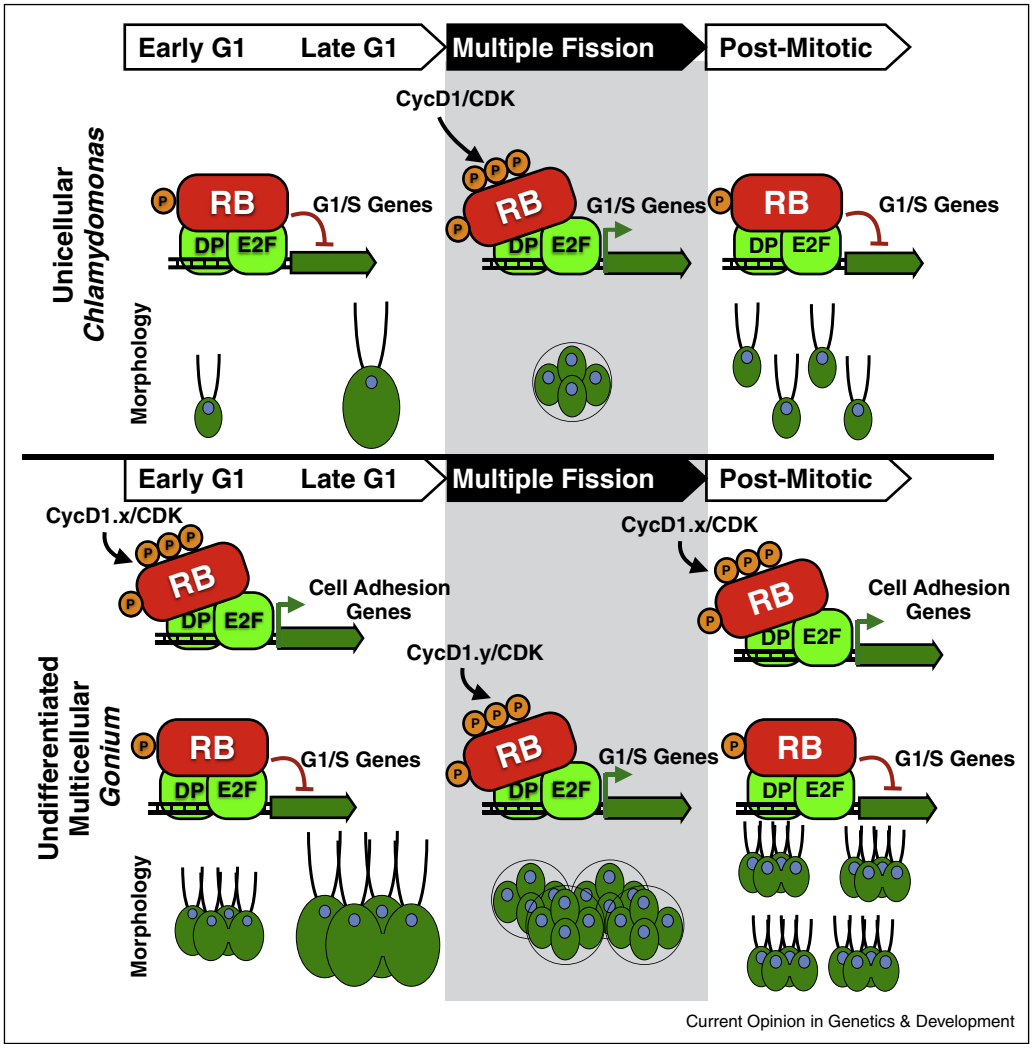
Recently, this hypothesis has been directly tested by expressing the *RB* gene from *G. pectorale* in *C. reinhardtii* cells lacking the *RB* gene [27••]. Surprisingly, *Chlamydomonas* cells complemented with the *Gonium RB* gene exhibited a multicellular phenotype. When these lines were crossed to mutants lacking a functional E2F-DP transcription factor, this phenotype was suppressed [27••]. These data support the hypothesis that RB-dependent regulation of existing cell adhesion genes present in the unicellular ancestor was important for multicellular evolution in this group. Because RB proteins are central to cell cycle regulation and development in many eukaryotes [69–75], this finding has significant implications for our understanding of the evolution of multicellularity in other eukaryotic taxa as well.

Co-option of environmentally induced stress responses for somatic cell differentiation in *V. carteri*

Generally, the evolution of germ-soma separation during the transition to multicellularity requires a change in the expression of vegetative and reproductive functions from a temporal pattern into a spatial context — resulting in these functions being differentially expressed between somatic and germ cells [76]. Mechanistically, it has been suggested that the evolution of soma involved the co-option of life-history genes that in unicellular lineages were induced by environmental cues as an adaptive strategy to enhance survival at an immediate cost to reproduction, by shifting their expression from an environmentally induced context into a developmental context [76,77].

In *V. carteri*, the segregation between the somatic and germ cell lines takes place early during embryonic development

Figure 2



Comparison between the cell cycles of the unicellular *Chlamydomonas* and the multicellular *Gonium*. **(a)** Growth and cell division in *Chlamydomonas* are coupled such that after an extended G1 phase in the light, they divide by multiple-fission in the dark. Chromatin bound RB-E2F-DP complexes transcriptionally regulate the multiple-fission cell cycle. During G1 phase, RB is hypo-phosphorylated and binds to, and inhibits transcription by, E2F-DP. During mitosis, cyclin D1 dimerized with a CDK hyper-phosphorylates RB, causing a conformational change in the chromatin bound complex, leading to the transcription of genes required for mitosis. After mitosis, RB becomes hypo-phosphorylated and cell cycle related genes are no longer transcribed, causing mitotic exit. **(b)** *Gonium* divides by multiple fission as well. RB also regulates the *Gonium* cell cycle except that during G1 phase the several cyclin D1 genes and their CDK partner phosphorylate RB at loci different than in *Chlamydomonas*, regulating genes required for cell–cell adhesion during G1 phase. During mitosis, cyclin D1 family genes with their CDK partner activate the cell cycle just as in *Chlamydomonas*; however, after mitosis, genes required for G1 phase cell–cell adhesion are up-regulated just before mitosis ends, keeping post-mitotic cells attached to each other and leading to the production of multicellular daughter colonies.

and involves a series of asymmetric cell divisions limited to one hemisphere of the embryo. Differences in cell size, not cytoplasmic composition, are thought to be solely responsible for the early establishment of distinct cell fates in this species [59]. Somatic cell specialization involves the differential expression of a master regulatory gene — known as *regA*, thought to encode a transcriptional repressor; mutations in this gene alone result in somatic cells regaining reproductive capabilities [47,78]. The proposed DNA binding domain of RegA is a SAND domain that is also found in other transcription factors such as ULTRAPETALA in *Arabidopsis thaliana* and SP100 in humans, where they are involved in regulating cell proliferation and differentiation [79,80]. *RegA* is only induced in cells whose size falls below a threshold size at the end of embryogenesis; the mechanism is unknown, but likely involves *cis*-regulatory elements identified in three of its intronic sequences [34,59]. *RegA* is thought to act by repressing

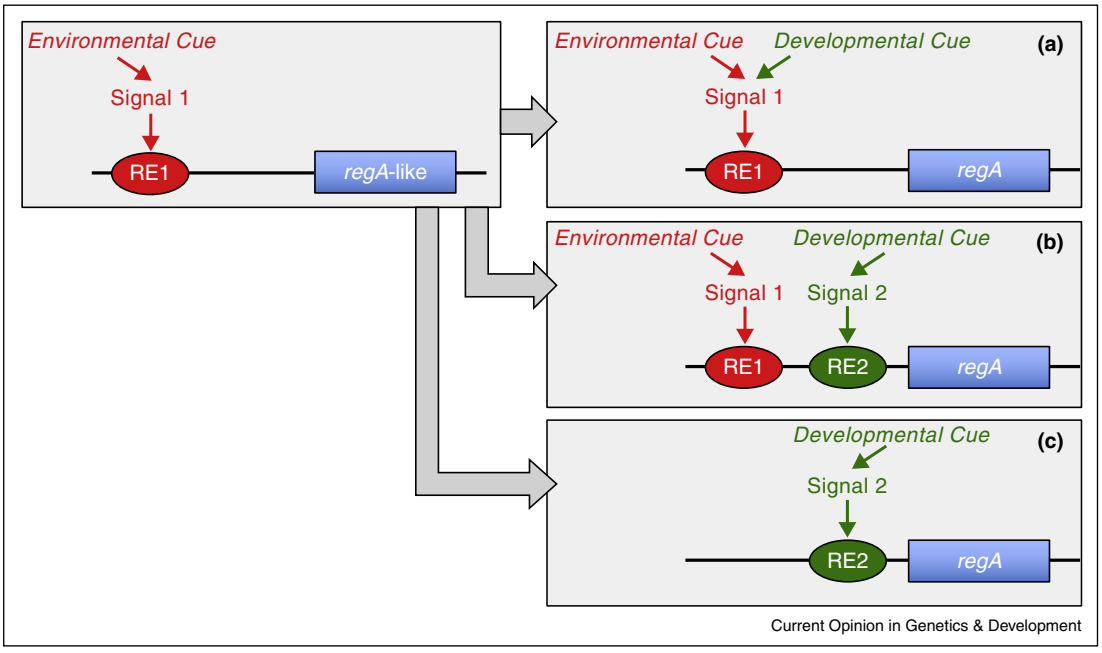
the expression of nuclear-encoded chloroplast proteins [81], which affects the ability of these small cells to photosynthesize, and ultimately to grow and divide.

RegA belongs to a large and diverse gene family known as the *VARL* gene family with members in both unicellular and multicellular volvocine species [77,82,83••,84••]. Interestingly, orthologs of *regA* have been identified in several distant *Volvox* species that evolved somatic cell differentiation independently suggesting that *regA* originated before the evolution of somatic cell differentiation, although whether *regA* is involved in somatic cell differentiation in these *Volvox* species is not known [82,84••]. However, a direct ortholog of *regA* has not been found in either *C. reinhardtii* or *G. pectorale* genomes [27••,84••]. The closest homolog of *regA* in *C. reinhardtii* is known as *RLS1* and its expression is induced under nutrient limitation (including phosphorus-deprivation and sulfur-deprivation), light deprivation and during stationary phase [77,85]. On the basis of these findings we proposed a hypothesis for the evolution of somatic cells in *V. carteri* involving the co-option of an ancestral environmentally induced *RLS1*-like gene, by switching its regulation from a temporal/environmental into a spatial/developmental context [77,85].

Three potential scenarios, each with distinct predictions, can be envisioned for such a change in regulation (Figure 3) [86]. In the first scenario, no new regulatory elements evolved; rather, the same ancestral environmentally induced signaling pathway was also induced during development in early multicellular volvocine algae with somatic cells (that is, the developmental signal simulated the environmental signal) (Figure 3a) [85]. This scenario would predict that *regA* in *V. carteri* is induced both environmentally and developmentally via the same signaling pathway (Figure 3a). A second scenario requires that an additional layer of regulation evolved as part of a new, developmentally induced signaling pathway, and both mechanisms have been maintained in *V. carteri*. This would predict that *regA* is induced both environmentally and developmentally, but the signaling pathways are different (Figure 3b). The third scenario assumes that the ancestral regulation of *regA* was replaced or lost and new regulatory elements evolved in *V. carteri* (Figure 3c). Notably, the former two scenarios predict that *regA* can still be induced in an environmental context. Indeed, *regA* is now known to be expressed outside its developmental context, in response to environmental stresses such as light following extended darkness [87]. This suggests that *regA* maintained some of is putative ancestral environmental regulation. However, the current data cannot distinguish between the first two scenarios presented above (Figure 3).

Understanding how an environmentally induced *RLS1*-like gene was co-opted into a regulator of cell differentiation will provide new insights into the evolution of novel

Figure 3



Three potential scenarios for the co-option of an ancestral, environmentally induced *regA*-like gene during the evolution of *regA* in the lineage leading to *V. carteri*. **(a)** No new regulatory elements (RE) evolved; both environmental and developmental cues converge on the same signal and ancestral regulatory element (RE1). **(b)** An additional layer of regulation (RE2) evolved as part of a new (developmentally induced) signaling pathway. **(c)** The ancestral regulation was replaced or lost and new regulatory elements (RE2) evolved.

Table 1

Summary of our current understanding of the genes involved in major morphological and developmental traits in volvocine algae and the potential genetic mechanisms underlying their evolution. For gene abbreviations see text

Species	Multicellularity			ECM			Complete inversion		Asymmetric division		Somatic cells		
	Presence	Genes		Presence	Genes		Presence	Genes	Presence	Gene	Presence	Genes	
		RB	*CYCD1*		*PHERs*	*MMPs*		*invA, B, C*		*glsA*		*VARLs*	*regA*
Chlamydomonas reinhardtii	N	1	1	N	31	44	N	1	N	1	N	12	0
Gonium pectorale	Y	1	4	N	35	36	N	1	N	1	N	8	0
Volvox carteri	Y	1	4	Y	78	98	Y	1	Y	1	Y	14	1
Co-opted gene		Single copy	Duplicated		Duplicated			Single copies		Single copy		Duplicate	
Likely change underlying the co-option event		Coding	Regulatory or binding partner		Coding and regulatory			Regulatory or in binding partners		Regulatory or in binding partners		Regulatory	

morphological traits. In addition, understanding how a pathway involved in responses to environmental changes has been co-opted into a developmental program will contribute to the growing interest in re-evaluating the role of environment in developmental evolution [88].

Co-option of structural genes for developmental complexity in the Volvocales

The two major leaps in organismal complexity discussed thus far, multicellularity and somatic cell differentiation, involved the co-option of transcriptional regulators, all of which are likely to impact the expression of many downstream genes. However, structural genes whose products are known to be involved in specific developmental and morphological traits in *V. carteri* have also been co-opted from single-celled ancestors. For example, four of the genes underlying two *V. carteri* specific developmental processes, asymmetric division and embryonic inversion, have orthologs in *C. reinhardtii* and *G. pectorale* (Table 1). Remarkably, *V. carteri* mutants in two of these genes, *invA* coding for a kinesin [40], and *glsA* coding for a co-chaperone involved in spindle placement [33,60,89] can be complemented by their *C. reinhardtii* orthologs [40,90], suggesting that these genes have been directly co-opted into developmental processes by either changes in their regulation (involving *cis* or *trans* elements) or changes in their protein interacting partners. Other structural genes involved in multicellular traits in the volvocine algae appear to have evolved by gene duplication followed by diversification; these include the genes coding for matrix metalloproteases (MMPs) and a class of hydroxyproline-rich glycoproteins called pherophorins (PHERs), all involved in the structure and function of the extracellular matrix (ECM) [27••,28••,91–96] (Table 1). Interestingly, although *C. reinhardtii* and *G. pectorale* have roughly the same total number of these ECM related genes (Table 1; though not all are direct orthologs to each other), *V. carteri* has significantly more ECM related genes than *Chlamydomonas* (Table 1; [41,91]), consistent with an increased amount of ECM in this species [27••].

Conclusions and perspective

The volvocine algae are reaping significant advances in our understanding of the genetic mechanisms underlying the evolution of multicellularity and developmental complexity. Several key traits associated with the evolution of multicellularity and developmental complexity in this group involved co-option events (Table 1). First, the cell cycle was reprogrammed via co-option of *RB* and cyclin D1 genes to promote the evolution of undifferentiated multicellularity by modifications to the multiple fission division pattern as observed in the extant *Gonium* (Figure 2 and Table 1). Interestingly, the ancestral multiple fission type of division has been further modified in distinct *Volvox* lineages, contributing to the four developmental programs known in this group [97]. In the lineage leading to *V. carteri*, a series of additional co-option events took place, including co-option of genes involved in the structure and function of ECM, embryonic inversion, asymmetric cell division, and establishing the somatic cell fate (Figure 1 and Table 1). Of particular interest is the co-option of *regA* in the differentiation of soma. Because soma evolved independently in several volvocine lineages [23••,41,48,97], including species whose developmental programs do not involve asymmetric divisions and multiple fission, further sequencing of volvocalean genomes and genetic analyses should reveal whether somatic cell evolution involved similar or distinct genetic mechanisms in this group.

The Volvocales are emerging as an important model-system in which to address the contribution of the many postulated types of genetic mechanisms contributing to the evolution of multicellularity and developmental complexity (Figure 1 and Table 1). Indeed, some co-option events involved changes in the regulation of genes (e.g., *regA*, *CYCD1*) while others involved changes to coding

sequences (e.g., *RB*) or possibly the binding potential of the encoded proteins (e.g., *glsA*, *invA*). Likewise, the co-option events included both single-copy genes (*inv*, *glsA*) as well as multi-copy genes (*VARLs*, *PHERs*, *MMPs*); and both regulatory (*regA*, *RB*, *CYCD1*) and structural (*invA*, *glsA*, *PHER*, *MMP*) genes. Because of their relatively low but variable levels of complexity as well as simple underlying genetics and recent evolutionary history, the volvocine algae are living up to their potential by providing significant insight into our understanding of the genetics of adaptations and evolution of complex developmental and morphological traits.

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