Phenotypic analysis of two unicellular *Gonium pectorale* mutants defective in extracellular matrix assembly

by

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Abstract

The evolution of multicellularity is a major transition in the morphological organization of organisms, however, the molecular mechanisms important for this transition in any taxa are currently not well understood. In most taxa, the molecular signature of the transition to multicellularity is obscured by nearly a billion years of divergence. In contrast, multicellularity evolved recently in the volvocine algae. As a result, the genomes of member species remarkably similar, suggesting the transition to multicellularity only requires the evolution of a few genes. The volvocine algae include members that span the range of morphological complexity from unicellular (e.g. *Chlamydomonas reinhardtii*) to undifferentiated multicellular (e.g. *Gonium pectorale*), to species with differentiated tissues (e.g. *Volvox carteri*). Corresponding to the morphological phenotypes, members of these organisms range from a simplified cell wall in the unicellular species into an expanded extracellular matrix important for multicellular group formation. To find genes important for multicellularity in undifferentiated multicellular *Gonium pectorale,* we performed a forward genetic screen for unicellular mutants. From this we identified two mutants, *uc-1C7,* where 99.6% of cells are unicellular and *uc-1H7* where 95% of cells are unicellular. Both mutants were found to be sensitive to detergent lysis suggesting these mutants have defects in extracellular matrix assembly. Additionally, total cell wall extracts were prepared from *uc-1C7* and wild-type strains. These were subjected to tandem mass spectrometry to identify which proteins were present in both lines, which demonstrated that the *uc-1C7* mutant is missing major components of its cell wall. Using an antibody that is specific for *Chlamydomonas* cell walls, preliminary immunofluorescence tests show a reduced signal in the *uc-1C7* and *uc-1H7* mutants compared to wild-type. Our results point to two unicellular *Gonium* mutants that have defects in assembling a functional extracellular matrix. Because these mutants have a unicellular phenotype, it demonstrates that cell wall of *Gonium* is essential for undifferentiated colony formation.

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Chapter 1 - Introduction

The evolution of multicellularity

How multicellular lineages arise is a fundamental question in biology [2], yet the transition from ancestral unicellular species to extant multicellular species is poorly understood. This transition has occurred ~25 times independently in various domains of life [1,2], yet little is known in each lineage how this transition occurred, particularly at the molecular level. The main reason for this gap in knowledge is that the molecular signature of this transition is typically hidden by a billion years of divergence. This makes extant multicellular organisms widely different from their unicellular ancestors at the genome level and can obscure the subtle differences in gene expression patterns and protein makeup.

As multicellularity requires the cooperation of cells for the maintenance, development, and reproduction of a multicellular organism, the structures that facilitate these processes are key for the transition from a unicellular to a multicellular state. It has been known from morphological studies that the extracellular matrix (ECM) is greater in multicellular Volvocales compared to unicellular *Chlamydomonas* [6] The ECM is highly dynamic structure, changing with developmental and environmental cues that provides both structural and nonstructural support for the organism [3,5,6]. Structurally, the ECM provides a physical scaffold critical for cell adhesion, rigidity, and support [4]. The ECM also provides a boundary between tissues in differentiated multicellular organisms and is known to play a role in the specialization of tissue types during development. In terms of nonstructural support, this structure is responsible for the defense response against pathogens as well as controlling intracellular communications [6].

Evolutionarily, the ECM likely evolved from a simplified cell wall to a more complex extracellular matrix to recognize, adhere, and communicate with surrounding cells [6] Cell

adhesion is important for multicellular eukaryotes, but in multicellular Volvocales the molecular mechanisms of cellular adhesion is not well understood. While this structure is key for the evolution of a multicellular organism, little is known at the molecular level how a simplified cell wall in unicellular organisms can evolve into a complex ECM in multicellular organisms.

Volvocales as a model system for the evolution of multicellularity

An order of green algae known as the Volvocales provides a unique system for studying the molecular mechanisms important for the evolution of multicellularity. Members of this group include closely related organisms that display a diverse array of morphological phenotypes yet are highly similar at the genome level [9,10]. Members of the Volvocales exhibit a stepwise increase in morphological complexity, ranging from the unicellular *Chlamydomonas reinhardtii* (hereafter *Chlamydomonas*) to the undifferentiated multicellular *Gonium pectorale* (hereafter *Gonium*) to the fully differentiated multicellular *Volvox carteri* (hereafter *Volvox*) (Fig 1).

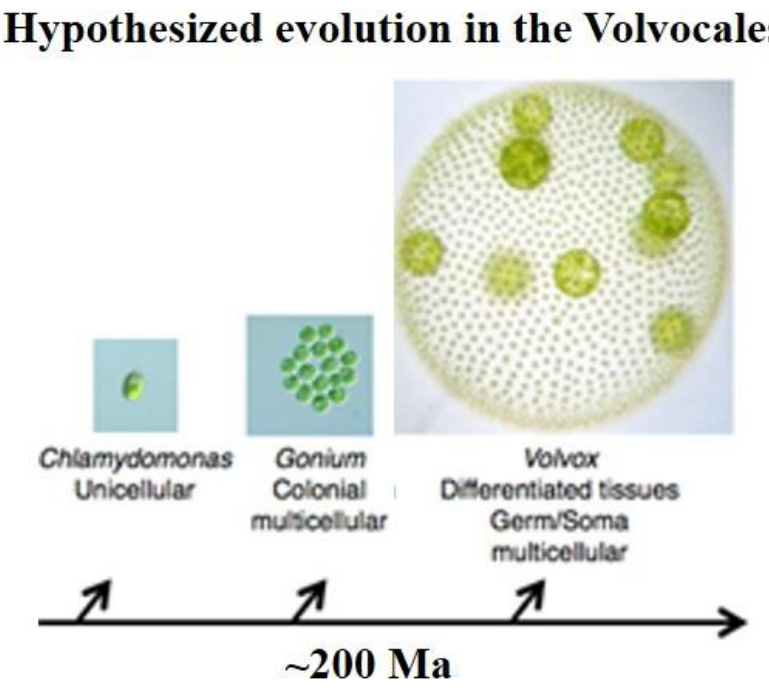


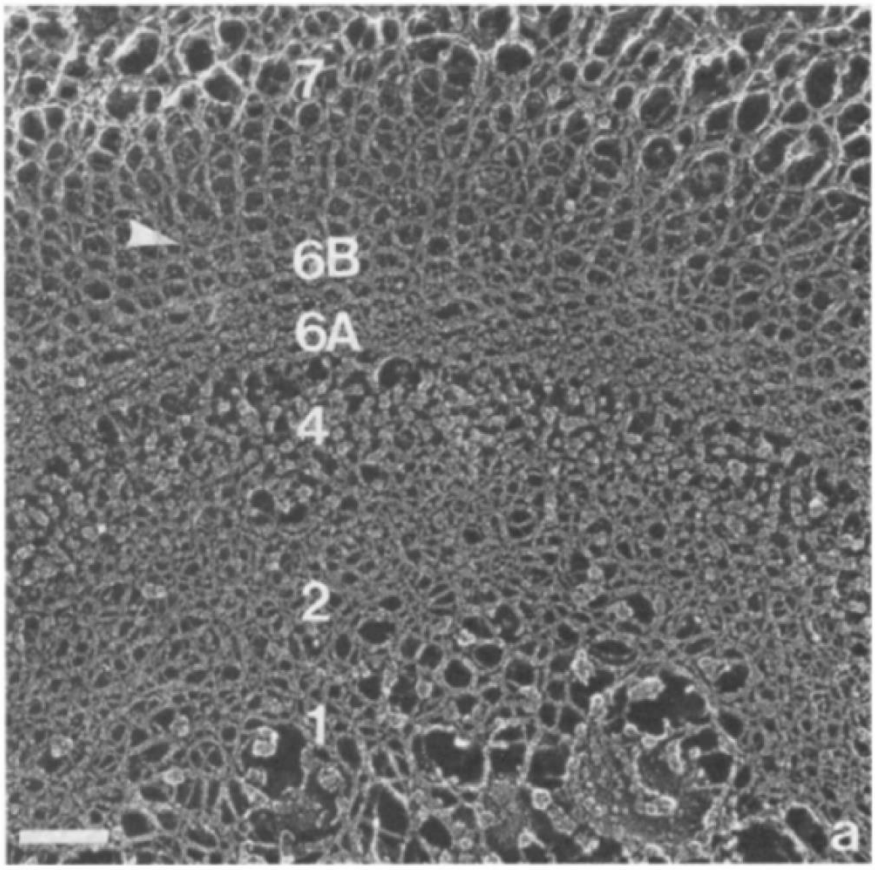
Fig. 1. Proposed evolutionary transition in the Volvocales adapted from Umen, J. G., & Olson, B. J.S.C. (2012) *Adv Bot Res.*

Corresponding to the morphological phenotypes, members of the Volvocales exhibit a stepwise evolutionary transition from a simplified cell wall in the unicellular species into an expanded extracellular matrix responsible for cell-cell communications and maintaining either colonial or differentiated cellular states after cell division [6].

Unlike most lineages, the Volvocales diverged relatively recently (~200 mya) [12], providing the opportunity to study the transition to multicellularity with minimal evolutionary noise. Additionally, the genomes of Volvocales species *C. reinhardtii, G. pectorale,* and *V. carteri* have been shown to be highly similar [8,9,10] suggesting that a major overhaul of the genome is not required to evolve multicellularity. The genomes of members of the Volvocales have revealed that *Chlamydomonas* and *Gonium* are especially comparable, sharing a similarity in GC-content of ~64% [9]. The genomes have also revealed that with increasing organismal complexity, there is an increase in average intron length and a decrease in gene density.

The cell cycles of members of the Volvocales also provide a unique opportunity to study the evolution of multicellularity as these organisms undergo multiple fission when they divide. During *Chlamydomonas* proliferation, the mother cell divides at 2^n producing 2, 4, 8, 16, or 32 daughter cells. This is similar to *Gonium* which undergo the same number of rounds of multiple fission except that these cells remain attached after cell division, suggesting cell adhesion is important for multicellular development. In addition, the cell cycles can be synchronized to a light/dark cycle to assess the growth and division of these members. This makes the Volvocales an ideal model system for the study of the evolution of multicellularity.

a



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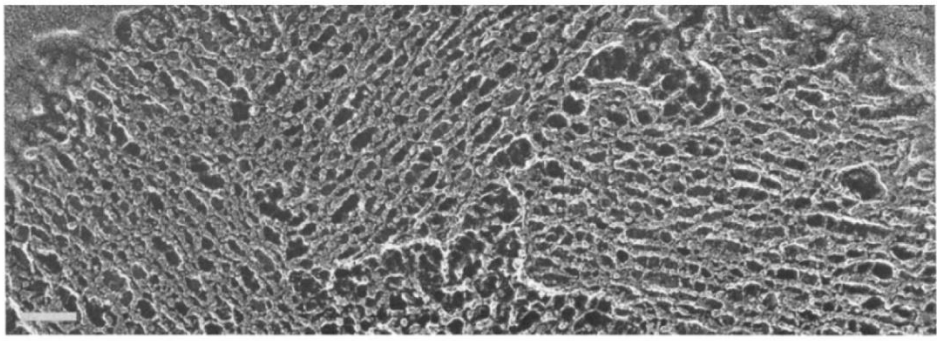

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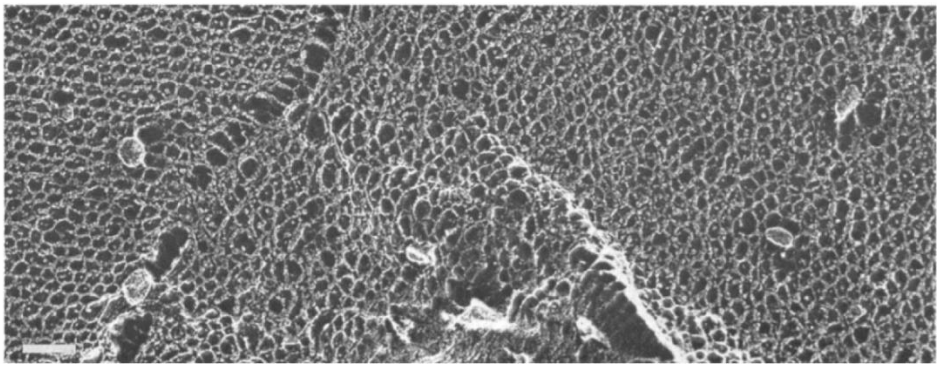


Fig. 2. a. wild-type *Chlamydomonas* cell wall showing the tripartite wall layers 2, 4, and 6; b. *Chlamydomonas* reconstituted with *Volvox* crystal components; c. *Volvox* reconstituted with *Chlamydomonas* crystal components

The extracellular matrix of the Volvocales

For the evolution of differentiated cells to occur, unicellular organisms must have first cooperated with one another which is facilitated by an extracellular matrix [6,7]. Therefore, the development and expansion of a complex extracellular matrix is one of the first prerequisites for the evolution of multicellularity. While this is an important step in the formation of a multicellular organism, little is known about how the extracellular matrix evolved to facilitate cell-cell adhesion. The Volvocales provide a remarkable opportunity to study the evolution of a simplified cell wall into a complex extracellular matrix that functions in keeping cells adhered after cell division.

In the Volvocales, the unicellular *Chlamydomonas* has a simple cell wall which was expanded in *Gonium* to allow for keeping colonial cells together after cell division. This extracellular matrix further expands and increases in complexity as cell size increases in the Volvocales. The volume of extracellular matrix material in this family ranges from ~1% total volume in the unicellular species *Chlamydomonas* to >99% total volume in the differentiated multicellular species *Volvox*, suggesting that an increase in extracellular matrix biogenesis took place in the transition from unicellularity to multicellularity [6,25].

Little is known of the protein composition of the ECM of the Volvocales [23], however the ultrastructure of the extracellular matrix has been extensively studied and divided into four distinct zones: the flagellar zone, the boundary zone, the cellular zone, and the deep zone [25]. While the exact function of each zone is unknown, each zone is composed of hydroxyproline-rich glycoproteins (HRGPs) and do not have cellulose [24]. The flagellar zone is the outermost

zone, encompassing the flagella and flagellar components. The boundary zone is continuous over the surface of the organism but is not structurally continuous in the deeper zones. In unicellular species of the Volvocales the cellular zone and the boundary zone are synonymous with one another. The cellular zone and the deep zone are both composed of a fibrous layer and an amorphous layer differing mainly that the deep zone is the closest to the plasma membrane.

While the cellular zone and deep zones of the ECM are structurally different across members of the Volvocales, the boundary zone has been shown to be structurally conserved across species. Interestingly, in *Chlamydomonas* and *Gonium* the boundary layer surrounds each individual cell, however in *Volvox* the boundary layer is shared across the whole organism. The boundary zone, including the tripartite zone [zones 2, 4, and 6 in Fig 2a] which is highly conserved across members of Volvocales, is highly structured. This region in addition to the flagellar and cellular zone is soluble in chaotropic salts, making it easy to extract and observe. In contrast, the deep zone is amorphous and is salt insoluble making it difficult to isolate and characterize. Taken together, these results suggest that the ECM evolved to promote cell adhesion in the Volvocales, an idea supported by the fact that known ECM genes are evolutionary expanding in *G. pectorale* and *V. carteri* [8,17].

In addition, interspecies reconstitution experiments of the tripartite wall layer using both *Chlamydomonas* and *Volvox* have provided evidence that the ECM of the two species is highly similar [43]. Using this method, they discovered that the tripartite layer of *Volvox*, when added to tripartite-deficient *Chlamydomonas* cells, reassembled and recreated the lattice structure (Fig 2b) as seen in the wild-type *Chlamydomonas* cell-wall (Fig 2a). The same holds true if the tripartite layer of *Chlamydomonas* is added back to tripartite-deficient *Volvox* (Fig 2c), showing that the tripartite wall layer is highly conserved across species.

Though members of the Volvocales have been used extensively as model systems for various studies in flagella and photosynthesis, little is known about how multicellularity evolved in this family and the role that the extracellular matrix has played in this transition. To date ECM genes have been identified in the Volvocales based on sequence similarity, but very few of these genes have been experimentally confirmed to participate in the ECM [25]. Remarkably, the ultrastructure of the boundary zone of *Chlamydomonas* and *Gonium* are highly similar making them the ideal systems for studying the evolution of the extracellular matrix to hold undifferentiated cells together [16].

Gonium pectorale as a model system

Gonium pectorale provides a simplistic model system to study the evolution of multicellularity, specifically cell-cell adhesion. This organism exists in a colonial state throughout its life cycle and, interestingly, only remains in a colonial state under optimal growth conditions. Conditions such as nutrient deficiencies and dense cell count results in dissociation of the colonies into individual cells. This shows that specific cell adhesion molecules are important in maintaining the colonial state and that reversion to the unicellular state is possible with the exclusion of specific proteins. The life cycle of Gonium also includes a brief period of maintaining cytoplasmic bridges, a hallmark of multicellularity, however the final stage of development lacks these bridges showing that the cell adhesion proteins are important in maintaining the colonial state [39].

Knowing that *Gonium* requires cell-cell adhesion molecules to remain in a colonial state, unicellular mutants can be generated to identify the proteins and pathways responsible for cell adhesion in a simplified colonial organism. We can then use this information to determine how cell-cell adhesion molecules interact in other more complex multicellular species such as *Volvox.*

Chapter 2 - Characterization of two unicellular *Gonium* mutants

Introduction

The evolution of multicellularity is a major transition in the morphological organization of organisms that has occurred multiple times in all domains of life, yet the molecular basis of this evolutionary transition is poorly understood. In almost all systems, the molecular signature of this transition is typically hidden by a billion years of divergence. Multicellularity requires the cooperation of cells for the maintenance, development, and reproduction of a multicellular organism. For the evolution of differentiated cells to occur, unicellular organisms must have cooperated, which typically occurs first in a colonial fashion. This includes the utilization of cell communication and cell-cell adhesion which is facilitated by an extracellular matrix. Therefore, the development and expansion of a complex extracellular matrix is one of the first prerequisites for the evolution of multicellularity. While this is an important step in the formation of a multicellular organism, little is known about how the extracellular matrix evolved to facilitate cell-cell adhesion.

An order of green algae known as the Volvocales provide a unique system for studying the molecular mechanisms important for the evolution of the extracellular matrix and cell-cell adhesion. This family includes extant species that have a stepwise evolutionary transition from a simplified cell wall into an expanded extracellular matrix responsible for cell-cell communications and keeping colonial and differentiated cells together after cell division [16,17]. In addition to this, the volume of extracellular matrix material in this family ranges from ~1% total volume in the unicellular species *Chlamydomonas* to >99% total volume in the differentiated multicellular species *Volvox,* suggesting that an increase in extracellular matrix biogenesis must have taken place in the transition from unicellularity to multicellularity [16].

Additionally, the Volvocales diverged recently better preserving the molecular signature of this transition than most other multicellular lineages [1]. While members of the Volvocales have been used extensively as model systems for various studies, much is unknown about how multicellularity evolved in this family and the role that the extracellular matrix has played in this transition.

To identify genes important for the ECM in the Volvocales, we utilized the simplistic model organism *Gonium* which displays the evolutionary steps to cell-cell adhesion. Additionally, this organism only remains in a colonial state under optimal growth conditions. This suggests that reversion to the unicellular state is possible with the exclusion of molecules that play a role in cell adhesion. We performed a forward genetic screen on *Gonium pectorale* mutants that are unicellular and isolated two unicellular mutants, *uc-1C7* and *uc-1H7*. Here we describe in depth the phenotypic data collected on these two mutants.

Results

Generation of uc-1C7 and uc-1H7

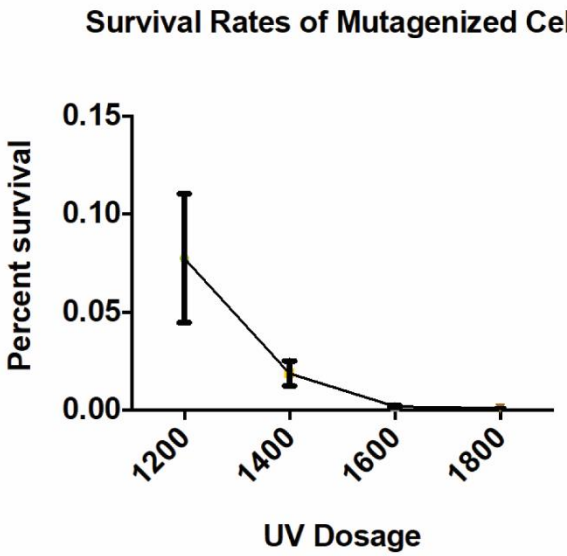
As an unbiased approach for identifying genes important for the evolution of multicellularity in the Volvocales, a forward genetic screen was performed by Katherine Johnson to identify unicellular *Gonium* mutants.

To isolate the mutants that were unicellular, a method was established utilizing soft agar plates (0.75% agar) to distinguish from unicellular and multicellular strains. When plated on soft agar plates, the multicellular strains display a punctate phenotype whereas unicellular strains display a spreading phenotype (Fig 3b*)*. This is due to the limited mobility that multicellular strains have in comparison to unicellular strains which are able to spread on the agar plate. Mutants were then generated by UV mutagenesis (Fig 3a) and phenotypically characterized to

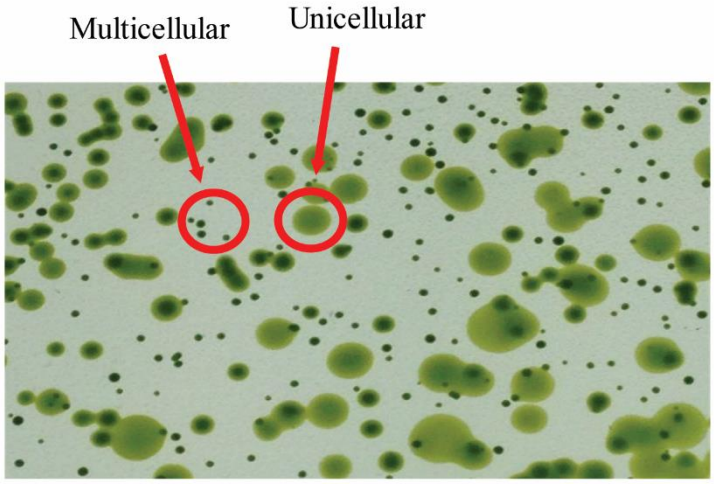
isolate unicellular mutants by plating on the soft agar plates. Using this method, Katherine Johnson isolated six unicellular mutants, two of which are characterized here, *uc-1C7* and *uc-1H7*.

In contrast to the wild-type strain *K3* (Fig 3c), both mutants are unicellular throughout their life-cycle where 99.6% *uc-1C7* cells are unicellular (Fig 3d) and where 95% of *uc-1H7* cells 95% unicellular (Fig 3e) upon phenotypic evaluation. When backcrossed to *wild-type*, *uc-1C7* segregated 1:1, showing that only one gene is responsible for the mutant phenotype. The mutant *uc-1H7* is more complex as when backcrossed to wild-type as this mutant segregates in a 1:3 pattern suggesting that two or more genes are responsible for this mutant phenotype.

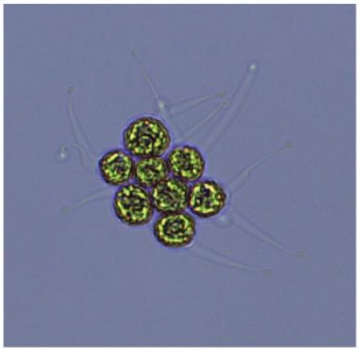
a


Survival Rates of Mutagenized Cells
Percent survival
0.15
0.10
0.05
0.00
1200
1400
1600
1800
UV Dosage

b


Multicellular
Unicellular

c



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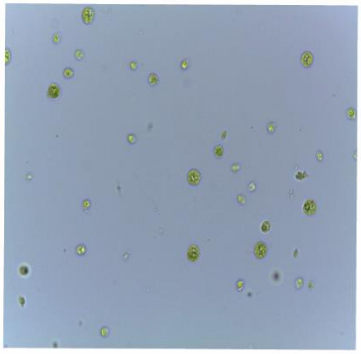


e

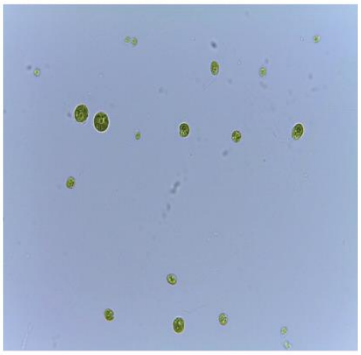
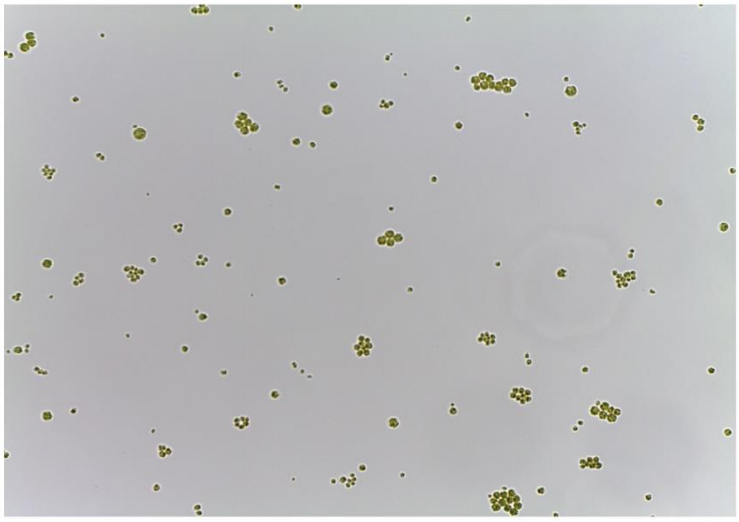


Fig. 3. a. Kill curve of UV mutagenesis for *Gonium pectorale*; b. Spreading vs. punctate phenotype of *Chlamydomonas* vs. *Gonium*, respectively, at a 50:50 ratio plated on soft agar plates optimized by Sarah Cossey and Katherine Johnson; c. wild-type *K3*; d. *uc-1C7*; e. *uc-1H7*

The tripartite wall layer is important for multicellularity

Knowing that the tripartite layer can be extracted and examined, we wanted to know the importance of this conserved layer in relation to multicellularity. To answer this question, wild-type *Gonium* was subjected to treatment with chaotropic agent (potassium perchlorate) followed by quantification of the cell morphology. The control treatment without the chaotropic agent resulted in ~50% of cells being unicellular, whereas the potassium perchlorate treatment resulted in ~95% of cells being unicellular (Fig 4). Surprisingly, potassium perchlorate extraction of the cells walls of wild-type cells not only resulted in a unicellular morphology (Fig 4), but cells were observed to still be swimming, indicating that cell wall extraction did not interfere with cell viability. This suggests that the tripartite layer is important for maintaining cell-cell adhesion in *Gonium pectorale.*

a



b

c

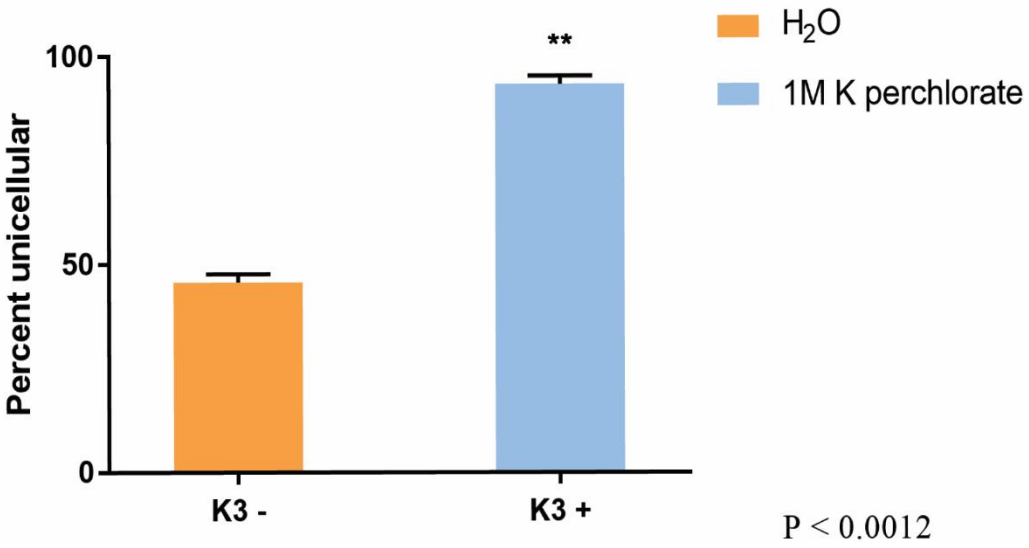

H2O
1M K perchlorate
**
100
50
0
Percent unicellular
K3 -
K3 +
P < 0.0012

Fig. 4. a. *K3* control; b. K3 treated with potassium perchorate; c. Quantification of the percent unicellularity with or without potassium perchlorate

uc-1C7 and uc-1H7 have a compromised cell wall

The *Gonium* mutants *uc-1C7* and *uc-1H7* are unicellular, suggesting that they are missing components important for maintaining the wild-type colonial form. Apart from these two mutants lacking cell-cell adhesion, their life cycle is identical to that of the wild-type. To test if these mutants were lacking components important for the cell wall we performed a detergent lysis test using the non-ionic detergent Nonidet P-40 (NP-40). NP-40 denatures proteins and breaks apart protein-lipid interactions, disrupting the ECM and causing cells that have a compromised ECM to lyse. For this test cells were treated with or without detergent and quantitatively measured for cellular intactness.

When wild-type cells were treated with detergent there was no significant decrease in the number of intact cells (Fig 5a). In contrast, when *uc-1C7* was treated with detergent, the number of intact cells decreased by almost 50% and cellular debris was noticeable. The mutant *uc-1H7* behaves similarly to *uc-1C7* when treated with detergent with a decrease in 25% cellular intactness when compared with wild-type (Fig 5b). These results indicate that *uc-1C7* and *uc-1H7* have a compromised cell wall leading to defects that result in their unicellular form.

uc-1C7 is missing components of its ECM

Knowing that *uc-1C7* has a compromised cell wall, we wanted to determine if this mutant was lacking components of its extracellular matrix. To answer this, we extracted total cell walls from wild-type and the *uc-1C7* mutant then compared equal protein content of *uc-1C7* to wild-type *K3* by SDS-PAGE. Prior to SDS-PAGE it was observed that *uc-1C7* has much less extractable protein form its ECM than wild-type when equal numbers of cells were subjected to

extraction (Fig 5c). Moreover, when separated by SDS-PAGE it was found that *uc-1C7* lacked many protein bands that are present in wild-type (Fig 5c). This shows that *uc-1C7* is missing major components of its tripartite wall layer.

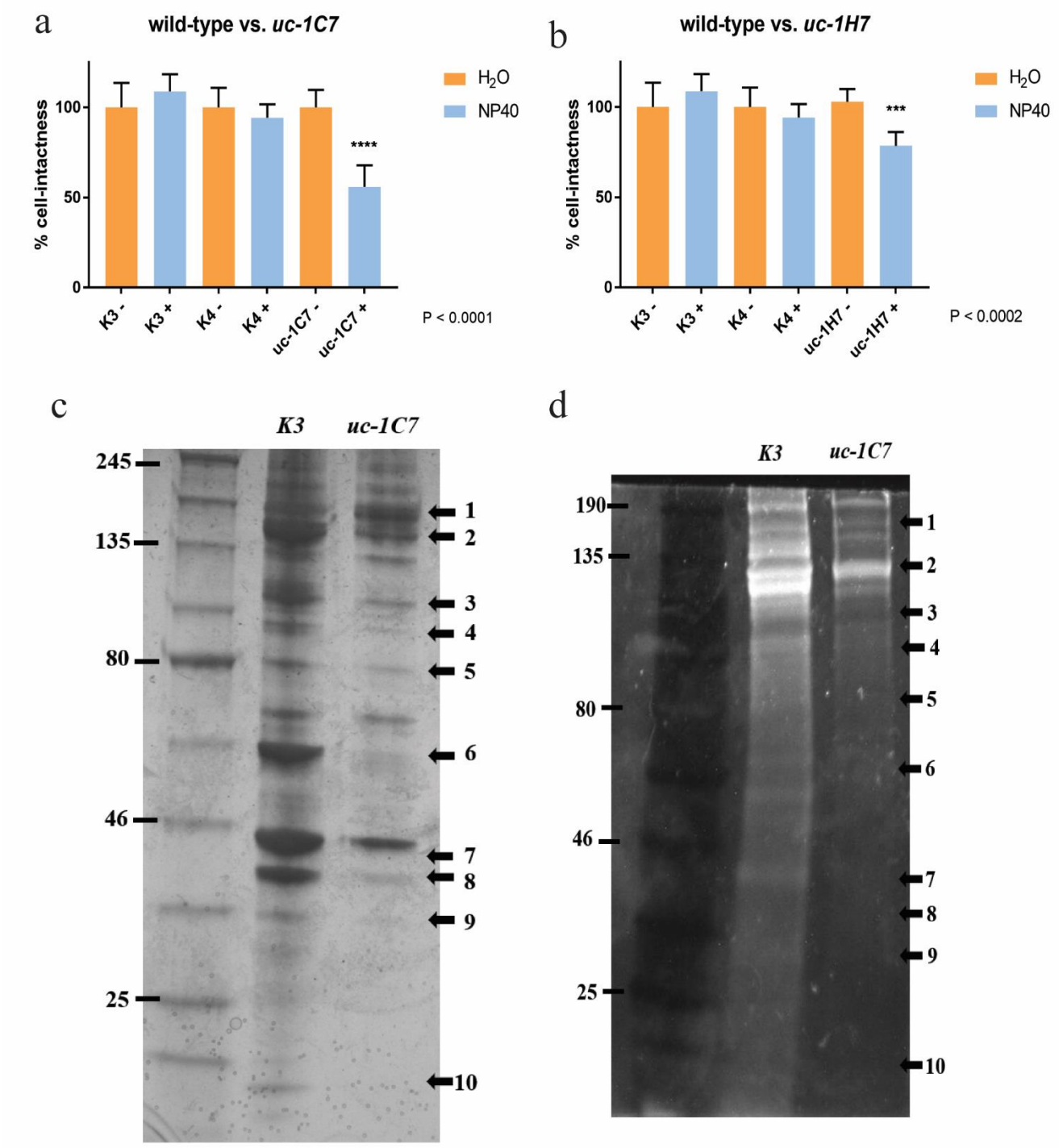

a
wild-type vs. uc-1C7
% cell-intactness
100
50
0
H2O
NP40
****
K3 -
K3 +
K4 -
K4 +
uc-1C7 -
uc-1C7 +
P < 0.0001
b
wild-type vs. uc-1H7
% cell-intactness
100
50
0
H2O
NP40
***
K3 -
K3 +
K4 -
K4 +
uc-1H7 -
uc-1H7 +
P < 0.0002
c
K3
uc-1C7
245
135
80
46
25
1
2
3
4
5
6
7
8
9
10
d
K3
uc-1C7
190
135
80
46
25
1
2
3
4
5
6
7
8
9
10

Fig. 5. a. Quantification of wildtypes *K3* and *K4* comparerd to *uc-1C7* after treatment with or without NP40; b. Quantification of wildtypes *K3* and *K4* comparerd to *uc-1H7* after treatment with or without NP40; c. Silver stained SDS-PAGE of wild-type *K3* vs. *uc-1C7* cell wall extracts, arrows indicating bands that were excised for MS/MS; d. Sypro Ruby stained SDS-PAGE of wild-type *K3* vs. *uc-1C7* cell wall extracts, arrows indicating bands that were excised for MS/MS

Protein identification by tandem mass spectrometry

To determine what proteins were missing in the *uc-1C7* mutant, we took a comparative approach by excising bands from both the wild-type and *uc-1C7* (Fig 5d) from the SDS-page as indicated by arrows 1-10 and sequencing the proteins by MS/MS. Contamination was then cleaned out of the data received and any proteins found in the mutant was removed from the wild-type list, leaving only proteins that were missing from the mutant *uc-1C7.* This comparative analysis revealed matches for proteins localized to the flagellar and boundary zones (table 1). Notably, when comparing proteins found in the wild-type but absent in the mutant, we identified major components of the ECM such as GP3, matrix metalloproteases, and c-type lectin. GP3 is a major glycoprotein of the tripartite layer and is found to interact with other proteins to form a lattice structure on the outermost layer of the ECM [40]. The matrix metalloprotease proteins are orthologous to the matrix metalloproteinase family which are known to break down portions of the ECM and play a central role in processes such as embryogenesis and wound repair [41]. Finally, the c-type lectins are carbohydrate binding proteins known to play a role in cell-cell adhesion [42].

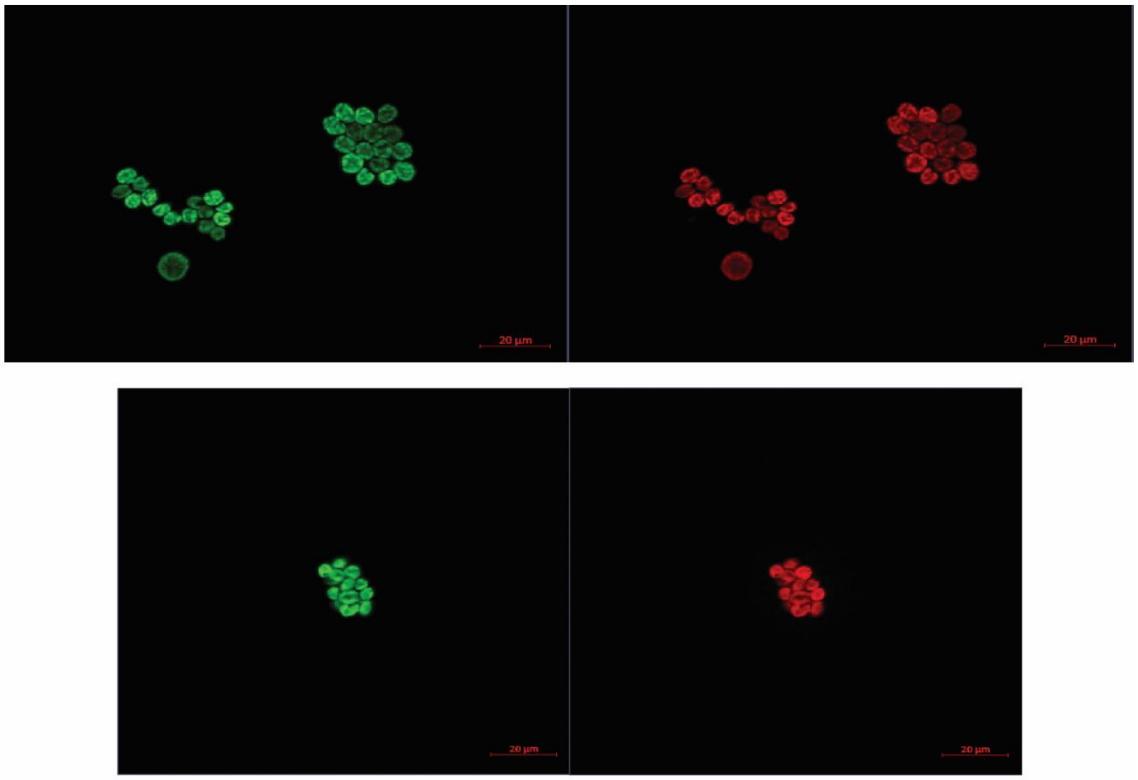
Size	Gene ID	Description from Geneious Blast
A1 180 kDa	scaffold00008.g58.t1	c-type lectin 2
	scaffold00048.g409.t1	protein
A2 145 kDa	scaffold00001.g189.t1	5-oxoprolinase
	scaffold00446.g336.t1	Cell Wall Glycoprotein GP3 Precursor
A5 80 kDa	scaffold00001.g876.t1	HSP70-HSP90
	scaffold00013.g774.t1	matrix metalloproteinase
	scaffold00016.g725.t1	HSP70C
	scaffold00019.g332.t1	flagellular associated protein
	scaffold00068.g385.t1	metalloproteinase of vmp family
A6 58 kDa	scaffold00003.g370.t1	calreticulin calcium-binding protein
	scaffold00004.g823.t1	chaperone 60B
	scaffold00014.g105.t1	basal body protein
A9 30 kDa	scaffold00002.g1344.t1	2-c-methyl-d-erythritol 4-phosphate cytidylyltransferase
	scaffold00023.g40.t1	flagellar associated protein
A10 20 kDa	scaffold00001.g773.t1	calmodulin
	scaffold00006.g718.t1	centriole proteome protein/flagellar protein

Table 1. Cell wall candidates of *uc-1C7* identified by tandem mass spectrometry

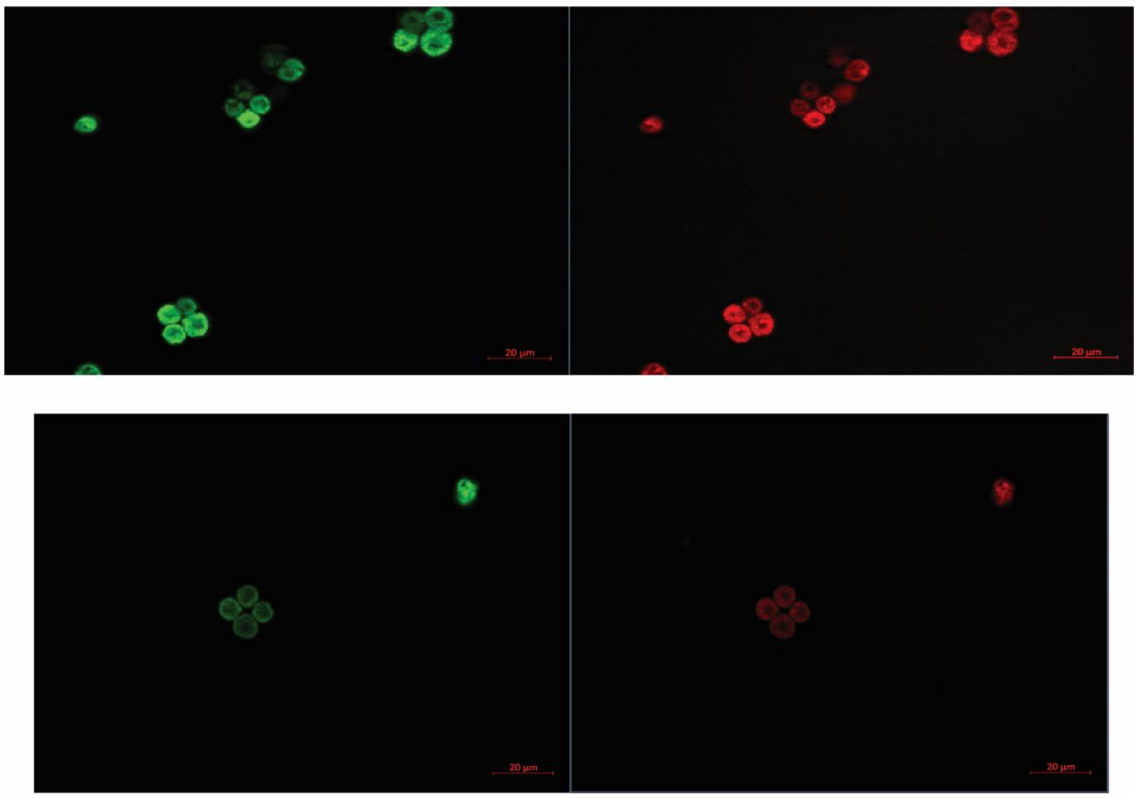
uc-1C7 and uc-1H7 show a weak affinity to a cell-wall antibody

A recombinant camelid nano-antibody fused to a 6X histidine tag and mCherry (clone B11) was previously shown to specifically recognize the cell walls of members of the Volvocales [37]. While the identity of its antigen in cell wall components has not been resolved, it is known that immunoprecipitation of cell walls with this antibody result in enrichment of known ECM proteins such as GP3 and many hydroxyproline rich glycoproteins (Bradley Olson, personal communication, not shown). This anti-cell wall mCherry antibody was used to quantify the amount of cell wall components in wild-type and unicellular mutants. After incubation with the anti-cell wall mCherry antibody, cells were mounted and observed by fluorescence microscopy. Signal for the anti-cell wall antibody was measured in the XX nm channel and normalized to the chlorophyll auto fluorescence from the cells in the XX nm channel. Chlorophyll is known to be proportional to cellular biomass in *Chlamydomonas*, and this was also found to be true in *G. pectorale* (Christopher Berger, personal communication). When both *uc-1C7* and *uc-1H7* are compared to wild-type *K3* and *K4*, both unicellular mutants showed a reduced amount of normalized anti-cell wall fluorescence signal from the antibody compared to wild-type controls (Fig 6). This supports the conclusion that *uc-1C7* and *uc-1H7* are missing components of their cell walls that promote undifferentiated colony formation.

a



b



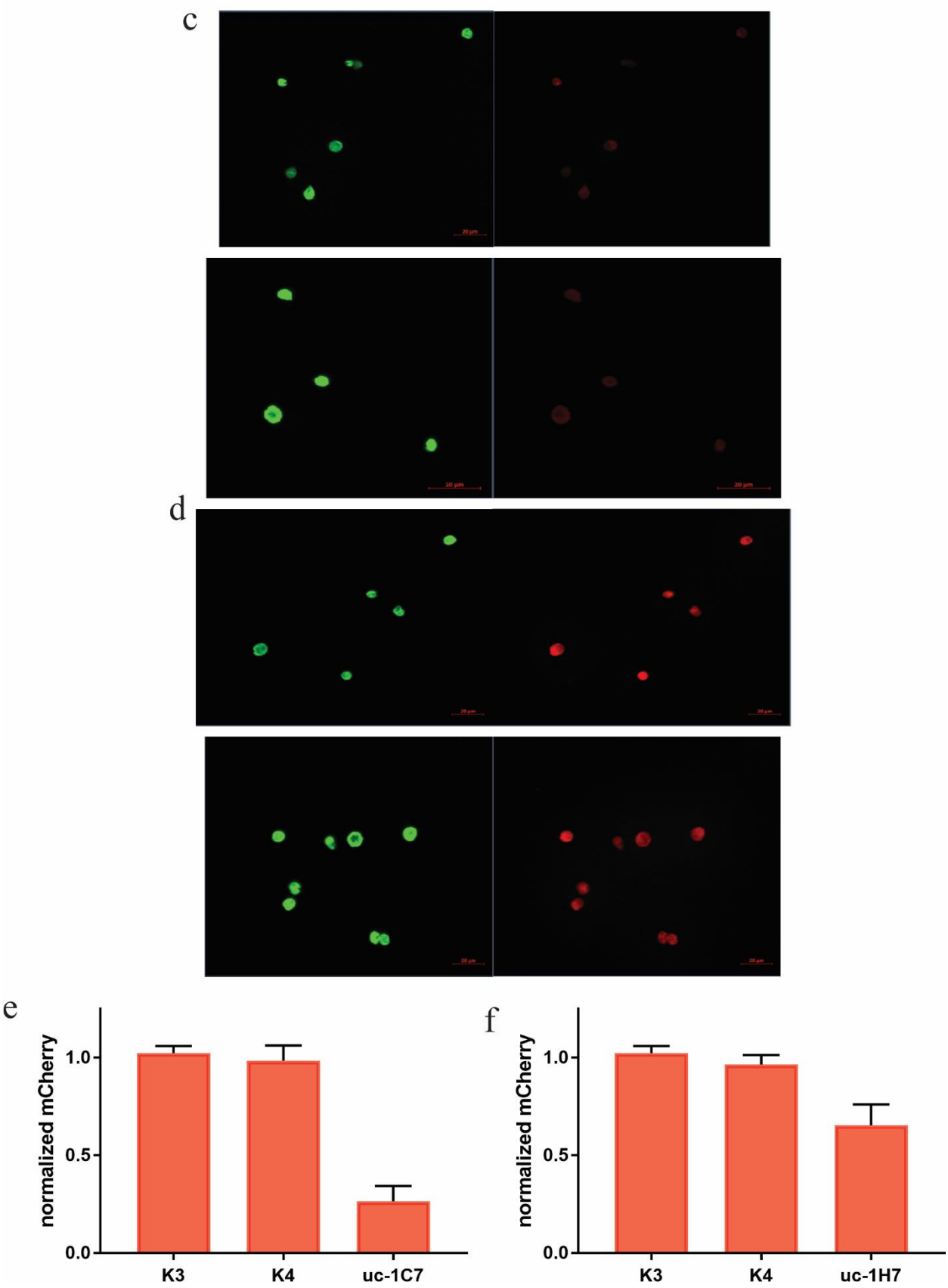

c
d
e
normalized mCherry
1.0
0.5
0.0
K3
K4
uc-1C7
f
normalized mCherry
1.0
0.5
0.0
K3
K4
uc-1H7

Fig. 6. a. *K3* chlorohyll auto-fluorescene (green) and anti-cell wall mCherry (red) b. *K4* c. *uc-1C7* d. *uc-1H7* e. Normalized mCherry signal comparing wild-types *K3* and *K4* to *uc-1C7*; f. Normalized mCherry signal comparing wild-types *K3* and *K4* to *uc-1H7*

Discussion

In this project, we have characterized in depth the phenotypes of two unicellular *Gonium* mutants. These mutants have a defect in extracellular matrix assembly providing proof that the ECM is important for multicellularity. In these experiments we focused on the ECM of *Gonium pectorale,* an undifferentiated member of the Volvocales that shares a close relation with the unicellular *Chlamydomonas* and the fully differentiated multicellular *Volvox.* While the ultrastructure of the ECM has been shown to be highly similar in previous studies, there is little molecular evidence connecting the similar phenotypes of the ECM to the genotypes of the morphologically different species. While this study only focuses on defining the phenotypes of two unicellular mutants, there is ample room to extend the knowledge attainable from these mutants.

To further analyze the phenotypes of these mutants, it would be worthwhile to examine the ultrastructure of both mutants to determine if the various layers are visibly altered in relation to the wild-type. This would allow us to isolate the layers of the ECM that are missing proteins important for cell-cell adhesion and, therefore, determine which of the four zones have undergone modifications to facilitate this important evolutionary requirement for multicellularity.

In the future, it is imperative to identify the mutation loci of these two mutants in order to characterize the genes important for ECM assembly and, furthermore, for multicellularity. Additionally, the genes identified from this study should be compared to orthologs of other

members of the Volvocales to determine not only how the genes have evolved with varying morphologies but to understand how the genes are regulated across species throughout the cell cycle. This can be accomplished by comparing the genes of interest from known transcriptome data of *Gonium pectorale* and to the expression patterns of *Chlamydomonas* and *Volvox.*

While we have yet to scratch the surface of the *uc-1H7* phenotype, the large number of tripartite wall components missing from *uc-1C7* as well as the sensitivity of this mutant to detergent lysis suggests that this mutant is lacking proteins from its ECM. Because of this, we can infer that the pathway responsible for that biogenesis of ECM components is functionally deficient. If this proves to be true, this mutant can provide insight into the genes involved in the ECM biogenesis pathway in the Volvocales.

Overall, *Gonium pectorale* is a simplistic model system that can provide insights into the modifications required to evolve a complex ECM responsible for maintaining cell-cell adhesion and facilitating cell-cell communications. This model system should be further exploited by taking advantage of cell cycle transcriptome data, genomic comparisons, and continuing the exploration of isolated unicellular *Gonium* mutants to better understand the role of the extracellular matrix for the evolution of multicellularity.

The Volvocales provide a unique opportunity to study the importance of the evolution of the extracellular matrix for multicellularity in eukaryotic cells. In humans and other eukaryotic organisms, faults in the production of the extracellular matrix typically leads to defects in skeletal and neuronal generation and can lead to lethality of the organism [45]. While this is an interesting subject to study in vertebrate systems, the complexity of the many cell types in these organisms can often make it difficult to study minute modifications to the ECM [46]. In contrast, the Volvocales provide a simplistic model for studying the importance of the ECM for

multicellularity with, at most, two different cells types in the differentiated *Volvox*. The range of morphological phenotypes, genome level similarity, and well established molecular protocols make it the ideal system for studying the evolution of multicellularity.

Materials and Methods

Strains and mutagenesis

The wild-type strains of *Gonium pectorale* used throughout this study were *K3* and *K4*. Cells were grown in either Standard Volvox Media (SVM) or Standard Volvox Media with Acetate (SVMA) at 30 C under continuous light. Mutants were generated by UV mutagenesis. After treatment, mutants were plated on soft agar SVMA plates (0.75%) and placed on a light shelf until colonies began forming. Colonies were picked and grown in a 96-well plate, where they were then sorted according to size by flow cytometry. Those that were selected were then phenotyped by light microscopy for unicellularity.

Removal of the tripartite wall layer

Wild-type *K3* cells were harvested by centrifuging at 600g for 3 minutes. Cells were resuspended in either deionized water or 1M potassium perchlorate for 10 minutes and then fixed using 1% paraformaldehyde. Cells were then examined by light microscopy and counted up to 100 in triplet. The percent unicellularity for each treatment was then calculated and graphed using GraphPad Prism software.

Detergent lysis

For detergent lysis, wild-type *K3*,*uc-1C7,* and *uc-1H7* cells were grown in SVM under continuous light at room temperature. Cells were then harvested by centrifuging for 5 minutes at 600g resuspended in either 0.1% Nonidet P40 (NP-40) or water as a control for 30 minutes. Cells

were then fixed in 1% paraformaldehyde and counted on a hemocytometer for cell intactness. An unpaired t test was performed comparing the control to the treatment to identify significance.

Cell wall extraction

Both *uc-1C7* and wild-type *K3* strains were grown in SVM and harvested by centrifuging at 3000g for 5 minutes. The SVM was decanted and the pellet was washed twice with deionized water. Cell walls were extracted by treating cells with 1M potassium perchlorate for 20 minutes with occasional gentle agitation. Cultures were then pelleted at 3000g for 3 minutes and the supernatant was transferred to a fresh tube. The supernatant was then centrifuged again at 40,000g for 10 minutes to pellet any unwanted debris. The clear supernatant was saved and measured for protein content using the Bradford assay and equal protein content was used for SDS-PAGE which was silver stained to resolve protein bands.

Tandem mass spectrometry

Cell walls were extracted, and total protein content measured using the Bradford assay [36] for protein concentration. Equal protein content (15ug) was loaded onto an SDS-PAGE and stained using SYPRO Ruby according to the protocol provided by the manufacturer (Thermofisher Scientific cat no. S12000). Protein bands of interest were excised and sent to the Oklahoma State University Proteomics Center for testing on an Orbitrap Fusion Tribrid. The programs used for fragment identification were MaxQuant/Perseus and Scaffold Proteome Software.

Analysis of MS/MS data

The output for MS/MS was a file for the program Scaffold. The file format was extracted and opened in Microsoft Excel where all further analyses were carried out. From this we simplified the data table by eliminating uninformative content for use with the scaffold software that was irrelevant for the analysis. The candidate list was narrowed down by eliminating

proteins that were present in both the wild-type and *uc-1C7* bands, leaving only proteins that were present in the wild-type but absent in *uc-1C7*. From this filtered list, proteins were narrowed down by selecting proteins that were only identified in a single band or those identified in adjacent bands, ex. Band 1 and 2 or band 2 and 3. From the singles and doubles isolated, we retrieved the accession numbers and used them to find the corresponding BLAST2GO hits. Using the BLAST2GO descriptions, candidates were manually selected based on their role in extracellular matrix localization and assembly. Protein sequences for the candidates were obtained and submitted to BLASTP and the best hit for *Chlamydomonas reinhardtii* were selected. Out of the Volvocales, the *Chlamydomonas reinhardtii* genome is the most well annotated and thus provided the most knowledge as to the function of orthologous genes from *Gonium pectorale*. We then matched the hits found from the BLAST with the GO analysis descriptions and from these annotations, the candidate list was further manually narrowed down to those proteins known to localize to the extracellular matrix.

Cell wall antibody

E. coli Rossetta cells were transformed using heat shock to uptake the mCherry VHH B11 plasmid obtained from and placed in autoinduction media [32] overnight. Cells were harvested and the cell wall antibody was extracted by immobilized metal affinity chromatography REF-Weeks paper on antibody.

Fluorescence microscopy

Gonium cells were grown in SVM and harvested by centrifugation. Cells were incubated in 1:1000 VHH B11 antibody for 1 hour with gentle shaking at room temperature. Cells were washed twice in SVM and incubated again with DAPI at 1:5000. Cells were mounted using Aqua-Poly/Mount from Polysciences, Inc. as described in the protocol by the manufacturer.

After 24 hours, cells were imaged on a microscope at 200x magnification. The excitation wavelengths were 552 and 584, for chlorophyll auto-fluorescence and mCherry, respectively, and the emission wavelengths were 602 and 590, respectively.

Analysis of fluorescence microscopy

Images were processed using ZEN Zeiss software to export multi-channel images of colonies and/or cells labelled with the anti-cell mCherry to that of the chlorophyll auto-fluorescence to normalize for biomass per cell or colony. Images were analyzed in ImageJ (version 1.8.0_112) to measure the amount of fluorescence per cell according to Burgess and McCloy [33,34]. The measurements were exported to excel to calculate the corrected total cell fluorescence for both auto-fluorescence and mCherry. The mCherry intensity was then normalized by dividing the corrected total cell fluorescence of mCherry by the chlorophyll auto-fluorescence and then plotted as a bar graph using GraphPad Prism software.

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