DISTAG/TBCCd1 Is Required for Basal Cell Fate Determination in Ectocarpus

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DISTAG/TBCCd1 IS REQUIRED FOR BASAL CELL FATE DETERMINATION IN *ECTOCARPUS*

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ABSTRACT

Brown algae are one of the most developmentally complex groups within the eukaryotes. As in many land plants and animals, their main body axis is established early in development, when the initial cell gives rise to two daughter cells that have apical and basal identities, equivalent to shoot and root identities in land plants, respectively. We show here that mutations in the *Ectocarpus DISTAG* (*DIS*) gene lead to loss of basal structures during both the gametophyte and the sporophyte generations. Several

abnormalities were observed in the germinating initial cell in *dis* mutants including increased cell size, disorganisation of the Golgi apparatus, disruption of the microtubule network and aberrant positioning of the nucleus. *DIS* encodes a TBCCd1 protein, which has a role in internal cell organization in animals, *Chlamydomonas* and trypanosomes. Our study highlights the key role of subcellular events within the germinating initial cell in the determination of apical/basal cell identities in a brown alga, and emphasizes the remarkable functional conservation of TBCCd1 in regulating internal cell organization across extremely distant eukaryotic groups.

SHORT TITLE

Basal cell fate determination in *Ectocarpus.*

ONE SENTENCE SUMMARY

Mutations in the *Ectocarpus DISTAG* gene, which encodes a TBCCd1 protein, disrupt the subcellular architecture in the germinating initial cell leading to loss of all basal structures from the developing alga.

INTRODUCTION

Events in the zygote leading up to and including the first cell division play a key role in the developmental patterning of multicellular organisms. In many plants and animals, asymmetric division of the zygote establishes the principal body axis of the early embryo, representing the first major patterning event during embryogenesis (Gönczy and Rose, 2005; Ueda and Laux, 2012). In land plants, the first cell division usually coincides with the establishment of apical and basal cell identities (Ueda and Laux, 2012) (but note that some fern gametophytes have tripolar germination patterns, Schneider, 2013). In *Arabidopsis* the asymmetrical division of the zygote is associated with a number of cellular events including movement of the nucleus to the apex of the cell, repositioning of other major organelles, formation of a large vacuole in the basal part of the cell and reorganisation of the microtubules into transverse cortical arrays associated with cell

outgrowth in the apical direction (Jeong et al., 2011a; Kimata et al., 2016; Lau et al., 2012). The establishment of the apical-basal axis in *Arabidopsis* has been shown to be controlled by a complex genetic network involving both auxin-dependent and auxin-independent pathways (Lau et al., 2012). The auxin-dependent network involves the transcription factor MONOPTEROS/AUXIN RESPONSE FACTOR 5, the auxin response inhibitor BODENLOS/INDOLE-3-ACETIC-ACID 12 and the auxin efflux carrier PIN-FORMED 7, whereas the auxin-independent pathway involves EMBRYO SURROUNDING FACTOR 1 (ESF1), the receptor-like cytosolic kinase SHORT SUSPENSOR, the MAP kinase kinase YODA, the MAP kinases MPK3 and MPK6 and several transcription factors including GROUNDED/RKD4 and WOX2 (Jeong et al., 2011b; Waki et al., 2011; Costa et al., 2014; Lau et al., 2012; Rademacher et al., 2012). However, it is not yet clear how these genetic networks implement the cellular events that underlie the formation of the apical-basal axis and the establishment of apical and basal cell identities.

The brown algae have evolved complex multicellularity independently of land plants but the two lineages share several key developmental characteristics such as the importance of the establishment of the apical/basal axis during development, extensive post-embryonic developmental patterning and developmental programs constrained by the absence of cell migration. The apical and basal structures of brown algae and land plants can also be considered to be analogous, with the basal systems of brown algae being functionally equivalent to rooting structures in land plants. In both lineages, the basal systems are composed of tip-growing filamentous rhizoid cells that extend into the substrate or air/water surrounding the plant and are used for anchoring (Jones and Dolan, 2012). In both brown algae and flowering plants apical/basal polarity is usually determined very early, before the first cell division (Fritsch, 1935). However, this process may be less complex in brown algae, because the development of initial cells (e.g. the zygote) does not occur within maternal tissues. Consequently, axis formation is expected to occur in a cell autonomous manner (but see (Whitaker, 1931) and not to be influenced by signals emitted by nearby parental cells (Costa et al., 2014).

The brown alga *Fucus* has been used for many years as a model system to study apical-basal axis formation during embryogenesis (e.g. Coelho et al., 2002; Brownlee and Bouget, 1998; Kropf et al., 1988). As in land plants, the zygote cell divides asymmetrically to establish the apical-basal axis (Bouget et al., 1998; Goodner and Quatrano, 1993). The two products of this division go on to produce the apical and basal systems of the alga, the thallus and the rhizoid, respectively (Brownlee and Bouget, 1998). The establishment of the apical-basal axis in *Fucus* has been studied in detail at the cellular level and has been shown to involve position dependent information from the cell wall (Berger et al., 1994). Apoplastic diffusible gradients appear also to be involved in pattern formation in the multicellular *Fucus* embryo (Bouget et al., 1998).

Whilst the large zygote and the external fertilisation process (fusion of male and female gametes released from the parent thallus) of *Fucus* makes it well adapted for the study of the cell biology of axis formation, this genus is not amenable to genetic approaches and, consequently, the genetic networks that control apical-basal axis formation have not been characterized in the brown alga lineage. Recently, the filamentous alga *Ectocarpus* sp. has emerged as a genetic model for the brown algae (Coelho et al., 2012c; Cock et al., 2014). A high-quality genome sequence is available for this species (Cock et al., 2010; Cormier et al., 2017), together with extensive transcriptomic data (e.g. Lipinska et al., 2015; Luthringer et al., 2015) and genetic tools including a dense genetic map (Heesch et al., 2010; Avia et al., 2017). Genetic transformation is not yet possible but gene knockdown using RNA interference has been demonstrated (Macaisne et al., 2017). These various tools were employed in a recent study that was the first to identify a brown algal developmental gene using a forward genetic approach (Macaisne et al., 2017).

Ectocarpus has a complex life cycle involving alternation between multicellular sporophyte and gametophyte generations (Coelho et al., 2012c; Peters et al., 2008) (Supplemental Figure 1). Unlike land plants, both the sporophyte and the gametophyte are derived from single cells, which are released into the seawater medium (gametes and meio-spores, respectively) greatly facilitating the analysis of early events during development. This characteristic also means that any parental influence on developmental

processes is essentially limited to information provided to the propagules before their release (but see also Arun et al., 2013).

Both the gametophyte and the sporophyte possess an apical-basal axis and clearly defined apical and basal filamentous systems (Coelho et al., 2011; Peters et al., 2008). In the gametophyte, the basal system consists of rhizoids and the apical system is composed of upright filaments that bear the gametangia. In the sporophyte, the basal system is more extensive, consisting of a network of firmly attached basal filaments, whereas the apical system resembles that of the gametophyte, consisting of branched upright filaments that bear the sexual structures. The general similarities between the gametophyte and sporophyte generations, in terms of their size and overall morphologies, make *Ectocarpus* an ideal system to investigate how two distinct developmental programs can be deployed from the same genome.

In this study, we report the identification of the *DISTAG* (*DIS*) locus, which is required for the formation of basal structures during both the sporophyte and gametophyte generations of the *Ectocarpus* life cycle. *DIS* encodes a Tubulin Binding Co-factor C (TBCC) domain protein of the TBCCd1 class. Mutations in the *Ectocarpus DIS* gene are associated with several modifications at the initial cell stage: disorganisation of the Golgi apparatus, increased cell size, disruption of the pattern of the microtubule network and aberrant positioning of the nucleus. *dis* mutants therefore link subcellular events within the initial cell with the acquisition of apical/basal cell identities. The phenotypes of *dis* mutants also confirm that the basal filament system of the sporophyte is developmentally equivalent to the rhizoid of the gametophyte generation, providing insight into the evolutionary events that led to the emergence of the sporophyte and gametophyte developmental programs in this species.

RESULTS

dis mutants lack a basal system

During the *Ectocarpus* gametophyte generation the apical/basal axis is established in the initial cell, prior to the first cell division. The two cells derived from the division of

142 the initial cell grow, in the form of two germ tubes, to establish a rhizoid (basal, root-like
143 organ) and a filamentous thallus (apical, shoot-like organ) (Figure 1). A UV mutagenesis
144 screen identified two mutant strains (Ec722 and Ec799, Supplemental Table 1;
145 Supplemental Figure 2) that failed to develop any of the basal structures normally
146 observed in the wild type gametophyte generation, i.e., the initial cells of the mutants
147 immediately developed as apical upright filaments.

148 During the early development of the wild type sporophyte generation, establishment of
149 apical structures is delayed and an extensive system of prostrate filaments consisting of
150 round cells is formed before the apical thallus filaments develop (Peters et al., 2008;
151 Figure 1). Several observations indicate that this basal network of prostrate filaments is
152 equivalent to the basal system of the gametophyte generation, represented by the rhizoid:
153 1) both structures develop at the base of the alga (in contact with the substratum), 2) both
154 structures serve an anchoring function and 3) in sporophytes carrying the *immediate*
155 *upright (imm)* mutation the system of prostrate filaments is homeotically replaced by a
156 rhizoid similar to that of the gametophyte (Peters et al., 2008; Macaisne et al., 2017).

157 The sporophyte generations of the Ec722 and Ec799 mutants failed to produce a
158 network of prostrate filaments and therefore also lacked basal structures (Figure 1). The
159 establishment of reproductive structures on apical systems was unaffected in both
160 generations, and the mutants were fully fertile as both gametophytes and sporophytes
161 (Figure 1).

162 The absence of a basal system significantly affected the capacity of the mutants to
163 adhere to the substratum (Figure 2) and consequently Ec722 and Ec799 were named
164 *distag-1* (*dis-1*) and *distag-2* (*dis-2*), respectively ('distag' means detached in the Breton
165 language). The loss of basal systems during both the gametophyte and sporophyte
166 generation in *dis* mutants is consistent with these two systems (rhizoids and prostrate
167 filaments, respectively) being developmentally equivalent between the two generations.
168 Developmental equivalence between the basal systems of the two generations was further
169 supported by the observation that when *dis-1 imm* double mutants were constructed these
170 lacked the rhizoid that replaces the prostrate filaments in the *imm* mutant (Figure 2).

Analysis of a segregating family generated from a cross between *dis IMM* and *DIS imm* parents indicated that *DIS* is epistatic to *IMM* (Supplemental Table 2).

In wild type *Ectocarpus*, secondary rhizoids are produced from the apical system cells at a late stage of development (Peters et al., 2008) (Figure 1). These rhizoids can be considered to be analogous to the adventitious roots produced from the stems of some land plants (Atkinson et al., 2014). The *dis-1* and *dis-2* mutants failed to produce secondary rhizoids during both the sporophyte and the gametophyte generations. Hence, production of all basal structures, both primary and secondary, was blocked in these mutants.

In fucoid brown algae, the apical system (the thallus), responds to damage by producing rhizoids (e.g. (Bouget et al., 1998), and we observed a similar phenomenon in wild type *Ectocarpus*. In contrast to the wild type, wounded apical filaments of the *dis* mutants failed to regenerate rhizoids, and instead produced new cylindrical apical filament cells (Figure 2; Supplemental Figure 3). Taken together, our results indicate that basal system formation fails to occur, at all developmental stages, in the absence of a functional *DIS* gene product.

One consequence of the loss of basal structures in the *dis* mutants is that the sporophyte and gametophyte generations are more similar than in the wild type because in both cases the vegetative thalli consist uniquely of upright filaments made up of cylindrical cells. Despite this resemblance, the sporophyte and gametophyte generations of the *dis* mutants retained generation-specific features. First, morphometric measurements showed that the length and width of the filament cylindrical cells and the angle at which secondary upright filaments branch off from primary filaments statistically distinguished sporophyte from gametophyte thalli in both wild type and *dis* mutant strains. In contrast, no significant differences were detected when these parameters were compared between the same generation of wild type and mutant strains (Supplemental Table 3). Second, at maturity *dis* gametophytes produced plurilocular gametangia, which contained gametes that were able to fuse with gametes of the opposite sex to produce zygotes. The sporophyte generation of the *dis* mutants, on the other hand,

produced plurilocular sporangia containing spores (incapable of fusion) and unilocular sporangia with haploid, meiosis-derived meio-spores. Finally, the expression patterns of generation-specific marker genes (Peters et al., 2008) were consistent with assigned life cycle generations in both wild type and *dis* individuals (Supplemental Figure 4).

Analysis of the *dis* transcriptome

To further characterize the Dis$^-$ phenotype, an RNA-seq approach was employed to study gene expression in the *dis-1* mutant compared with wild type basal and apical tissues (Supplemental Table 4). Consistent with the fact that *dis* has lost the ability to produce a basal system and is composed exclusively of apical system cells, analysis of *dis* transcriptional profiles indicated that they were more similar to apical than to basal transcriptomes of the wild type strain (Figure 3A, 3B; Supplemental Figure 5).

Several analyses were carried out to characterize the set of genes that were differentially expressed in the three tissues analysed. These included an analysis of the putative functions of clusters of genes with shared patterns of regulation (Supplemental Table 5 and 6), an analysis of the 200 most differentially expressed genes (100 most up- and 100 most down-regulated) in these comparisons (Supplemental Table 7 and 8), an analysis of the differentially regulated genes that coded for proteins with putative signal peptides (representing putative secreted proteins, Supplemental Table 9 and 10) and a KEGG pathway analysis (Supplemental Table 11). Global patterns in the changes in gene expression were analysed using a set of manually assigned functional categories (based on the functional categories used in Tarver et al. (2015). GO term enrichment analysis (Blast2Go; Conesa and Götz, 2008) and KEGG analysis were then used to provide more detailed information about the processes and pathways affected. The relative frequency of the manually assigned functional categories was visualized using a word cloud (Figure 3C). Note that about half of the genes in the transcriptome datasets were of unknown function, and these were excluded from our analysis.

Putative basal and apical system effector genes. The gene cluster analysis focused on genes that were either up- or downregulated in both the *dis-1* mutant and wild type apical tissues compared to wild type basal tissues (Figure 3A, 3B; Supplemental Table 5).

The aim of this approach was to identify potential basal and apical system effector genes. The set of genes that was upregulated in *dis* and the wild type apical system compared to the wild type basal system (potential apical system effectors) were enriched in the GO categories 'kinase activity', 'protein modification', 'cell differentiation' and 'cell communication' (Fisher's exact test, $p<0.05$) while the genes that were downregulated in both *dis-1* and the apical system compared to the basal system (potential basal system effectors) were enriched in categories related to 'transcriptional activity', 'signal transduction' and 'extracellular' or 'membrane-located processes' (Supplemental Table 6). Accordingly, the assignment of basal and apical effector genes to manually curated functional groups highlighted an important proportion of genes coding for proteins putatively involved in membrane functions and transport, cell wall biosynthesis and modification, vesicle trafficking, adhesion, cell regulation and signalling (Figure 3C upper panel, Supplemental Table 12).

Top-200 most differentially regulated genes. GO term enrichment analysis of the 200 most differentially regulated genes in the *dis-1* mutant compared with wild type basal and apical systems highlighted a range of GO term categories (Supplemental Table 8), but there was a particular abundance of genes with functions related to 'membrane', 'oxidation-reduction process' and 'carbohydrate metabolic processes'. The cellular components highlighted by the GO term analyses were 'membrane' and 'extracellular region'. Manual assignment to functional categories revealed that the common functional groups represented within this set of genes included cell wall biosynthesis and organization, membrane function and transport, cell regulation and signalling, adhesion, vesicle transport and cytoskeleton (Figure 3C middle panel, Supplemental Table 12).

Secreted proteins. The set of differentially expressed genes that were predicted to encode secreted proteins were analysed in detail because the phenotypes of the *dis* mutants are associated with disruption of the Golgi apparatus. Interestingly, a disproportionate number of genes coding for proteins with a putative signal peptide were present in the pool of genes that were differentially regulated in *dis* versus wild type basal system (Fisher test, p-value=0.00002877 and p-value=0.01992 for up and downregulated genes, respectively; Supplementary Table 4). Analysis of the GO term associated with

these genes indicated enrichment for range of GO categories including carbohydrate transport, transmembrane transport, extracellular components, integral components of membrane, transmembrane signalling and Golgi associated vesicles (Supplemental Table 10). The most commonly represented manually annotated functional categories represented within this dataset included, as above, cell wall, adhesion, extracellular processes, membrane function and transporters, cytoskeleton, and cellular regulation and signalling (Figure 3C lower panel).

KEGG analysis. Analysis of the assignment of the differentially expressed genes to KEGG pathways (Xie et al., 2011) revealed a number of processes that were differentially regulated in *dis* versus wild type samples. Pathways involved in interactions with the extracellular-matrix, protein processing in the endoplasmic reticulum and protein export were upregulated in the *dis-1* mutant compared with the wild type basal system, whereas signalling pathways and protein processing in the endoplasmic reticulum were downregulated (Supplemental Figure 7; Supplemental Table 11).

Taken together, comparison of the *dis-1* transcriptome with wild type basal and apical tissues suggested that carbohydrate/cell wall-related processes, membrane transport, cellular signalling and secretory activity may be affected in *dis* mutants.

Note that transcript abundance was also measured using an independent method (RT-qPCR) for 12 of the differentially regulated genes to verify that the differential expression patterns observed were robust (Supplemental Figure 6). Globally, the results of the RT-qPCR analysis confirmed those obtained based on the RNA-seq data (Spearman's correlation R^2=0.619, p-value = 0.0012).

Loss of bipolar germination from the initial cell in *dis* mutants

The vast majority of initial cells of both the sporophyte and gametophyte generations of the *dis* mutants produced a single germ tube rather than the two germ tubes normally observed with wild type strains (Peters et al., 2008). This phenotype was observed more consistently with the *dis-2* mutant than with the *dis-1* mutant (85% and 95% for *dis-1* and *dis-2* sporophytes respectively, n=591; 82% and 95% for *dis-1* and *dis-2* gametophytes

respectively, n=54) (Figure 2). We also noted that, whilst the *dis-2* mutant completely failed to produce a second germ tube, a proportion of *dis-1* individuals (65%, n=235) produced one or more enlarged and abnormally shaped cells at the end where the second germ tube would normally emerge, possibly corresponding to an aborted basal system (Figure 2).

Genetic analysis and identification of the *DIS* gene

Sporophytes can be propagated asexually through the production of mito-spores (Figure 1). The Dis$^-$ mutant phenotype was stable through 30 rounds of asexual generations via mito-spores.

A male *dis-1* gametophyte (Ec722) was crossed with an outcrossing wild type female gametophyte of the strain Ec568 (Coelho et al., 2011; Peters et al., 2008) (Supplemental Table 1, Supplemental Figure 2). The resulting sporophyte (Ec653) exhibited a wild type pattern of development indicating that the *dis-1* mutation was recessive. A segregating population of 200 individuals derived from this cross consisted of 92 and 108 phenotypically wild type and mutant individuals, respectively, consistent with a 1:1 segregation ratio and Mendelian inheritance of a single-locus recessive mutation (Chi-square test = 1.28, df = 1, p-value = 0.257). A female individual from this progeny that carried the *dis-1* mutation was crossed with a male gametophyte of the *dis-2* strain (Ec799). The resulting sporophytes (Ec808, Ec809) exhibited a Dis$^-$ phenotype indicating that the two mutations *dis-1* and *dis-2* were allelic.

A cloning-by-sequencing approach (Schneeberger et al., 2009) identified a candidate locus on chromosome 05 for the location of the mutation in the *dis-1* mutant (Figure 4A) and sequencing of this genomic region identified a single nucleotide mutation at position 3330523 that was present in the *dis-1* mutant (Ec722) but absent from the Ec32 and Ec568 wild type strains (Figure 4B). This mutation was located in intron 15 of the gene Ec-05_001860. A cleaved amplified polymorphic sequence (CAPS) marker, developed based on the candidate causal mutation, showed absolute co-segregation of this mutation with the Dis$^-$ phenotype in 265 individuals of the segregating family derived from sporophyte Ec653. Re-sequencing of the Ec-05_001860 gene in the *dis-2* mutant

identified a point mutation in exon 13 that results in the introduction of a stop codon into the coding region of the gene (Figure 4B). Taken together, these analyses provide strong evidence that Ec-05_001860 corresponded to the *DIS* gene.

A protocol for genetic transformation does not exist for *Ectocarpus* (or for any other brown alga), but injection of double stranded RNA into zygotes of the brown alga *Fucus* has been shown to induce an RNA interference (RNAi) response, leading to knockdown of target gene expression (Farnham et al., 2013). We have recently adapted this *Fucus* RNAi protocol for *Ectocarpus,* using synthetic siRNA molecules instead of long double stranded RNA molecules and a lipofectant specifically adapted for dsRNA delivery to introduce the siRNAs into the cell rather than the microinjection procedure used for *Fucus* (Macaisne et al., 2017).

Following simultaneous introduction of three siRNA molecules targeting the *DIS* gene, a small proportion (about 2.5%) of the parthenogenetic gametes exhibited a pattern of development that closely resembled the phenotypes of the *dis* mutants (Figure 4C); the gametes emitted a germ tube in a unipolar manner. Germination gave rise to an upright filament consisting of the cylindrical cells typical of the apical system. The gametes that showed unipolar germination occasionally produced an aborted second germ tube with an abnormally shaped cell similar to the enlarged and abnormally shaped cells that are sometimes produced by *dis-1* mutants. This phenotype was not observed in the control treatments, where initial cells were incubated with siRNA molecules directed against another *Ectocarpus* sp. gene (Ec-13_001890). These observations indicated that RNA-interference-induced knockdown of *DIS* gene expression had the same developmental consequences as the *dis* mutation in a proportion of the treated individuals, further confirming that Ec-05_001860 corresponds to the *DIS* gene.

Expression pattern of the *DIS* gene during the life cycle

RT-qPCR analysis indicated that, as expected, *DIS* transcript abundance was significantly reduced in both *dis-1* and *dis-2* mutants (Supplemental Figure 8).

We used RNA-seq to assay the abundance of the *DIS* transcript throughout the life cycle of *Ectocarpus*. Specifically, we compared the expression of *DIS* at several stages of the ontogeny of the gametophyte and in several tissues of the sporophyte generation (Supplemental Figure 1B). This analysis indicated that transcription of *DIS* was upregulated in the basal system compared with the apical upright system during the sporophyte generation (pairwise analysis between apical system and basal system, FC=1.9; p_{adj}= 7.66E^{-06}). The *DIS* transcript was detected at low abundance during the gametophyte generation (which consists predominantly of apical filament cells), similar to the level detected in the apical system of the sporophyte (Supplemental Figure 8). Taken together, the expression pattern of *DIS* during the life cycle of *Ectocarpus* is consistent with a role in the regulation of the basal system, both during the gametophyte and the sporophyte generations.

DIS **encodes the conserved protein TBCCd1**

The *DIS* gene is predicted to encode a 774-amino acid protein with a tubulin binding cofactor C (TBCC) domain (accession PF07986 in Pfam database; Supplemental Table 13). Three classes of TBCC proteins have been described in other eukaryotic lineages: canonical TBCC, retinitis pigmentosa protein 2 (RP2) and TBCCd1. Phylogenetic analysis indicated that *DIS* encodes a TBCCd1 protein (Figure 4D; Supplemental Figure 9; Supplemental Table 14). *DIS* is the only TBCCd1-encoding gene in the *Ectocarpus* genome. Note that the stop codon in *dis-2* is located within the region encoding the TBCC domain (Figure 4B).

Canonical TBCC functions together with several other tubulin cofactors/chaperones in αβ-tubulin assembly and therefore plays a key role in microtubule dynamics (Tian et al., 1996; Nithianantham et al., 2015). RP2 is also thought to be involved in microtubule assembly but the role TBCCd1 is less clear. Phenotypic analyses have shown that TBCCd1 plays an important role in positioning organelles within the cell in diverse organisms (Feldman and Marshall, 2009; André et al., 2013) but the molecular mechanism through which this protein acts is unclear and may not involve direct effects

on microtubule dynamics (Goncalves et al., 2010). These previous studies prompted us to compare intracellular features of germinating *dis* initial cells with those of the wild type.

No defects in flagella structure and function were detected in *dis* mutants

Chlamydomonas mutants affected in the *ASQ2* gene (which encodes a TBCCd1 protein) have a variable number of flagella (Feldman and Marshall, 2009). In contrast, we observed no variation in flagella number in *dis* mutant individuals and both the posterior and the anterior flagella of *dis* gametes were morphologically similar to the wild type equivalents and were positioned normally, being inserted asymmetrically on the “ventral" side, close to the eyespot (Maier, 1997; Supplemental Table 15). *dis* gametes exhibited no abnormal swimming behaviour that might have been indicative of loss of flagella functionality; both wild type and *dis* gametes were positively phototactic (Supplemental Table 15). Moreover, male gametes of *dis* and wild type strains showed no difference in fertilization success, evidence that the former can swim towards female gametes in response to pheromone release as efficiently as wild type gametes (Supplemental Table 15). In summary, we did not find any evidence that DIS is required for flagella functionality.

Initial cells of *dis* mutants exhibit increased cell size, modified Golgi architecture, abnormal positioning of the nucleus and modification of the microtubule network

The germinating initial cells of *dis* mutants were significantly larger than wild type initial cells (mean areas of 53.9 μm^2, 54.1 μm^2 and 32.0 μm^2 for *dis-1*, *dis-2* and wild type, respectively; Supplemental Table 15; Figure 5D) and analysis of transmission electron microscopy (TEM) images indicated an unusually abundant trans Golgi network in the initial cells of *dis* mutants, suggesting a perturbation of secretory activity (Figure 5A-F). Moreover, Golgi cisternae of *dis-1* and *dis-2* cells were significantly shorter than those of the wild type (Wilcoxon test, p-value=$1.22e^{-13}$; Figure 5H). A similar phenotype has been observed in TBCCd1-depleted human cells, which are enlarged and exhibit a disorganized Golgi (Goncalves et al., 2010). The cellular phenotype of the *dis* mutants is therefore consistent with TBCCd1 having a conserved role in maintaining the structural

integrity of the Golgi during early development across extremely distant eukaryotic groups. Note that depletion of the tubulin binding co-factor TBCE also causes disorganisation and fragmentation of the Golgi (Haase and Rabouille, 2015).

The microtubule network plays an important role in the positioning of cellular organelles and has been implicated in the organisation and positioning of the Golgi apparatus close to the nuclei (reviewed in Rios and Bornens, 2003). We therefore investigated whether the microtubule network was altered in *dis* mutants. In wild type initial cells, a well organised interphase microtubule network was visible during initial cell germination. Microtubules nucleated from the pole opposite the first germination tube and microtubule bundles were gently curved and oriented parallel to the germination axis towards the germination pole (Figure 6A, B; Supplemental Figure 10). In *dis* mutant cells, microtubule bundles were more abundant compared with the wild type (Figure 6; Wilcoxon test, p=0.000156 and p=0.0069 for wild type versus *dis-1* and wild type versus *dis-2* comparisons, respectively) and the architecture of the microtubule network was disturbed. *dis* mutant microtubules were organised in wavy bundles with a criss-cross pattern (Figure 6A, B; Supplemental Figure 10). The perturbations of the microtubule architecture were only observed in the germinating initial cell, before the first cell division. No abnormalities of the microtubule network were detected in *dis* mutant filament cells at or after the two-cell stage, compared with wild type cells (Figure 6C). Cells of wild type and *dis* 15 day old germlings exhibited similar microtubule networks with bundles of a variety of thicknesses.

In addition to the above-mentioned flagellar phenotype, the *Chlamydomonas asq2* mutant exhibits defects in centriole positioning and number (Feldman and Marshall, 2009). We examined two aspects of centriole positioning in the *Ectocarpus dis* mutants: the degree with which centrioles were associated with the nucleus and the positioning of the associated centriole and nucleus with respect to the germination axis. Regarding the first aspect, centrioles were positioned close to the nuclei in both wild type and *dis* mutant cells and there was no evidence of detachment of centrioles from the nuclear envelope in *dis* mutants (Figure 5A-C). In contrast, we detected marked differences between wild type and *dis* mutant cells with respect to the position of the centrioles in

relation to the germination pole. In wild type cells, centrioles (which co-localise with microtubule nucleating sites, Figure 6E) were located close to the nucleus on the side opposite the germination pole ('distal'), whereas in *dis* mutant cells, we observed microtubule nucleation both laterally and on the proximate side of the nucleus (Figure 6F).

More than 200 *dis* mutant germlings were inspected at the two-cell stage to determine whether the aberrant positioning of microtubule nucleation sites in germinating initial cells leads to defects in the plane of cell division when these cells divides. This analysis failed to detect any individuals showing signs of aberrant patterns of cell division, i.e. there were no individuals in which the new septum was laid down obliquely, in a division plane that was not perpendicular to the growth axis.

We also analysed the position of the nucleus within the germinating cell. In the majority of wild type germinating initial cells the nucleus was positioned near the pole opposite the first germination tube (Figure 6E). In contrast, the majority of the nuclei in *dis-1* and *dis-2* initial cells were positioned centrally and in up to 30% of the cells the nucleus was located at the germinating end, a configuration that was only observed very rarely (about 2%) in wild type germinating initial cells (Figure 6E, F). Analysis of individuals from the two-cell stage onwards did not detect any aberrations in the positioning of the nuclei in *dis* mutants compared to the wild type (Wilcoxon test, p-value=0.837 and P=0.207 for wild type vs *dis-1* and wild type vs *dis-2* respectively), indicating that the phenotype observed in initial cells is limited to that stage of development (Figure 6E).

Taken together, these analyses indicate that loss of the DIS protein leads to several defects in subcellular architecture of the initial cell and that these defects are associated with alterations in the microtubule network.

To further examine the role of microtubules during early development of *Ectocarpus* initial cells, we analysed the effect of drugs that disrupt both microtubule polymerisation and depolymerisation (although note that the increased number of microtubule bundles in *dis* mutants is more consistent with a depolymerisation defect). Oryzalin, which induces

458 microtubule depolymerisation, significantly inhibited elongation of wild type initial cells
459 and delayed the first cell division but did not result in perturbations of the microtubule
460 network resembling those observed in the *dis* mutants (Supplemental Figure 11;
461 Supplemental Table 16). Furthermore, oryzalin treatment did not lead to aberrant
462 positioning of the nuclei, compared with wild type untreated control cells (Wilcoxon test,
463 W= 101.5, p-value = 0.3818, Supplemental Figure 11). Similarly, treatment of wild type
464 initial cells with taxol, a microtubule stabilizing drug, inhibited germination and cell
465 division but did not produce a cellular phenotype similar to that of the *dis* mutants. Taxol
466 treated cells had thick microtubule bundles that were mostly oriented parallel to the cell
467 membrane (Supplemental Figure 10, 11). This pattern differed from those of both
468 untreated wild type cells and *dis* cells. Taxol treatment did not have any significant effect
469 on the position of the nucleus in germinating cells (Wilcoxon test, W= 95, p-
470 value=0.1978, Supplemental Figure 11D).

471 The lack of any detectable effect of pharmacological perturbation of the microtubule
472 network on the positioning of the nucleus within the initial cell suggests that alternative
473 cytoskeletal components, such as actin filaments, may be involved in positioning the
474 nucleus, as observed in plant root hair cells (Ketelaar et al., 2002).

475 Brefeldin A (BFA) has been shown to disrupt the Golgi and to inhibit polarisation of
476 the germinating *Fucus* embryo (Bisgrove and Kropf, 2001; Hable and Kropf, 1998).
477 Treatment of *Ectocarpus* sp. initial cells for 24h with BFA weakened the cell wall
478 (leading to the release of cytosolic material) and inhibited cell elongation, but did not
479 reproduce the Dis$^-$ phenotype (Supplemental Table 16, Supplemental Figure 11).

480 Taken together, the results of these pharmacological experiments suggest that the
481 phenotypes associated with the *dis* mutations are not simply due to defects in microtubule
482 polymerisation or depolymerisation nor to an overall deficiency in secretion.

483 ## DISCUSSION

484 ### ***DIS* is required for the establishment of basal structures in *Ectocarpus***

Analysis of the phenotypes of the two *dis* mutants described in this study indicated that the *DIS* gene is required for the development of basal structures throughout the *Ectocarpus* life cycle. In wild type *Ectocarpus* gametophytes, the initial cell divides asymmetrically to produce two cells with apical and basal identities. Further divisions in opposite directions of each of these cell types generate an apical thallus and a basal rhizoid, respectively. In the gametophytes of *dis* mutants the basal germ tube that gives rise to the rhizoid is not produced. The consequent unipolar germination gives rise to individuals with an apical system but no basal structures. We suggest that the basal system is not produced because the *dis* mutants fail to deploy a putative basal determinant factor ('B') in the initial cell and, as a result, the first division produces an apical cell and a quiescent undifferentiated cell (Figure 7).

In the wild type sporophyte, the first division of the initial cell is symmetrical and the two germ tubes form the two ends of a basal filament, which is strongly attached to the substratum. In the absence of the DIS protein, the initial cell produces just one germ tube, which gives rise to the apical system. We interpret the failure to initiate a second germ tube as being a consequence of the failure to establish basal cell identity in the sporophyte of this mutant. We suggest that, in the absence of the basal determinant factor 'B', the initial cell divides to produce an apical cell and a quiescent undifferentiated cell in the same manner as observed in the gametophyte generation.

The *dis-2* mutation creates a premature stop codon within the region that encodes the TBCC domain and is therefore likely to be a null mutation. The *dis-1* mutation, on the other hand, is located in an intron (Figure 3). This mutation causes a reduction in transcript abundance but does not modify the open reading frame of the transcript. Analysis of the RNA-seq data did not detect any evidence that the mutation affects splicing of the transcript. Therefore, *dis-1* may not represent a null mutation. If *dis-1* is not a null allele, then under the model shown in Figure 7, the abortive structures produced instead of the second germ tube in *dis-1* mutant sporophytes can be interpreted as arising due to incomplete penetrance of the *dis-1* mutation, allowing a small amount of the putative basal determinant 'B' to accumulate, and the production of an abortive basal system.

The model proposes that the observed phenotypes are due to variations in concentration, rather than the subcellular distribution, of the putative 'B' factor. One possible scenario would be that the abnormal Golgi apparatus of the initial cell is unable to produce sufficient amounts either of the putative 'B' factor itself or of a protein that generates the 'B' factor enzymatically. Interestingly, comparison of the *dis-1* transcriptome with those of wild type apical and basal tissues identified an enrichment in genes coding for secreted proteins and highlighted a putative role for *DIS* in, among other processes, cell wall modification processes, secretion, transport and vesicle trafficking. Secretion and diffusion of cell fate determinants, particularly from the cell wall, have been shown to play a crucial role in pattern formation in brown algae (Bouget et al., 1998; Arun et al., 2013). Interestingly, mutant analysis indicates that cell wall synthesis, cell wall integrity sensing, and vesicle trafficking are also important processes during the development of *Marchantia polymorpha* rhizoids and *Arabidopsis* root hairs (Honkanen et al., 2016).

In *Ectocarpus*, the apical and basal developmental programs appear to be independent, in the sense that removal of the basal system in the *dis* mutants had no visible effect on patterning of the apical system. In contrast, *Arabidopsis* mutations that result in the loss of basal structures, such as *monopteros* and *bodenlos* for example, also tend to cause more or less subtle modifications to the apical structures (Hardtke and Berleth, 1998; Hamann et al., 1999). This difference may reflect the simpler developmental program of *Ectocarpus* compared to *Arabidopsis* but may also be related to the fact that the primary function of brown algal basal systems is to act as a holdfast, whereas the aerial tissues of land plants are dependent on the root system not only for anchorage but also for water and nutrient supply. In land plants, therefore, there may be more of a tendency for the developmental programs of the apical and basal systems to be interdependent.

DIS **encodes a TBCC protein with a conserved role in intracellular organisation**

The *DIS* gene encodes a TBCCd1 protein. TBCCd1 is a member of the TBC group of proteins, which also includes the retinitis pigmentosa protein 2 (RP2) and TBCC, a component of the chaperone complex (TBCA to TBCE) that catalyses the formation of

α,ß-tubulin heterodimers (Tian and Cowan, 2013; Steinborn et al., 2002). All three TBCC proteins have been strongly conserved across the eukaryotic tree (Figure 4D, Supplemental Table 13) suggesting that each has a specific function in the cell. However, whilst the role of TBCC has been described in detail, the exact cellular and molecular function of TBCCd1 remains unclear.

The role of TBCC in the α,ß-tubulin heterodimerisation pathway is to trigger GTP hydrolysis catalysing the release of newly formed αβ-tubulin heterodimers. Importantly, this pathway is reversible so that TBCC plays a role in both microtubule assembly and disassembly (Nithianantham et al., 2015). Both TBCC and RP2 have GAP activities, mediated by their TBCC domains, and RP2 can complement a TBCC mutant (Schwahn et al., 1998; Schwarz et al., 2012). In contrast, the TBCC domain of TBCCd1 lacks several conserved residues including a critical arginine residue and TBCCd1 is thought not to be a GAP protein (Bartolini et al., 2002; Scheffzek et al., 1998), although it has been suggested that a nearby conserved arginine residue may substitute for the lost arginine residue (Feldman and Marshall, 2009) (Supplemental Figure 9). Therefore, although TBCCd1 probably interacts with the cytoskeleton in some way to mediate its cellular functions, it is currently unclear whether this specifically involves a direct role in microtubule polymerisation/depolymerisation.

TBCCd1 has been localised to the centrosome and the Golgi in organisms as diverse as humans, *Chlamydomonas* and trypanosomes and loss-of-function experiments have indicated a conserved and important role for TBCCd1 in various aspects of the organisation of cellular architecture in these diverse eukaryote groups (André et al., 2013; Feldman and Marshall, 2009; Goncalves et al., 2010). Loss or knockdown of TBCCd1 has severe consequences for cell morphology, for example leading to alterations in the shape and motility of human cells (Goncalves et al., 2010), mitotic defects in trypanosomes (André et al., 2013) and defects both in flagella number and functioning and during cell division due to misorientation of the mitotic spindle in *Chlamydomonas* (Feldman and Marshall, 2009). Although we did not detect any defects in flagella structure or centriole positioning in *Ectocarpus dis* mutants, the initial cells of these mutants did show defects in the positioning of the nucleus, and an atypical microtubule

network during germination. Moreover, *dis* initial cells were markedly larger than the wild type equivalents and their Golgi network was more abundant and fragmented (shorter cisternae). Increased cell size and disorganisation of the Golgi apparatus have been observed in TBCCd1-depleted human and trypanosome cells (Goncalves et al., 2010; André et al., 2013). The role of TBCCd1 in regulating organelle positioning, microtubule and Golgi architecture, highlighted by our study, can therefore be traced back to the crown divergence of the major eukaryotic groups, more than a billion years ago (Eme et al., 2014).

The Golgi defects in the *dis* mutants are particularly interesting with regard to the model described in Figure 7, which proposes that the Dis$^-$ phenotype is due to failure to deploy a hypothetical basal-cell-fate-determining factor 'B' during the first cell division. In animals, the Golgi is critical for cellular differentiation and morphogenesis because it spatially constrains developmental pathways and it is required for the establishment of cell polarity and normal subcellular organization (Copeland et al., 2016; Vinogradova et al., 2009; Zhong, 2011). Moreover, in the embryo of the brown alga *Fucus*, targeted secretion from the Golgi to the cell wall has been implicated in axis fixation and establishment of the basal versus the apical system (Shaw and Quatrano, 1996).

In *Arabidopsis*, asymmetrical division of the zygote is preceded by reorganisation of the cytoskeleton and re-positioning of the nucleus from a central position to the apical region of the cell (Kimata et al., 2016; Ueda et al., 2011). Mutation of the zinc finger transcription factor gene *WRKY2* not only disrupts the asymmetrical division of the zygote cell but also leads to partial loss of basal identity in the basal cell lineage (Ueda et al., 2011). Interestingly, the *Ectocarpus dis* mutants not only fail to produce a basal system but also exhibit disrupted organisation of the microtubule cytoskeleton and defects in nucleus positioning in the initial cell. This suggests that, as in flowering plants, the establishment of the basal cell lineage in brown algae depends critically on events leading up to and including the first division of the initial cell.

It is not clear how loss of the TBCCd1 protein leads to disorganisation of the microtubule cytoskeleton in the *dis* mutants (whether this is a direct or an indirect effect

603 for example) but this phenotype does not appear to be simply due to defects in
604 microtubule polymerisation or depolymerisation because treatment with oryzalin or taxol
605 did not result in a phenotype that resembled that of the *dis* mutants. The observed
606 modifications to the microtubule architecture in *dis* mutants may be due to more subtle
607 changes in nucleating activity, rates of assembly and disassembly of bundles,
608 modifications of microtubule dynamic instability and/or tubulin or microtubule
609 posttranslational modifications (Mitchison and Kirschner, 1984; Song and Brady, 2015).

610 Based on the phenotypes associated with TBCCd1 loss in other eukaryotes and on the
611 phenotypes of the *dis* mutants, we propose that acquisition of basal cell identity in
612 *Ectocarpus* depends critically on features of the initial cell architecture, that are disrupted
613 in the absence of a functional DIS/TBCCd1 protein. Further work is required to
614 understand the exact cellular role of TBCCd1 in *Ectocarpus* but the identification of this
615 component will allow new insights into the cellular mechanisms involved in establishing
616 apical/basal identity.

617 ***DIS* and the evolution of sporophyte and gametophyte developmental programs**

618 The pattern of early development of the *Ectocarpus* sporophyte, which involves
619 deployment of an extensive basal system before the establishment of the apical/basal
620 axis, is unusual because most brown algal sporophytes exhibit early establishment of the
621 apical/basal axis leading directly to the production of a thallus cell and a rhizoid,
622 respectively (Fritsch, 1935). In the *Ectocarpus immediate upright* (*imm*) mutant the
623 extensive basal system of the sporophyte fails to form and this structure is replaced by a
624 rhizoid (Peters et al., 2008). Based on the phenotype of the *imm* mutant, we have
625 suggested that the pattern of early sporophyte development in extant *Ectocarpus* evolved
626 from a simpler, ancestral developmental program that more closely represented that of
627 the gametophyte, i.e. involving an asymmetrical initial cell division that gave rise directly
628 to apical and basal organs (Macaisne et al., 2017). The *dis* mutants provide further
629 support for this hypothesis because they exhibit complete loss both of the rhizoid during
630 the gametophyte generation and of the extensive network of prostrate filaments during
631 the sporophyte generation, suggesting that the two structures are indeed equivalent.

Moreover, in a *dis-1 imm* double mutant sporophyte the rhizoid that normally replaces the prostrate filament system fails to develop, providing further evidence that the rhizoid that forms in the *imm* mutant sporophyte is developmentally equivalent to the basal prostrate filament system of the wild type sporophyte.

In land plants, which also have haploid-diploid life cycles, there has been considerable interest in the evolutionary origins of the sporophyte and gametophyte developmental programs; specifically whether each generation has independently evolved its own developmental pathways or, alternatively, whether there has been recruitment of developmental programs from one generation to the other during evolution (Dolan, 2009; Pires and Dolan, 2012; Shaw et al., 2011). Current evidence indicates that the developmental networks that implement land plant sporophyte programs were mainly recruited from the gametophyte generation, which was initially the dominant generation in the land plant lineage (Dolan, 2009; Niklas and Kutschera, 2010) but there is also evidence that there have been sporophyte-specific innovations (Szovenyi et al., 2011; Sano et al., 2005). Analyses of the *imm* (Macaisne et al., 2017) and *dis* (this study) mutants suggest that the evolution of developmental systems in the brown algae also involved both co-opting of programs from one generation to the other and generation-specific developmental innovations. The phenotypes of the *dis* mutants suggest that, despite their clear morphological differences, the basal systems of the sporophyte and gametophyte generations share underlying mechanistic features because the TBCCd1 protein is necessary for the deployment of both types of basal structure. In this respect, therefore, the sporophyte and gametophyte basal structures appear to be homologous. On the other hand, it is only in the sporophyte that the rhizoid has been modified to produce an extensive system of prostrate filaments (note that gametophytes carrying the *imm* mutation are indistinguishable from wild type gametophytes; Peters et al., 2008). The developmental program that depends on the IMM protein therefore appears to have been a sporophyte-specific innovation, which was presumably built onto a more ancient program shared with the gametophyte generation that requires the action of the *DIS* gene.

MATERIAL AND METHODS

UV mutagenesis and isolation of mutant strains

Strain cultivation, genetic crosses, raising of sporophytes from zygotes and isolation of meiotic families were carried out as described in (Coelho et al., 2012a, 2012d). Gametes of *Ectocarpus* are able to develop parthenogenetically to produce haploid partheno-sporophytes, which are identical morphologically to the sporophytes that develop from diploid zygotes (Peters et al., 2008; Coelho et al., 2011). This phenomenon was exploited to screen directly, in a haploid population, for mutants affected in early sporophyte development. UV mutagenesis of gametes was carried out as described previously (Godfroy et al., 2015) and mutant partheno-sporophytes lacking basal structures were identified by visual screening under a light microscope.

Genetic mapping of the *DIS* locus

To obtain an approximate map position for the *DIS* gene, the *dis-1* mutant (Ec722) was crossed with the outcrossing line Ec568 to generate a mapping population of 265 individuals. Each of the 265 individuals was derived from a different unilocular sporangium (each unilocular sporangium contains 50-100 meiospores, derived from a single meiosis followed by at least 5 mitotic divisions). The meio-spores germinated to produced gametophytes, which were isolated and allowed to germinate parthenogenically. The resulting partheno-sporophytes were then observed under a light microscope to determine whether they exhibited the Dis^- phenotype. The *dis-1* mutation was then approximately mapped using 21 Dis^- individuals from this segregating population and 74 microsatellites markers from the *Ectocarpus* microsatellite-based genetic map (Heesch et al., 2010). Using this approach, we localised the *dis-1* mutation to chromosome 05 at 9.5 cM from the M_114 marker.

A cloning-by-sequencing strategy (using the SHOREmap approach,(Schneeberger et al., 2009) was then used to precisely localise the *dis-1* mutation. Genomic DNA was extracted from 87 wild type and 96 *dis* individuals from the mapping population using

687 NucleoSpin® 96 Plant II (MACHEREY-NAGEL, Düren, Germany) and pooled into two
688 wild type and Dis$^-$ pools. An Illumina HiSeq2500 platform (Fasteris, Genève, Suisse) was
689 the used to generate 44.9 Gb of sequence data, corresponding to 117 and 104 million 2x
690 100 bp paired-end reads for the wild type and Dis$^-$ pools, respectively (SRA accession
691 numbers SRR3710253 and SRR3710254, respectively). The same DNA extraction
692 protocol and sequencing platform were employed to sequence the *dis-2* mutant strain
693 Ec799, producing 49 million 2x125 paired-end reads (SRA accession number
694 SRR3714421). Reads were cleaned to remove nucleotides with quality scores of less than
695 20 from both ends using Prinseq (Schmieder and Edwards, 2011). Reads were then only
696 retained if they were longer than 50 nucleotides, had a mean quality of at least 25 and no
697 non-determined nucleotides. About 3.6% and 4.3% of the raw wild type and *dis* reads
698 were removed, respectively. Bowtie2 (Langmead and Salzberg, 2012) was used to map
699 71.9% and 74.7% of the wild-type and *dis* cleaned reads, respectively, onto the Ec32
700 reference genome. The read mapping was then improved by read realignment and base
701 quality recalibration using the GATK software suite. Variant calling was carried out
702 using the "consensus" tool of the SHORE pipeline in order to analyse allele frequency.
703 Since we had only one reference genome sequence for *Ectocarpus*, corresponding to the
704 wild type strain that was mutagenised to generate the *dis* mutants, we were not able to
705 apply the strategy described in the SHOREmap publication to generate the list of markers
706 (association of SHOREmap extract and create). We therefore selected genetic markers by
707 comparing SNPs called in wild type and Dis$^-$ pools and extracted SNPs that segregated as
708 expected, i.e., common SNPs for which the sum of the wild type and *dis-1* allele
709 frequencies was between 0.8 and 1.2. In order to prevent loss of markers close to the
710 mutation, we also included SNPs that were specific to one of each of the pools with a
711 frequency of more than 0.9 and less than 0.1. Using this marker list, and crossing
712 SHOREmap annotation results with manual selection of mutations from SHORE
713 consensus, SHORE qVar and samtools/VarScan (Koboldt et al., 2013; Schneeberger et
714 al., 2009) identified a unique SNP specific to the *dis-1* pool at position 50914 of scaffold
715 sctg_184 (position 3330523 on chromosome 5). This A to G transition mutation was
716 located in intron 14 of the *DIS* gene 15 base pairs after the end of exon 14.

A cleaved amplified polymorphic sequence (CAPS) marker (Supplemental Table 2), developed based on the single nucleotide polymorphism at position 3330523 of chromosome 05, was used to genotype the 265 individuals of the mapping population (which included 90 wild type and 175 *dis-1* individuals). This analysis confirmed that the presence of the A to G transition was 100% correlated with the Dis$^-$ phenotype in this large family. In addition, PCR amplification and Sanger sequencing of the genomic region containing the mutation confirmed its presence in the *dis-1* mutant (Ec722) and its absence in the Ec32 and Ec568 strains (the original strain used for the mutagenesis and the outcross female line used to generate the segregating population, respectively).

Resequencing of the *DIS* gene in the *dis-2* mutant identified a point mutation (a C to T transition) in exon 13 that results in the introduction of a stop codon into the coding region of the gene (chr05, position 3332908).

Genetic interaction between *DIS* and *IMM*

An *imm* mutant female (Ec602) was crossed with a *dis-1* male (Ec722) and the resulting sporophyte (Ec649) gave rise to a 40-gametophyte progeny, all derived from independent meiotic events. The phenotypes of the progeny were scored under an inverted microscope, and genotypes were assessed using CAPS markers for *IMM* and *DIS* loci (Supplemental Table 2).

RNAi knockdown of *DIS* expression

Small interfering RNAs (siRNAs) directed against the *DIS* gene transcript were designed using version 3.2 of E-RNAi (Horn and Boutros, 2010). The specificity of the designed siRNAs was determined by comparing the sequence (Blastn) with complete genome and transcriptome sequences. Candidates that matched, even partially, genomic regions or transcripts in addition to *DIS* were rejected. Three siRNAs with predicted high specificity corresponding to different positions along the *DIS* transcript were selected (Supplemental Table 17). The control siRNA was directed against the *Ectocarpus* sp. gene Ec-13_001890 which is located within the sex-determining region of the male sex chromosome and therefore not an essential gene for sporophyte function. siRNAs were

introduced into *Ectocarpus* sp. strain Ec32 gametes using the transfection reagent HiPerFect (Qiagen, Valencia, USA). One microlitre each of 0.5 µg/µl solutions of siRNAs in 1X Universal siMAX® siRNA Buffer (MWG Eurofins, Ebersberg, Germany) was mixed with 12 µl of HiPerFect transfection reagent in a final volume of 100 µl of natural seawater, vortexed to mix and incubated for 10 min at room temperature before being added dropwise to 100 µl of freshly released gametes in natural seawater in a Petri dish. After rotating gently to mix, the Petri dish was incubated overnight at 13°C. The following day, 10 ml of culture medium was added (Coelho et al., 2012c) and incubation continued at 13°C. RNAi-induced phenotypes were observed under a light microscope and the number of individuals that resembled the *dis* mutant were scored in at least 400 individuals for three experimental replicates. Control treatments were carried out in the same manner using an siRNAs directed against the endogenous gene Ec-13-001890.

Unfortunately, it is currently not possible to determine whether the siRNA treatment reduces *DIS* transcript abundance because, based on the observed phenotypes, gene expression is affected in only a small percentage of the treated cells. However, the use of an siRNAs directed against the endogenous gene Ec-13-001890 showed that the effect was sequence-specific. It would therefore seem highly unlikely that the observed phenotypes were due to processes that did not involve interference with the expression of the *DIS* gene.

Comparative transcriptome analyses

RNA-seq analysis was carried out to compare the abundances of gene transcripts in the *dis-1* mutant with those in basal and upright filaments of the wild type sporophyte generation. Duplicate synchronous cultures (with more than 100 individuals each) were prepared for each sample under standard conditions (Coelho et al., 2012c). Upright filaments of adult (4-week old) wild type partheno-sporophytes were dissected from prostrate, basal filaments using the sharp end of a Pasteur pipette under a binocular microscope. Visual inspection under the microscope ensured the absence of contaminating basal filaments. Wild type basal filaments were obtained by harvesting immature individuals before the emergence of the upright filaments, two weeks after

initial cell release from plurilocular gametangia. The *dis-1* mutant was grown under the same conditions as the wild type tissue and was similarly used at two weeks after release of initial cells from plurilocular gametangia. Tissue samples were rapidly frozen in liquid nitrogen and processed for RNA extraction. Total RNA was extracted from each sample using the Qiagen Mini kit (http://www.qiagen.com) as previously described (Lipinska et al., 2015). For each replicate, cDNA was produced by oligo-dT priming, fragmented and prepared for 2x 100 bp paired end sequencing on an Illumina Hiseq 2000 platform by Fasteris (CH-1228 Plan-les-Ouates, Switzerland). TopHat (v2.0.8) was used to map the RNA-seq reads to the reference genome. Supplemental Table 18 provides details of the sequencing, mapping statistics and accession numbers. Pairwise differential expression analysis between *dis-1*, basal and upright filaments was performed with the DESeq2 package (Bioconductor) using an adjusted p-value cutoff of 0.1 and a minimal fold-change of 2.

Analysis of predicted gene functions

InterProScan (Zdobnov and Apweiler, 2001) and Blast2GO (Conesa and Götz, 2008) were used to recover functional annotations for *Ectocarpus* proteins. For Blast2GO, a Fisher exact test with a p-value cutoff of 0.05 was used to detect enrichment of specific GO-terms in the various groups of genes. KEGG pathway enrichment was analysed using the KOBAS 2.0 platform (Xie et al., 2011). Signal peptides were predicted using Hectar (Gschloessl et al., 2008) implemented through the Galaxy platform (http://webtools.sb-roscoff.fr/). A gene-by-gene manual curation was used to associate each gene in the differential transcriptomic datasets to a functional category. Enrichment in specific functional categories in relation to the *Ectocarpus* sp. genome was carried out by statistical analysis using Chi-squared test ($P<0.05$).

Phylogenetic analysis of TBCC proteins

A multiple alignment of the TBCC domains of diverse TBCC, RP2 and TBCCd1 proteins was generated with Muscle (http://www.ebi.ac.uk/Tools/msa/muscle/). Based on this alignment, an unrooted maximum likelihood phylogenetic tree was built with the

LG+G model using MEGA7 (http://www.megasoftware.net/mega.php) and 1000 bootstrap replicates. Model testing was carried out in MEGA7. The coordinates of the TBCC domains within the protein sequences were determined using the NCBI Conserved domains tool (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi). Accession numbers and domain coordinates are provided in Supplemental Table 14.

Microdissection and regeneration of *dis* mutants and wild type filaments

Microdissection was performed with the sharp end of a Pasteur pipette under an inverted microscope. After dissection, the material was kept at 13°C under standard culture conditions (Coelho et al., 2012e). Wild type and *dis* sporophyte apical filament cells were isolated by micro-dissection 7 days after the emergence of the first upright filaments (20 days after gamete release); gametophyte apical filament cells were isolated by micro-dissection 15 days after meio-spore release.

Morphometric analysis of germinating partheno-sporophytes

Germinating initial cells of *dis-1*, *dis-2* and wild type partheno-sporophytes 36h after release from gametangia were photographed under an upright microscope (BX41, Olympus, Tokyo, Japan). Cell areas were measured for a total of 57 cells using ImageJ (http://imagej.nih.gov/ij). Morphological measurements and analysis of images of adult filaments were also performed under an inverted microscope (CKX41, Olympus, Tokyo, Japan) using ImageJ (http://imagej.nih.gov/ij/, 1997-2016). Between 25 and 100 adult filament cells were measured in each of eight different wild type or *dis-1* and *dis-2* individuals. The statistical analysis was performed in R.

Electron microscopy analysis of cellular ultrastructure

Medium containing mature *Ectocarpus dis* partheno-sporophytes was pipetted onto a plastic film (gel support films, ATTO CO., Tokyo, Japan). The film was cut into <1 cm side triangles, and these were attached to Petri dishes by adhesive tape. Two days after the release of mito-spores from plurilocular sporangia, the resulting germlings, which were attached to the triangles, were rapidly immersed in liquid propane cooled to -180˚C

by liquid nitrogen, and immediately transferred into liquid nitrogen. The samples were submerged in substitution solution containing 2% osmium tetroxide with acetone at -80˚C for two days, at -40°C for 2 h and at 4°C for 2 h. Finally, the temperature of the samples was gradually allowed to rise to room temperature, and they were then washed with acetone several times. The gel support films were infiltrated and embedded in Spurr's low-viscosity resin (Polysciences Inc., Warrington, PA, USA) on aluminium foil dishes. The films with the samples were turned inside out on the upper surface of the resin. Serial sections were cut with a diamond knife on an ULTRACUT ultramicrotome (Reichert-Jung, Depew, NY, USA) and mounted on formvar-coated slot grids. Sections were stained with TI blue (Nisshin EM Co. Ltd, Tokyo, Japan) and lead citrate, and observed using an electron microscope (JEM-1011, JEOL Ltd., Tokyo, Japan). The images shown in Figure 5 are representative of 14 initial cells.

Golgi fragmentation was quantified using a method adapted from (Tang et al., 2011)). Transmission electron microscope photographs were used to measure the lengths of cisternae. Measurements were performed on 14 different individuals, and a total of 113 cisternae from *dis-1, dis-2* and wild type individuals were analysed. Data from *dis-1* and *dis-2* were pooled. Cisternae were defined as long membranous structures with a length greater than twice their width, the latter not exceeding 60 nm. Normal cisternae ranged from 20 to 30 nm in width and were longer than 400 nm. Statistical significance was assessed by a Wilcoxon test, implemented in R.

Quantitative reverse transcriptase PCR analysis

The abundance of gene transcripts during the *Ectocarpus* life cycle was assessed using RT-qPCR. RT-qPCR was performed as previously described (Coelho et al., 2011; Lipinska et al., 2013). Primer pairs were designed to amplify regions of the 3'UTR or the most 3' exon of the gene to be analysed (Supplemental Table 19). *In silico* virtual PCR amplifications were carried out using the e-PCR program (Rotmistrovsky et al., 2004) and the male Ec32 genome sequence to check the specificity of oligonucleotide pairs.

The Plant RNeasy extraction kit (Qiagen, Courtaboeuf, France) was used to extract total RNA from at least three biological replicates for each of the stages of the life cycle:

gametes, young sporophytes, immature gametophytes, mature gametophytes, partheno-sporophytes and diploid heterozygous sporophytes. The RNA was treated with RNAse-free DNAse-I according to the manufacturer's instructions (Qiagen) to remove any contaminating DNA and stored at -80°C. The concentration and integrity of the RNA was checked using a NanoDrop 2000 spectrophotometer (ThermoScientific, www.thermofisher.com) and by agarose gel electrophoresis. A control PCR without reverse transcriptase was performed to ensure absence of contaminating DNA. For each sample, up to 1 µg of RNA was reverse-transcribed to cDNA using oligo-dT and the Superscript II RT kit (Life Technologies, Gaithersburg, MD, USA) according to the manufacturer's instructions and the cDNA was diluted with water to 1.2 ng equivalent RNA.µL-1. cDNAs were amplified using the IQ Sybrgreen supermix (Biorad Laboratories, Hercules, CA, USA) on a Chromo4 System thermocycler (BioRad Laboratories). The amplification efficiency was tested using a genomic dilution series and was always at least 80%. The specificity of amplification was checked with a dissociation curve. The EF1α gene (Ec-21_002980) or the Arp2/3 gene (Ec-09_003710) were chosen as a constitutively expressed controls based on (Le Bail et al., 2008) and their constitutive expression was validated by the RNAseq datasets. The normalised data were expressed as the mean±s.d. calculated from three independent biological experiments.

Inhibitor treatments

Stock solutions of inhibitors were prepared in DMSO (oryzalin at 100 mM, taxol (paclitaxel) at 5 mg/ml, and brefeldin A at 25 mg/ml) and working solutions were diluted in half strength Provasoli-enriched seawater (PES)(Coelho et al., 2012d). Freshly released *Ectocarpus* sp. gametes were settled in Petri dishes and treated for 24h with 0.01-1 µM oryzalin, 5-20 µM paclitaxel or 2.5-25 µg/ml brefeldin A (BFA). Controls were treated with 0.002% DMSO. Cells were allowed to develop after removal of the inhibitor and observations were performed four and seven days later, using an inverted Olympus CKX41 microscope. For each experiment, between 19 and 310 individuals

were counted in triplicate dishes (529, 1071 and 2142 cells scored in total in each of the oryzalin, paclitaxel and brefeldine A treatments, respectively; Supplemental Table 16).

Immunostaining

Ectocarpus samples were processed as described by (Coelho et al., 2012b) using an adapted protocol from (Bisgrove and Kropf, 1998). Briefly, *Ectocarpus* cells were settled on coverslips and at appropriate times after settlement were rapidly frozen in liquid nitrogen and fixed in 2.5% glutaraldehyde and 3.2% paraformaldehyde for 1h, then washed in PBS and treated with 5% Triton overnight. Samples were then rinsed in PBS and 100 mM NaBH4 was added for 4h. Cell walls were degraded with cellulase (1% w/v) and hemi-cellulase (4% w/v) for 1h, and the preparation was then rinsed with PBS and blocked overnight in 2.5% non-fat dry milk in PBS. Samples were treated with anti-tubulin antibody (1/200th, DM1A, Sigma-Aldrich) at 20°C overnight, and then treated with the secondary antibody (AlexaFluor 488-conjugated goat anti-mouse IgG, Sigma-Aldrich; 1:1000 in PBS) at 20°C overnight. The preparation was rinsed with PBS and blocked overnight in 2.5% non-fat dry milk in PBS and then treated with anti-centrin antibody (1/1000th anti-centrin 1 ab11257, Abcam) at 20°C overnight, followed by the secondary antibody (1/1000th AlexaFluor 555-conjugated goat anti-rabbit IgG, Sigma Aldrich) for 8h. Samples were stained with 4', 6-diamido-2-phenylindole (DAPI, 0.5 µg/ml in PBS) for 10 min at room temperature, and finally mounted in ProLong Gold (Invitrogen).

Confocal microscopy

Confocal microscopy was conducted using an inverted SP8 laser scanning confocal microscope (Leica Microsystem) equipped with a compact supply unit which integrates a LIAchroic scan head, several laser lines (405 nm, 488 nm) and standard photomultiplier tube detectors (PMT). We used the oil immersion lens HC PL APO 63x/1.40 OIL CS2. The scanning speed was set at 400 Hz unidirectional. The pinhole was adjusted to one Airy unit for all channels. The spatial sampling rate was optimized according to Niquist criteria, generating a 0.058x0.058x0.299 µm voxel size (xyz). The Z-stack height fitted

914 the specimen thickness. A two-step sequential acquisition was designed to collect the
915 signal from three or four channels. The first step recorded the anti-tubulin fluorescence
916 signal (excitation 488 nm/emission 530 nm), and the transmitted light. The second step
917 acquired the DAPI fluorescence signal (excitation 405 nm/emission 415-480 nm) and the
918 anti-centrin signal (excitation 552 nm/emission 560-590 nm). Signal intensity was
919 averaged three times. The Fiji software was used to optimize the raw images, including
920 maximum intensity projection (MIP), and denoising (3*3 median filter). For any given
921 data, both wild-type and mutant images were analysed simultaneously with similar
922 settings. The Fiji software was also used to estimate the number of microtubule bundles
923 in each cell, before the first cell division. Tracking of bundles was performed on
924 maximum intensity projections of z-planes covering the whole thickness of the cells. We
925 drew three lines transversely, perpendicular to the growth axis of the cell: one in the
926 centre of the cell, and other two half way between the centre and the cell extremities (see
927 diagram in Figure 6D). Peaks corresponding to the microtubule bundles were then
928 identified in plots of intensity profiles at each of the three positions in the cell and
929 counted. The three values were averaged for each cell to derive an estimation of the
930 number of microtubule bundles in each cell. Note that in the *dis* mutants, due to the
931 disorganised nature of the microtubule network, average bundle numbers may be
932 somewhat underestimated. This is because this method is well adapted for tracking
933 microtubule bundles oriented with their long axis parallel to the image plane, but we may
934 have missed bundles that were perpendicular to the plane of the transection.

935 **Measurement of the positions of nuclei and microtubule nucleation sites**

936 Nuclei were stained *in vivo* with Hoechst 33342 (Thermofisher), imaged with an
937 Olympus BX microscope with a x40 objective, and distances were measured using Fiji
938 software. In germinating initial cells, the position of the nucleus was calculated by
939 measuring the distance from the nucleus to the germinating pole (i.e. the end
940 corresponding to the emerging germ tube) and the distance from the nucleus to the
941 opposite pole of the cell and dividing the latter by the former. Nuclei were scored as
942 being anterior (located near the emerging germ tube), central or posterior (located nearer

the pole opposite the germination pole). At the 2-cell stage, the positions of nuclei were similarly measured in a directional manner by measuring the distance from the nucleus to the cell boundary at the germinating end of the filament and the distance from the nucleus to the opposite pole of the cell and dividing the latter by the former. For cells in multicellular filaments at a later stage of development, it was not possible to assign a direction of growth. Filaments were therefore orientated arbitrarily and the position of each nucleus was calculated by dividing the distance from one end of the cell (x) by the distance from the other end of the cell (y) as illustrated in Figure 6H.

Microtubule nucleation site positions were measured by dividing each cell into four diagonal quadrants and scoring the position of the nucleating sites with respect to these quadrants: proximal (located on the same pole as the emerging germ tube), lateral or distal (located nearer the pole opposite the germination pole) as illustrated in Figure 6F.

Accession numbers

Accession numbers are provided in Supplemental Table 18.

Supplemental Data

Supplemental Data Set – Supplemental Tables S1 to S19

Supplemental Figure 1. The haploid-diploid life cycle of *Ectocarpus*.

Supplemental Figure 2. Pedigree of the strains used in this study.

Supplemental Figure 3. Cell regeneration in *dis* versus wild type filaments.

Supplemental Figure 4. Expression patterns, measured using RT-qPCR, of generation-specific marker genes (Peters et al., 2008) during the gametophyte and sporophyte generation of *dis-1* individuals compared with the wild type.

Supplemental Figure 5. Venn diagram comparison of the set of genes expressed in *dis-1* with those expressed in wild type basal (Basal) and apical (Apical) filaments (TPM>1).

Supplemental Figure 6. Correlation between RNA-seq and RT-qPCR results.

968 Supplemental Figure 7. KEGG pathway analysis.

969 Supplemental Figure 8. Patterns of expression of *DIS*.

970 Supplemental Figure 9. Alignment of the TBCC domains of diverse TBCCd1 (green),
971 TBCC (orange) and RP2 (blue) proteins and predicted secondary structure representation
972 of the overall fold of the TBCC domain of the *Ectocarpus* sp. DIS/TBCCd1 protein.

973 Supplemental Figure 10. Additional representative confocal maximum z-projections of
974 untreated wild type cells, untreated *dis-1* and *dis-2* mutant cells andwild type cells treated
975 for 4h with 10 µM taxol at several stages of early development of the initial cell.

976 Supplemental Figure 11. Effects of inhibitors treatment on the microtubule
977 cytoskeleton and early development.

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