Title: A novel, lineage-primed prestalk cell subtype involved in the morphogenesis of *D. discoideum*

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Abstract

Dictyostelium morphogenesis requires the tip, which acts as an organizer and conducts orchestrated cell movement and cell differentiation. At the slug stage the tip region contains prestalk A (pstA) cells,which are usually recognized by their expression of reporter constructs that utilize a fragment of the promoter of the ecmA gene. Here, using the promoter region of the o-methyl transferase 12 gene (*omt12*) to drive reporter expression, we demonstrate the presence, also within the pstA region, of a novel prestalk cell subtype: the pstV^{A} cells. Surprisingly, a sub-population of the vegetative cells express a pstV^{A} : GFP marker and, sort out to the tip, both when developing alone and when co-developed with an excess of unmarked cells. The development of such a purified GFP-marked population is greatly accelerated: by precocious cell aggregation and tip formation with accompanying precocious elevation of developmental gene transcription. We therefore suggest that the tip contains at least two prestalk cell subtypes: the developmentally-specified pstA cells and the lineage-primed pstV^{A} cells. It is presumably the pstV^{A} cells that play the dominant role in morphogenesis during the earlier stages of development. The basis for the lineage priming is, however, unclear because we can find no correlation between pstV^{A} differentiation and nutrient status during growth or cell cycle position

at the time of starvation, the two known determinants of probable cell fate.

Keywords

morphogenesis, organizer, *Dictyostelium*, prestalk cell, lineage-priming, heterogeneity

Introduction

Multicellular morphogenesis involves co-ordinated cell migration, cell differentiation and cell sorting. The organizer region controls these morphogenetic events spatiotemporally in a wide range of multicellular organisms, not only vertebrates and invertebrates but also plants and lower eukaryotes. Molecular studies have clarified the importance of the organizer formation and function in animal development (Bode, 2012; De Robertis, 2009; Thisse and Thisse, 2015). It is suggested that evolution of the organizer was crucial for the emergence of complex morphogenesis (Gerhart, 2001). However, the organizer region and its role in 3D structure formation remain largely unclear in primitive multicellular organism such as *Dictyostelium discoideum*.

D. discoideum is a model organism for multicellular morphogenesis in amoebozoa. The

amoebae proliferate unlimitedly under abundant nutrients. But when foods are exhausted, the amoeboid cells form a fruiting body, comprised of differentiated stalk and spore cells. In early development, starved cells aggregate by collective migration of up to 10^5 individual cells towards cAMP oscillations emanating from an aggregation center. The initiation of collective cell behavior depends on the elevation of extracellular cAMP concentration and its oscillations (Gregor et al., 2010). After the aggregated cells form tight-mound structures, the cells begin to differentiate into prespore and prestalk cell subtypes, resulting in "salt-and-pepper" mixture. Prestalk cells differentiate at random within the mound and subsequently migrate to the apical region to form the tip. The mound elongates upward to form a first finger then migrates as a slug, in which a distinct pattern of anterior prestalk cells and posterior prespore cells is established.

The tip acts as an organizer that is required for morphogenetic events throughout development. When the tip region is severed from a migrating slug, development pauses until the emergence of a new tip. Moreover, transplantation of a tip to the flank of another slug results in generation of a secondary slug, that separates from the original (Raper, 1940). Therefore *D. discoideum* is well-suited for studying the function and differentiation of organizer

1 cells in a primitive multicellular organism.

2 The anterior prestalk cells in the slug consist of two distinct subtypes, defined using

3 sub-fragments of the paradigmatic prestalk *ecmA* promoter (Early et al., 1993). Cells in the

4 front-half of the prestalk region are called prestalk A (pstA) cells, while cells in the rear-half of

5 the prestalk region are called prestalk O (pstO) cells. The cap site proximal promoter region of

6 *ecmA* (the ecmA region) is functional in pstA cells, whereas the cap site distal promoter region

7 (the ecmO region) is functional in pstO cells and also in the anterior-like cells (ALCs) that lie

8 scattered throughout the prespore region. An additional cell subtype expressing the transcription

9 factor CudA is located in a cone within the tip region (Fukuzawa and Williams, 2000).

10 However, the involvement of these cell types in the initial stages of 3D tip formation remains to

11 be determined.

12 DIF-1 (Differentiation Inducing Factor-1), a small-chlorinated signal molecule, induces

13 prestalk cell differentiation (Early et al., 1995; Morris et al., 1987). DIF-1 is synthesized by

14 des-DIF-methyltransferase, the product of the *dmtA* gene. When *dmtA* is disrupted, the mutant

15 cannot produce DIF-1 and shows several defects in development, including pstO cell

16 differentiation (Thompson and Kay, 2000b). Another DIF-synthesis gene *stlB*-, that is involved

in the initial step of DIF-1 synthesis, exhibits a major defect in differentiation of lower cup cells and basal disc cells which belong to the pstO subtype (Austin et al., 2006; Saito et al., 2008). However, these mutants were still able to form a tip and exhibited normal pstA cell differentiation. Therefore tip formation and pstA cell differentiation are likely to be independent of DIF-1 signaling.

MrfA (the homolog of human myelin-related factor) is a transcription factor that regulates pstA cell differentiation (Senoo et al., 2012). MRFa was purified as a binding protein using ecmA-specific promoter elements, and the null mutant strain, with a genetic inactivation of *mrfA*, did not express the ecmA-derived promoter fragment specific for pstA cells. The *mrfA*-mutant cells exhibited a multi-tip phenotype, implying that pstA has a role in tip dominance, but the mutant still formed a normal tip and slug, even though their size was reduced. These observations suggest that formation of the tip may not require authentic pstA cells, and that other yet unidentified cell population(s) may be involved.

The comprehensive *in situ* hybridization analysis of Maeda et al. reported three pstA specific genes (*ecmF* (SLF308), BCS1-like protein coding gene (SLI271) and *omt12* (SSK861)) whose expressions are independent of DIF-1 (Maeda et al., 2003). *ecmF* encodes an

1 extracellular matrix protein and is regulated by the transcription factor Dd-STATa (Shimada et
2 al., 2004a; Shimada et al., 2004b). The product of SLI271 is similar to mitochondrial chaperone
3 BCS1 that is necessary for formation of the ubiquinol cytochrome c reductase (bc1) complex.
4 *omt12* encodes an o-methyl transferase (OMT) family 2 protein that is 75% identical to
5 des-DIF-methyltransferase.

6 OMT12 has the ability to modify the alkyl resorcinol ring to yield a variant of
7 4-methyl-5-pentylbenzene-1, 3-diol (MPBD) *in vitro* (Ghosh et al., 2008). MPBD is a bioactive
8 polyketide, which acts in cooperation with DIF-1 in prestalk/prespore pattern formation and
9 spore differentiation (Narita et al., 2011; Saito et al., 2006; Sato et al., 2013). Also MPBD has
10 been reported to be involved in cAMP signaling in early development (Narita et al., 2014). Such
11 a modification of MPBD by OMT12 could provide subtle variations of biological activities *in*
12 *vivo*. These lines of evidence led us to consider *omt12* as being important for tip cell
13 differentiation.

14 In this study we compare the expression patterns of the orthodox pstA marker derived from
15 *ecmA* and a novel marker constructed with *omt12* promoter sequences. We demonstrate that the
16 pstA cell population includes at least two subtypes, *omt12*-positive cells (we term pstV^{A} cells)

1 and ecmA-positive cells (which we continue to term pstA cells). Surprisingly, some

2 *omt12*-positive cells are detected in growing populations and these cells (the $pstV^A$ cells) sort

3 directly to the emerging tip region in mounds. When the $pstV^A$ cells were purified from growing

4 cells, they developed several hours faster than the unsorted population. These findings suggest

5 that the $pstV^A$ cells are lineage-primed in the vegetative phase and are the initiators of tip

6 formation.

7

8 **Material and Methods**

9

10 **Cell culture and development**

11 *Dictyostelium discoideum* wild-type strain Ax2 cells were grown in HL5 axenic medium (Watts

12 and Ashworth, 1970) either on petri dishes or in a shaken suspension at 22°C. Strains possessing

13 the neomycin-resistance gene were grown in HL5 supplemented with 10 μg/ml of G418. For

14 development, cells were washed twice with phosphate buffer (KK2: 16.5 mM KH_2PO_4, 3.9 mM

15 K_2HPO_4, pH 6.1) before plating at a density of 4.7 x 10^5 cells/cm^2 on 1.6% water agar plates. An

16 SZH10 microscope and DP71 camera (OLYMPUS) were used for observation of development.

Plasmid construction

For generating ecmA:RFP and *omt12*p (*omt12* promoter):GFP vectors, we prepared pDdGFP and pDdRFP plasmids based on the pDdGal vector (Harwood and Drury, 1990) by substituting the *lacZ* to GFP or RFP and then the promoter was inserted upstream of GFP or RFP as follows. The flanking regulatory region of *omt12* from a 5' end point at nucleotide -936 to nucleotide +24, numbered relative to the ATG initiation codon of *omt12*, was amplified by PCR and cloned into the BamHI site of pDdGFP. The ecmA element was amplified from nucleotide -743 to nucleotide +3, numbered relative to the ATG initiation codon of the *ecmA* promoter sequence (Zhukovskaya et al., 2006) by PCR and cloned in pDdRFP. For construction of a dual promoter vector, a fragment with *act15*p (*actin15* promoter) and RFP was amplified from pDM323 (Veltman et al., 2009) by PCR and integrated into the HindIII site of the *omt12*p:GFP vector. For generating *omt12*p:PKA-Rm and ecmA:PKA-Rm expression vectors, each promoter sequences was excised from *omt12*p:GFP and ecmA:RFP construct and cloned into the XbaI/BglII sites of PKA-Rm (Harwood et al., 1992a). Constructs were introduced into Ax2 cells by electroporation (Pang et al., 1999), and stable transformants were selected and maintained

1 under 10 µg/ml G418.

2

3 **Slug dissociation**

4 For measurement of the proportion of labeled cells in the slug, *omt12*p:GFP / ecmA:RFP double

5 labeled cells were developed on water agar in a slit chamber at 22 ºC to form migrating slugs.

6 The slugs were collected in KK2 containing 20 mM EDTA and dissociated by passing through

7 a 24G needle several times. Dissociated cells were pictured by epifluorescence microscopy at

8 the same exposure for the GFP or RFP channels in all experiments. All of the cells including

9 GFP and RFP positive cells were counted in the field of each image using Cell Counter plugin

10 (ImageJ, NIH).

11

12 **Fluorescence time-lapse imaging during early development**

13 For under-agar development, cells were washed twice with Development Buffer (9.5 mM

14 $NAHPO_4$, 5 mM KH_2PO_4, 200 µmM $CaCl_2$, 2 mM $MgSO_4$). Fifteen microliter of 2 x 10^6

15 cells/ml cell suspension was placed on a 35 mm glass-bottom dish (i.e., 1.5 x 10^5 cells/cm^2) and

16 then covered by 2% thin-water agar. For development on cover slip, cells were washed twice

with KK2 and resuspended to a cell density of 1 x 10^7 cells/ml in KK2. The cell suspension was spread over 1 cm^2 of an alkaline-treated, hydrophilic cover slip surrounded by a 3 mm width of plastic tape. After removing excess KK2 by pipetting, the glass was placed inverted onto a glass-bottom dish and incubated at 22 ˚C. A IX71 microscope and DP71 camera or FV1000D confocal microscope (OLYMPUS) was used for imaging.

Long-term fluorescence time-lapse imaging during vegetative phase

For observation of cell division, cells were washed twice with KK2 and resuspended at 1 x 10^7 cells/ml in KK2, and 1 μl of the cell suspension and 1 μl of heat-killed bacteria (Lloyd et al., 1990) were diluted in 200 μl of KK2 on a 35 mm glass-bottom dish (Iwaki, Japan). The dish was incubated at 22 ˚C for 6 h for adaptation to bacterial culture. Prior to observation, cells were overlaid with thin agar and filmed using an IX83 system (OLYMPUS) with an EMCCD camera (evolve, PHOTOMETRICS).

Fluorescence-Activated Cell Sorting analysis

A FACSAria II (Becton Dickinson) was used for cell sorting and analysis of fluorescent

1 intensity of the labeled cells. Various sorting parameters were adjusted using vegetative

2 unlabeled cells. Exponentially growing cells were harvested and resuspended in HL5 at a cell

3 density of 5 x 10^6 cells/ml and were kept on ice before sorting. The cells were washed with KK2

4 buffer and resupended with KK2 buffer containing 0.3 mM EDTA at a cell density of 5 x 10^6

5 cells/ml. Every FACS analysis was performed within 10 min with a flow-rate at around 2,000

6 cells/s at 22 ˚C.

7 For development, 1 x 10^6 of the sorted cells were collected into HL5 and then washed twice

8 with KK2 before plating on a water agar plate. For chimera development, 5 x 10^4 cells were

9 sorted and collected in HL5 containing unlabeled 1.95 x 10^6 cells, and developed as above. For

10 re-culture of the FACS-separated populations, 1~2 x 10^5 cells were collected in HL5 containing

11 10 μg/ml G418 at 22 °C. To confirm the sorting quality, a part of sorted cells were re-analyzed

12 through FACS in every experiment. We also verified viability of the separated cells by Trypan

13 blue staining and confirmed that there were no significant damages caused by FACS (data not

14 shown). Fluorescence compensation between GFP and RFP was performed properly before

15 FACS separation using *omt12*p:GFP or *act15*p:RFP single labeled cell lines.

16

1 **Reverse transcription PCR**

2 Cells were developed on water agar plates and total RNA was extracted using RNeasy Mini Kit

3 (QIAGEN) according to the protocol provided, followed by removal of contaminating genomic

4 DNA with RNase free DNase Set (QIAGEN). cDNA was synthesized by reverse transcription

5 of 0.6 μg total RNA with a random 9 mer primer and M-MuLV reverse transcriptase (New

6 England Biolabs). The PCR program was as follows: One cycle of 2 min at 94˚C followed by

7 25 cycles of 30 sec at 94˚C, 30 sec at 52˚C, 1 min at 72˚C. The primer sets used for amplifying

8 developmental genes are listed in the Supplemental table.

9

10 **Results**

11

12 **A novel prestalk marker**

13 *omt12* is one of several pstA specific genes that have been identified by *in situ*

14 hybridization (Maeda et al., 2003). It encodes an o-methyltransferase family 2 protein that has

15 the capability to synthesize a variant of MPBD (Ghosh et al., 2008). We created an *omt12* knock

16 out mutant by homologous recombination and found that it exhibits normal cell differentiation

and development, as judged by various markers (data not shown). This suggests the possibilities that the modified MPBD produced by OMT12 does not have a specific role in differentiation, or polyketide variant(s) produced by other methyltransferases could compensate the function of *omt12*.

To investigate *omt12* expression as a novel marker, we amplified its promoter region by PCR (including the ATG start codon and followed by 21 bp of the coding sequence). The product contained 936 bp of the upstream of *omt12,* which is the full intergenic region between *omt11* and *omt12* (i.e. they are lying in parallel in the genome: http://dictybase.org). It was cloned into a multicopy GFP expression vector to create *omt12*p:GFP.

To compare the expression patterns of *omt12* and ecmA (the cap-site proximal *ecmA* promoter sub-fragment generally employed as the pstA marker (Zhukovskaya et al., 2006)), an *omt12*p:GFP and a ecmA:RFP construct were introduced into Ax2 cells by co-transformation, and stable transformants showing tip-specific expression of both markers were obtained as clones. Since we confirmed similar expression of the markers among several clones, one clone was selected and used for all experiments.

Prestalk genes such as *ecmA* are not expressed in vegetative cells (Jermyn et al., 1987), and

1 we first confirmed that ecmA:RFP, the pstA reporter construct, was not expressed in growing

2 cells but was expressed at multicellular stages (Fig. 1A). Surprisingly, however, a *omt12*p:GFP

3 signal was clearly detected in some of the growing cells (Fig. 1A; Vegetative). In the mid-late

4 mound stage, both markers' expression was observed in scattered cells, which is a typical pstA

5 pattern of differentiation (N.B., in earlier stages such as the streaming aggregate stage pstA cells

6 are not detectable). It is notable that the GFP and RFP signals did not overlap in the mound,

7 except for in a minority of cells (Fig. 1A; Mound). Both markers subsequently showed the

8 expression patterns predicted for the emerging tip region in the tipped-mound and the slug

9 stages. In addition, weakly positive cells were seen scattered in the posterior region, suggesting

10 expression of *omt12* in ALCs (Fig. 1A; Slug). High magnification confocal images of the slug

11 tip also showed intermingling of individual cells that were positive for either the *omt12*p or

12 ecmA marker plus a few that express both markers (Fig. 1B). All of these classes of

13 marker-positive cells were eventually incorporated into the developing stalk of the culminant

14 (Fig. 1A).

15 In order to estimate the ratio of these cell subtypes, we counted the RFP and GFP single or

16 double positive cells in the fluorescence images of dissociated slugs using ImageJ (Fig. 1C).

1 The result showed that the slug includes about 3.5% each of the marker positive cells, in which

2 1.4% of double positive cells are included.

3 To examine the behavior of *omt12*p:GFP and ecmA:RFP-positive pstA subtypes in

4 development, chimerae with 10% of the marker strain and 90% of unlabeled parental strain

5 were developed under 2% thin water agar and filmed by confocal microscopy. In the early

6 mound, only *omt12*p:GFP positive cells were detected (Fig. 2A, supplemental movie S1). The

7 ecmA:RFP positive cells appeared at random locations after a few hours of mound formation.

8 Consistent with the analysis of 100% labeled populations (Fig. 1C), ecmA expression was

9 observed in a low proportion of the *omt12*p:GFP positive cells (Fig. 2A, white arrow).

10 Since tip formation is suppressed under thin agar development, we established a 3D

11 real-time fluorescent observation method. Cells were developed on a hydrophilic coverslip

12 under a thin layer of water; this enables observation of tip development followed by slug

13 formation (Fig. 2B, upper illustration). We obtained time-lapse fluorescence images using

14 confocal microscopy and observed the structure of the emerging tip (Fig. 2B, supplemental

15 movie S2). Both the *omt12*p:GFP and ecmA:RFP positive cells rotate around the axis of the

16 multicellular structure while sorting to the emerging tip (high power pictures for a mound are

also shown in Fig. 3C).

These results demonstrate the presence of two distinct prestalk subtypes within the pstA region. We term the *omt12*p:GFP positive cells as $pstV^A$ cells and the ecmA:RFP positive cells as pstA cells. There is also a minority of cells that express both markers but we do not know exactly how they originate and we will not consider them further.

_omt12_p:GFP positive, vegetative cells sort directly to the tip region of the mounds

*omt12*p:GFP showed heterogeneous expression in vegetative cells and tip specific expression in multicellular stages. These results imply a linkage between *omt12*p:GFP expression level in growing cells and $pstV^A$ cell fate. The simplest interpretation given that the reporter protein is stable is that most of the vegetative GFP positive cells sort into the tip region during development. To elucidate this, we tracked individual *omt12*p:GFP positive cells from the vegetative stage to tip formation. As long-term imaging for ~12 h was required, we filmed by dividing development into two phases.

Firstly, we performed fluorescent time-laps imaging from 0 h to 6 h of development, i.e. until mound formation under 2% thin agar (Fig. 3A). The GFP intensity of each cell in the

1 population did not significantly change throughout early development, and the cells contributed

2 equally to the aggregate regardless of the level of *omt12*p:GFP expression (Fig. 3A,

3 supplemental movie S3, see also supplemental movies S4 and S5 for low power view). We

4 measured GFP intensity of cells during the first 5 h of development, i.e. before mound

5 formation. We selected 7 cells showing different GFP intensities at 0 h, which did not undergo

6 cell division for a further 5 hours (i.e. there were cells showing a doubling time of less than 5

7 hours; see Fig. 7C). All of the cells measured exhibited a steady level of GFP intensity during

8 the period (Fig. 3B), suggesting that the heterogeneity of *omt12*p:GFP expression was stably

9 maintained through early development.

10 Secondly, we observed the behavior of *omt12*p:GFP positive cells from mound to tip

11 formation. The labeled cells were developed on a hydrophilic coverslip and images were

12 acquired as described in Fig. 2B. At the late-mound stage, the *omt12*p:GFP positive cells were

13 still scattered within the mound but subsequently sorted out to the emerging tip region with

14 rotational cell movement (Fig. 3C, supplemental movie S6).

15 Taken together, these results indicate that heterogeneous *omt12*p:GFP expression in

16 growing cells is maintained throughout early development, and cells expressing a higher level

of the marker gene have a strong tendency to sort out to the tip region as a subpopulation of the pstA cells.

To obtain further evidence of the linkage between the expression level of *omt12*p:GFP in the vegetative phase and the subsequent cell fate as pstV^{A} cells, we performed chimeric development of unsorted wild-type cells and FACS-separated *omt12*p:GFP positive cell populations. To this end, we constructed a dual-promoter vector carrying both *omt12*p:GFP and semi-constitutive, *actin15* promoter-driven RFP (*act15*p:RFP) on a single plasmid backbone (Fig. 4A). This vector introduces an equivalent copy number of both markers to individual cells and allows us to track cells by the RFP signal.

Cells bearing the dual-promoter construct showed heterogeneity of *omt12*p:GFP and *act15*p:RFP expression in vegetative stage as expected, but we found that the *omt12*p:GFP positive cells do not totally overlap with the *act15*p:RFP positive cells (Fig. 4A). FACS analysis of the vegetative cell population showed a simple bell-shaped curve for *omt12*p:GFP expression (Fig. 4B). We separated the cells as Top 2%, Middle 15% and Bottom 2% fractions according to the *omt12*p:GFP expression levels, and the sorting quality was verified by FACS re-analysis followed by epifluorescence microscopy (Fig. 4B). Each of the FACS-separated fractions was

1 subsequently mixed with unlabeled cells at a ratio of 2.5% (labeled cells) and 97.5% (unlabeled
2 cells) and observed at the slug and culminant stages by epifluorescence microscopy (Fig. 4C
3 and D). In a control experiment with labeled unsorted cells (Fig. 4C; top panels), GFP
4 expression was seen strongly in the tip and weaker expression in scattered cells in the rear
5 prespore region, while RFP expression was uniformly distributed in the entire slug. This
6 confirmed that the labeled cells retained the dual vector in the slug and mingled uniformly with
7 unlabeled cells, with enhanced activity of *omt12*p in the tip region. The top 2% population
8 preferentially distributed in the anterior prestalk region of the chimera with strong tip
9 expression of *omt12*p, and also in the prespore region as scattered cells (they are likely to be
10 ALCs, see below) (Fig. 4C; Top 2%). On the other hand, most cells derived from the Middle
11 15% and Bottom 2% populations were found scattered in the posterior region with an
12 undetectable level of *omt12*p:GFP expression (Fig. 4C). Maturing fruits exhibited the same
13 sorting tendency. Top 2% cells were mainly found in the tip prestalk region, as well as upper
14 and lower cups (derived from ALCs) in the culminant (Fig. 4D). In contrast, most of the Middle
15 15% and Bottom 2% populations distributed within the spore mass (Fig. 4D).
16 These results indicate that the sorting behavior of cells expressing high level of

*omt12*p:GFP in growing cells is consistent with the intrinsic expression of *omt12* in slugs, suggesting that the fate of the pstV^{A} cells had been lineage primed in the vegetative stage.

The pstV^{A} cells enriched from growing cells begin to aggregate early

Since the tip acts as an organizer to establish 3D structure, we hypothesized that the pstV^{A} cells may have an important role in tip-related functions. To examine this possibility, we compared the developmental phenotype of the vegetative *omt12*p:GFP subpopulations separated by FACS.

We performed FACS-separation of vegetative cells to obtain Top 5% and Bottom 5% of *omt12*p:GFP expression. Sorting quality was verified by FACS re-analysis followed by epifluorescence microscopy (data not shown). As a developmental control, an unsorted population was also collected through FACS. Each fraction was developed as a pure population on water agar plates under light.

We observed roughly a 6 h delay of mound formation in the control development compared with parental Ax2. This is probably due to damage incurred by passing cells through the FACS; however, once aggregation commenced, the developmental timing was soon

1 restored, and the FACS-separated cells developed successfully to form normal fruiting bodies

2 by 28 h of development. This is consistent with the initial delay of development because the

3 parental Ax2 forms fruits by 22 h (N.B. In our laboratory condition, the parental Ax2 cells

4 develop 2 h faster than general timing of fruiting body formation by 24 h).

5 Among these populations, the Top 5% developed ca 8 h faster than the control population

6 (Fig. 5A). There was no difference in the number or size of the aggregates between the unsorted

7 population and the Top 5%. Once aggregates had formed, the developmental speed of the Top

8 5% population was nearly the same as that of the unsorted population. On the other hand, the

9 Bottom 5% showed almost equivalent development with an unsorted population, though tip

10 formation was slightly delayed (Fig. 5A).

11 The expression level of *omt12*p:GFP in slugs reflected the initial strength of the marker;

12 the Top 5% showed the strongest *omt12*p:GFP signals in the tip region while the Bottom 5%

13 showed weak but specific tip expression (Fig. 5A, fluorescence images). Interestingly, the

14 Bottom 5% showed elevated *omt12*p:GFP expression by the slug stage, suggesting some

15 regulation of pstV^A differentiation during development.

16 As a biological control, we performed the same experiment using *act15*p:GFP-labeled,

vegetative cell populations. The Top 5% and Bottom 5%, derived from vegetative *act15*p:GFP heterogeneity, showed no significant differences in developmental timing from an unsorted population; although the early development of these populations was again delayed by several hours compared to the parental Ax2, presumably due to cell damage (data not shown).

To elucidate the cause of precocious development of Top 5% population, we performed RT-PCR at 8 h of development for *carA* (cAMP receptor A), *acaA* (adenylyl cyclase A) and *csaA* (contact site A); proteins that are functionally involved in early development (Fig. 5B). Each population showed almost the same *carA* expression level. On the other hand, Top 5% showed higher expression of *acaA* and *csaA* at 8 h, while other populations including the unsorted control showed a basal level of expression. At 12 h and 14 h of development there was no difference of expression levels among the sorted populations (data not shown). These results suggest that the precocious development of the Top 5% might be due to a more rapid increase of cAMP concentration, derived from the elevated level of *acaA* expression, which induces a precocious shift toward aggregation competence.

In order to obtain further insights for the role of pstV^A cells in cell aggregation, cell subtype-specific obstruction of the developmental process would be useful. For this purpose, we

1 performed pstA subtype-specific inactivation of cAMP dependent Protein Kinase A (PKA).

2 PKA regulates expression of early developmental genes and cell differentiation in

3 *D.discoideum* development (reviewed by Loomis, 2014), and a dominant-negative form of PKA

4 regulatory subunit (PKA-Rm), of which two point mutations were introduced in cAMP binding

5 sites, causes inactivation of the PKA catalytic subunit (Harwood et al., 1992a). The

6 over-expression of PKA-Rm using the *actin15* promoter or *ecmA* full promoter blocks

7 development prior to cell aggregation or culmination, respectively (Harwood et al., 1992a;

8 Harwood et al., 1992b). In the same way, we examined the effects of PKA-Rm expression

9 under the control of ecmA or *omt12* promoters. We observed that *omt12*p:PKA-Rm expression

10 under mild antibiotic selection (5 μg/ml of G418) blocked cell aggregation completely for more

11 than 20 h (Fig. 6). The aggregation minus effect is much more potent than cells expressing

12 PKA-Rm under a strong promoter, i.e. that of *actin15*; the block of aggregation was never

13 perfect (84% of the control), even at high concentrations of G418 (100 μg/ml) (Harwood et al.,

14 1992a) . The expression of ecmA:PKA-Rm permitted cell aggregation, but blocked the

15 following tip formation (Fig. 6). The block of tip formation at this stage could be consistent

16 with the presence of an overlapping population that expresses the pstV^{A} marker within pstA

1 cells; ecmA:PKA-Rm expressing cells include ca 40% of pstV^A cell population (Fig. 1C). These

2 results would further support the proposed function of pstV^A cells in cell aggregation.

3

4 **Heterogeneous expression of *omt12* in growing cells is dynamic and independent of cell**

5 **cycle**

6 Heterogeneous gene expression in proliferative cell populations arises at random or more

7 deterministically by asymmetric cell division (Chang et al., 2008; Mascre et al., 2012). We

8 therefore characterized the dynamics of *omt12* expression in proliferating cells.

9 To analyze the dynamics of *omt12* expression during cell division, we performed

10 time-lapse imaging and tracking of dividing cells until they became grand daughter cells. To

11 obtain high resolution fluorescence images with reduced background, we used an *omt12*p:RFP

12 labeled clone, placed underneath a thin agar with heat-killed bacteria to shorten the total imaging

13 time. Under these conditions, the cells doubled every 4.5 h- 8.5 h, and we could track several

14 lineages over three generations in a single experiment. When focusing on a single *omt12*p:RFP

15 positive cell, the fluorescence signal was observed passing from the mother cell through two

16 divisions (Fig. 7A, arrowheads).(Supplemental movie 7). When we tracked the RFP intensity

and cell size of other strongly and mildly-positive *omt12*p:RFP cell lineages, the RFP intensity in a single cell lineage was again maintained through two cell divisions (Fig. 7B). Moreover, we measured cell-division times of individual cells and we found no significant connection between division time and *omt12* expression levels (Fig. 7C). These results indicate that heterogeneous *omt12* expression state is not affected by the cell cycle but is moderately stable during cell growth.

As fluctuations in gene expression and response to various extrinsic factors lead to cell-cell heterogeneity (Eldar and Elowitz, 2010; Raj and van Oudenaarden, 2008), we examined the stability of *omt12* expression level in growing cells. We purified Top 5% and Bottom 5% cells from *omt12*p:GFP expressing populations and then regrew them in HL5 medium. When the culture reached near-saturation it was diluted to maintain exponential growth. Over 3 days of culture (approx. 4 doubling times), both the Top 5% and Bottom 5% populations gradually regenerated the shape of an *omt12*p:GFP histogram that was more similar to that of an unsorted population (Fig. 8A). After 14 days of culture, both Top 5% and Bottom 5% populations showed almost exactly the same shape histograms as an unsorted population (Fig. 8A). We also examined the developmental phenotypes for all populations from 3 days culture and found that

they had almost recovered normal developmental timing (Fig. 8B). These results indicate that the heterogeneity of *omt12* expression is re-established dynamically by slow random drift over many generations to reach the starting equilibrium.

Discussion

A novel prestalk marker

In primitive multicellular organisms the origin and the role of the organizer in morphogenesis are almost unknown. Our study suggests a new scenario for *Dictyostelium* morphogenesis. We hypothesise that it is directed by the pstV^A cells; cells which express a novel GFP reporter construct based on the promoter of the *omt12* gene. Both the pstA and the pstV^A subtypes exhibit similar cell behavior during aggregation and slug formation. However, a low proportion of weakly expressing *omt12*p:GFP positive cells exist during growth. Time lapse cinematography shows that these same cells increase marker expression and sort to the tip. Although *omt12*p:GFP and ecmA:RFP expression overlaps in a proportion of cells, the majority of cells express each marker independently of the other (Fig. 1C). The origin of the double expressers is unclear but some of them are derived from *omt12*-expressing cells (Fig. 2A).

1 They could be genuinely different, playing different roles, or, given the fact that the reporter

2 proteins are stable, they could be the result of trans-differentiation. The results also suggest that,

3 due to their predicted precocious cAMP production, the pstV^A cells might be the initiators of

4 cell aggregation.

5 Cells lacking the MrfA transcription factor show a defect in differentiation of the pstA

6 cells; there is a reduction of ecmA reporter expression and tip dominance is reduced (Senoo et

7 al., 2012). The *mrfA*- mutant is however still able to form a tip and there is tip-specific

8 expression of *omt12*p:GFP (Fig. S1), indicating that differentiation of pstV^A cells is independent

9 of MrfA signaling. This suggests that the function of pstV^A cells may be distinct from that of

10 pstA cells, although the two cell types are intermingled uniformly in the pstA region.

11 The prestalk cells occupy about 20 % of the anterior prestalk region in a slug (Takeuchi et

12 al., 1986), and the pstA region was suggested to be equivalent to half of the anterior region, i.e.

13 roughly 10 % of the slug length (Early et al., 1993). Our cell counting analysis gives a more

14 precise figure of the minimal fraction of cells within the region because the total (pstV^A + pstA)

15 is ca 5.5 % of the disaggregated slug cells (Fig. 1C). However the prestalk region includes other

16 prestalk subtypes. A cone in the slug tip includes cells expressing the CudA transcription factor

(pstA*/AB cells). Another transcription factor STATa (DSTa*)* is nuclear-localized at the periphery of the tip and induces *cudA* gene expression in pstA*/AB cells (Araki et al., 1998; Fukuzawa and Williams, 2000). Since *cudA* or *dstA* null mutants still form normal tips, these transcription factors are not likely to contribute to the initial stage of tip formation and it suggests that the "tip organizer", mainly composed of pstA*/AB cells, has a specific role in late morphogenesis. Our results suggest a role for pstV^{A} cells in early morphogenesis: cell aggregation and tip formation. Our findings provide evidence that the anterior tip region is composed of unexpectedly various cell types with diverged developmental functions.

The pstV^{A} cells are lineage-primed in vegetative phase

In addition to the tip expression of the *omt12* promoter in slugs, we unexpectedly detected heterogeneous *omt12*p:GFP signals in vegetative cells using a high-copy vector with G418 selection, which yields approx. 150 copies of the transgene per cell (Nellen and Firtel, 1985; Nellen et al., 1984). When a low-copy *omt12*p:GFP construct with blasticidin selection was employed, the GFP signal was observed in the tip region but not in vegetative cells (data not shown). The *omt12* transcription level is very low in vegetative cells, and increases towards

aggregation, peaking at 12 h of development (Fig. S2, see also dictyExpress). Therefore we assume that the reason the *omt12*p:GFP signal is detectable in vegetative phase is due to amplification of the basal heterogeneous *omt12* expression by virtue of the high-copy construct.

Importantly, we demonstrated the linkage between the heterogeneous vegetative *omt12* expression and $pstV^A$ cell fate by time-lapse imaging and chimeric development. Namely, vegetative *omt12*p strongly positive cells sort out to the pstA region as $pstV^A$ cells together with the canonical pstA cells. By contrast the *omt12*p mildly positive or negative cells do not sort to the prestalk region. Therefore the heterogeneous *omt12* expression in growing cells and their tendency toward $pstV^A$ cell differentiation in the multicellular stages suggests a lineage priming of the $pstV^A$ cell fate.

To date, several studies on vegetative heterogeneities affecting the prestalk/prespore cell fate have been reported. Growth history is one of the determinants of prestalk/prespore cell fate bias. Cells (G-) grown in medium without glucose tend to differentiate into prestalk cells when mixed with cells (G+) grown with glucose, while G+ cells preferentially become prespore cells (Blaschke et al., 1986; Leach et al., 1973; Noce and Takeuchi, 1985). Additionally, cell cycle position at the onset of starvation has been linked to prestalk/prespore biases. Cells starved

1 around S/M phase tend to differentiate into prestalk cells, while the cells at mid-late G2-phase

2 sort into the prespore region (Araki et al., 1994; McDonald and Durston, 1984; Weijer et al.,

3 1984). Furthermore, intracellular Ca^{+} concentration (Azhar et al., 2001; Azhar et al., 1996;

4 Baskar et al., 2000) and pH (Gross et al., 1983; Inouye, 1985; Kubohara et al., 2007) also have

5 been known to correlate with cell fate bias.

6 It has been suggested that the cell fate biases by growth history and cell cycle position

7 could both influence cellular sensitivity to DIF-1, a polyketide prestalk inducer (Thompson and

8 Kay, 2000a). A recent study reported that heterogeneous RasD expression in the vegetative

9 phase has a key role in fluctuation of DIF-1 sensitivity by glucose concentration alterations

10 (Chattwood et al., 2013). Cells showing a relatively high level of RasD expression exhibit an

11 increased tendency to become pstO and pstB cells. Similarly, intracellular Ca^{+} concentration

12 and pH affect the efficiency of DIF-1 induced stalk cell differentiation (Kubohara et al., 2007;

13 Kubohara and Okamoto, 1994). We found that the heterogeneous *omt12* expression was not

14 affected by glucose concentration (Fig. S3). The DIF-independency of *omt12* expression

15 (Maeda et al., 2003) suggests that the heterogeneity of *omt12* expression in vegetative phase

16 may not link to a fluctuation of DIF-1 sensitivity, but further investigation is needed to obtain

1 more insights.

2 Muramoto et al. have reported that histone H3K4 methylation stabilizes inheritance of the

3 *act5* gene transcriptional state through a single cell lineage (Muramoto et al., 2010), suggesting

4 that chromatin modification could control transcriptional stability during the growth phase. The

5 histone methyltransferase Set1 mediates H3K4 methylation and knockout of *set1* causes

6 destabilization of the transcriptional state (Chubb et al., 2006). Interestingly, *set1*- cells show

7 precocious cell aggregation with rapid expression of early developmental genes (*acaA* and

8 *carA*) immediately after starvation. Since similar traits are observed in the development of Top

9 5% *omt12*p:GFP-positive populations, the transcriptional stability of *omt12* expression in

10 growing cells might be related with H3K4 methylation state. To verify this connection, precise

11 detection of frequency and length of *omt12* transcriptional bursts in a single cell lineage would

12 be required.

13 The chimeric development showed that a fraction of the strongly *omt12*p positive cells

14 dispersed in the posterior region of the slug. These seem to be ALCs that eventually become

15 upper and lower cup cells (Fig. 4C and D, Top 2%). As ALCs consist of pstO, pstB and pstU

16 cells (Yamada et al., 2010), it suggests the possibility of an interaction of cell lineage priming

1 mechanisms between pstV^A and other prestalk subtypes. Moreover the various lineage-priming

2 systems probably enable *D. discoideum* amoeba to establish the heterogeneous prestalk

3 subtypes including the organizer cell population(s).

4

5 **The function of pstV^A cells**

6 Decomposition analysis of heterogeneous cell population provides a functional meaning

7 (Altschuler and Wu, 2010). Our study using FACS-mediated cell enrichment provides valuable

8 information to elucidate whether lineage-primed pstV^A cells have any specific functions in early

9 development. Populations enriched in *omt12*p positive cells initiated cell aggregation

10 significantly faster than the unsorted cell populations and showed a precocious increase in *acaA*

11 transcription in early development. These results suggest that, in normal development, the

12 lineage-primed pstV^A cells could take the lead over the remaining populations in cell

13 aggregation via precocious cAMP production. A previous study reported that the onset of the

14 cAMP pulses that direct cell aggregation is a collective behavior that depends on a rise of the

15 extracellular cAMP concentration (Gregor et al., 2010). Since the *omt12*p positive cell

16 population distributes evenly among other cells during aggregation, the population could

contribute to an increase in the overall extracellular cAMP concentration to initiate cell aggregation rather than to make aggregation center(s) by itself. On the other hand, the *omt12*p negative cell enriched population showed normal developmental timing with a slight delay in tip formation. Since the slug derived from the *omt12*p:GFP negative cells has a weak but tip-specific GFP signal, the recovery of development could reflect replenishment of $pstV^A$ cells from the *omt12*p:GFP negative cells. Inactivation of PKA in the *omt12p*-positive, limited number of cells blocked in aggregation, suggesting that the $pstV^A$ cells might be necessary for organizing aggregation. In summary, we propose that the $pstV^A$ cells are a novel prestalk cell subtype that seem likely to be centrally involved in constructing the 3D structure.

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Figure Legends

Figure 1. Expression patterns of ecmA:RFP and *omt12*p:GFP in growth and development.

A stable clone bearing both ecmA:RFP and *omt12*p:GFP marker constructs was analyzed. (A) Both marker's developmental expression patterns. The images were taken by epifluorescence microscopy with the same exposure time, except for the images in the vegetative phase which were taken independently. The white square bracket shows a group of GFP/RFP-positive cells situated in the anterior prestalk region. The white dotted lines indicate outlines of the developmental structures. Bar; 100 µm. (B) High power view of the anterior regions of two individual slugs. Each image is a maximum intensity projection (MIP) of Z=6 slices with 1.5 µm intervals, viewed by confocal microscopy. Bar; 50 µm. (C) The proportion of each pstA marker positive cells within the slug. The *omt12*p:GFP and/or ecmA:RFP positive cells in the fluorescence images were counted using Cell Counter plugin (ImageJ, NIH). The average value and SD of four independent experiments are shown.

Figure 2. Behavior of the two pstA subtypes in mounds and tipped aggregates.

(A) Chimerae with 90% unlabeled cells and 10% of the double labeled cells were

1 developed under 2% thin-water agar. All pictures are extracted from a movie, in which MIP

2 images of Z=6 slices with 2 μm intervals that were taken every 1 min by confocal microscopy.

3 The white arrow indicates one of the double positive cells originated from a *omt12*p:GFP single

4 positive cell, that begun to expresses ecmA:RFP at a later stage. Time-lapse images starting

5 from the late-streaming stage were acquired for 7 h; the time stamp represents elapsed time of

6 image acquisition (hh.mm.ss). Bar; 100 μm. (B) Time-lapse imaging of tip formation in

7 chimerae. The chimeric cell population used in (A) was developed on a hydrophilic coverslip

8 with a thin water film. The pictures were taken and processed as in (A), except that 14 μm

9 intervals were employed. The image acquisition was performed for 2 h from late-mound stage.

10 The time stamp represents elapsed time of filming (hh.mm.ss). Bar; 100 μm.

11

12 **Figure 3. The vegetative *omt12*p:GFP positive cells sort to the emerging tip of a mound.**

13 (A) Tracking of the vegetative *omt12*p:GFP positive cells from vegetative to mound stage.

14 The *omt12*p:GFP labeled clonal cells were developed under 2% thin-water agar and time-lapse

15 images were acquired by epifluorescence microscopy for 6 h. The magenta arrowhead indicates

16 the cell expressing *omt12*p:GFP strongly. The white dotted lines indicate the outline of the early

1 mound. The time stamp represents elapsed time of image acquisition (hh.mm.ss). Bar; 100 µm.

2 (B) Measurement of the *omt12*p:GFP intensity during five hours of early development. GFP

3 signal intensities in seven selected cells were measured at each developmental time point using

4 ImageJ (NIH). (C) Tracking of the *omt12*p:GFP positive cells during tip formation. Cells were

5 developed by the same method as in Fig. 2B. The pictures are MIP images of Z=4 slices with 18

6 µm intervals that were taken every 1.5 min by confocal microscopy. The image is a merger of

7 two channels (phase contrast and GFP). Images were acquired from the mid-mound stage for 2

8 h. The time stamp represents elapsed time of filming (hh.mm.ss). Bar; 100 µm.

9

10 **Figure 4. Sorting behavior of the vegetative *omt12*p:GFP positive, moderate and negative**

11 **cells.**

12 (A) A dual promoter vector carrying *omt12*p:GFP and *act15*p:RFP for FACS-sorting and

13 chimeric development. The schematic drawing shows that the two reporter genes share an

14 *actin8* terminator. Cells were transformed with the vector and stable clones bearing both marker

15 signals were isolated. The epifluorescence images of the GFP, RFP channels and their merge

16 taken from exponentially growing, clonal transformant cells are shown. Bar; 50 µm. (B) FACS

1 separation of *omt12*p:GFP positive, moderate and negative fractions in a growing cell

2 population. A transformant clone in (A) was sorted into three fractions according to the GFP

3 intensity indicated by the black-line rectangles; Top 2%, Middle 15%, and Bottom 2%. The

4 sorting-quality of each cell population was confirmed by FACS re-analysis followed by

5 epifluorescence microscopy as shown. These fluorescence images were taken with the same

6 light intensity and exposure time. Bar; 50 µm. (C, D) Chimeric development with 2.5% of the

7 FACS-separated, double labeled cells and 97.5% of unlabeled cells. Slug (C), Culminant (D).

8 The white dotted lines indicate the outline of the specimen. White square brackets in (C) and

9 (D) indicate approximate prestalk region in the slug and upper/lower cups, respectively. The

10 control experiment is a chimera with 2.5% of unsorted double-labeled cells and 97.5%

11 unlabeled cells. Bar; 100 µm.

12

13 **Figure 5. Vegetative pstV^{A} cells develop precociously**

14 *omt12*p:GFP-positive and negative populations were sorted from exponentially growing cells as

15 the Top 5% and Bottom 5%, respectively. For a control, the unsorted cell population was also

16 collected via FACS. The sorting quality of each population was confirmed by FACS re-analysis

and epifluorescence microscopy as in Fig. 4B. (A) Developmental phenotype of the Top 5%, the Bottom 5% enriched populations a and the unsorted population. Each population was developed on water agar plate for the time indicated. The images show the same developmental area for each population. The fluorescence images show the GFP pattern of each population at the slug stage. All images were taken with the same exposure time. Developmental timings of the three populations are summarized in a lower schematic diagram. Developmental stages are shown as follows: Aggregation (A), Mound (M), Slug (S), and Fruiting body (F). (B) Semi-quantitative RT-PCR analysis using early developmental genes for the FACS-enriched populations. Each FACS-enriched population in (A) was developed on a water agar plate and total RNA was prepared at 8 h of development. RNA samples were treated with DNase I prior to reverse transcription. *Ig7* (mitochondrial large rRNA) serves as a control.

Figure 6. The developmental phenotypes of cells expressing a dominant-negative form of the PKA-R subunit (PKA-Rm) under control of the *omt12* or the ecmA promoter.

PKA-Rm, which contains point mutations in the two cAMP binding sites (Harwood et al., 1992b) was expressed in wild-type cells under the control of either *omt12*p or ecmA promoter

1 and stable cell lines were selected in HL5 containing 5 μg/ml of G418. The transformants were
2 developed and observed on a water agar plate along with parental wild-type cells. Bar; 1 mm.
3
4 **Figure 7. *omt12* expression during cell growth.**
5 (A) Time-lapse images during cell division of *omt12*p:RFP labeled clonal cells. Cells were
6 cultured under 2% thin agar with KK2 and heat-killed bacteria. The images were taken every 15
7 sec The arrowheads indicate two cell divisions of a single RFP positive cell lineage. The time
8 stamp represents elapsed time of filming (hh.mm.ss). (B) Measurement of *omt12*p:RFP intensity
9 and cell size during two cycles of cell division. In the time-lapse imaging used in Fig. 7A,
10 two cell lineages showing strong (solid circle) or middle (open circle) RFP intensity
11 were analyzed using ImageJ (NIH). The red and blue colors indicate RFP intensity
12 (logarithmic scale) and cell size (linear scale), respectively. The dotted lines indicate
13 the timing of each cell division. (C) Correlation analysis between cell-division time and
14 *omt12*p:RFP expression. Cell lineages that underwent two cycles of division in (A) were
15 selected, and doubling times of 30 cells between daughter and granddaughter cells were
16 measured by cell tracking. The coefficient of correlation between doubling-time and RFP

intensity; *r* was calculated by the Pearson product-moment correlation coefficient formula. The correlation coefficient indicates that there is no correlation between doubling-time and RFP intensity.

Figure 8. Dynamics of *omt12*p:GFP expression heterogeneity

(A) FACS analysis of the Top 5%, Bottom 5% and unsorted cell populations of a clonal *omt12*p:GFP transformant after 3 days and 14 days culture. These cell populations were separated from exponentially growing cells, and re-cultured in HL5 containing 10 µg/ml G418. Each population was analysed for their GFP intensity by FACS at each time point. Unsorted cell population was analysed as a control. (B) Comparison of development of the Top 5%, Bottom 5% and unsorted cell populations after 3 days culture. All populations were developed on water agar plate as in Fig. 5A. Bar; 1 mm.

Legends for Supplementary Materials

Figure S1. *omt12*p:lacZ expression in *mrfA*- mutant cells

1 For construction of *omt12*p:*lacZ*, the *omt12* promoter sequence excised from the

2 *omt12*p:GFP was cloned into EcoRI/BamHI site of the pDdGal vector (Harwood and Drury,

3 1990). The transformant slugs were fixed and stained for β-galactosidase. The staining time for

4 each sample was controlled to obtain approximately the same intensity in the tip region. Bar;

5 100 μm.

6

7 **Figure S2. Expression of *omt12* during early development**

8 RT-PCR for *omt12* in vegetative and early development is shown. Wild-type Ax2 cells

9 were developed on filters after washing with KK2, and total RNA was prepared at 0 h, 4 h, 8 h

10 and 12 h of development. RNA samples were treated with DNase I prior to reverse

11 transcription. Note that there are basal levels of *omt12* expressions, albeit very weak, during

12 vegetative to 8 h. *Ig7* (mitochondrial large rRNA) expressions are shown as a control.

13

14 **Figure S3. The effect of glucose on *omt12*p:GFP expression in growing cells**

15 FACS analysis of *omt12*p:GFP labeled cells grown in HL5 medium with/without 75 mM

16 glucose for 2 days. The simple-bell shaped histograms of GFP intensity show a good match

between the two conditions, indicating that the heterogeneity is maintained irrespective of glucose in the medium.

Supplemental movie S1. Behavior of ecmA:RFP and *omt12*p:GFP double labeled cells during mound stage

The double labeled (10%) and unlabeled cells (90%) were mixed and developed under thin agar. Time-lapse images were recorded at 1 min intervals by confocal microscopy.

Supplemental movie S2. Behavior of ecmA:RFP and *omt12*p:GFP double labeled cells during tip formation

The cell mixture in Supplemental movie S1 was developed on a hydrophilic coverslip to allow for 3D morphogenesis. Time-lapse images were recorded at 1 min intervals by confocal microscopy.

Supplemental movie S3. Time-lapse images of *omt12*p:GFP labeled cells from vegetative to mound stage

The cells were developed under thin agar and time-lapse images were recorded at 1 min intervals with epifluorescence microscopy.

Supplemental movie S4. Time-lapse images of *omt12*p:GFP labeled cells from vegetative to stream stage

The labeled cells (10%) and unlabeled cells (90%) were mixed and developed under thin agar; the dark space in the field of view is filled with unlabeled cells. The cells began to form streams toward an aggregation center arising in the right side of the field after 7 h. The time stamp represents the elapsed time after nutrient removal (hh.mm.ss).

Supplemental movie S5. Time-lapse images of *omt12*p:GFP labeled cells from stream to early mound stage

Experiments were performed under thin agar as in Supplemental movie S4. Time-lapse filming was started after 8 h of development and filmed for 2 h. The time stamp represents the duration of development (hh.mm.ss).

Supplemental movie S6. Time-lapse images of *omt12*p:GFP labeled cells during tip formation

The labeled cells were developed on a hydrophilic coverslip. Time-lapse images were recorded at 1.5 min intervals by confocal microscopy.

Supplemental movie S7. Time-lapse images acquired during cell division of *omt12*p:RFP labeled clonal cells

The cells were cultured under the thin agar with KK2 and heat-killed bacteria. Time-lapse images were recorded at 15 sec intervals with epifluorescence microscopy.

1

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