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Cell type proportioning in *Dictyostelium* slugs: lack of regulation within a 2.5-fold tolerance range

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Abstract The proportion of prestalk and prespore cells in *Dictyostelium discoideum* slugs is often cited as an example of "almost perfect" regulation. The pattern is similar over a very wide range of cell number; furthermore, removal of either of the cell types leads to compensatory transdifferentiation. Several studies of *Dictyostelium* fruiting bodies, however, have suggested that proportioning in *Dictyostelium* differs systematically from true constancy. We have confirmed this in the slug stage using a short-lived β-galactosidase as a reporter of the prestalk specific *ecmA* gene expression: the prestalk proportion decreases from 24 ± 5% in slugs of 10^3 cells to 10 ± 3% when 10^5 cells are present. Regeneration experiments suggest that this difference is not due to a modulation of the proportioning set-point by size, as one might have expected; instead there appears to be a regulatory "tolerance zone" at all sizes. After amputation of the whole posterior region, transdifferentiation stops after the fraction of prestalk has been reduced from 100% to 28 ± 2%, well above the initial value of 10 ± 3%, while after anterior removal the transdifferentiation endpoint is about 10%. Most strikingly, we find *no regulation at all* after partial amputations of the prespore region. It seems that any prestalk proportion is stable between a ~10% lower threshold and a ~30% upper threshold. To explain this, we propose a regulation mechanism based on a negative feedback plus cell type bistability. In both intact and regenerating slugs we find that the slug morphology is regulated so that the *length-to-width* ratio of the anterior region is constant.

Key words *Dictyostelium* · cell-type proportioning · regeneration · transdifferentiation

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Introduction

In many organisms cell type differentiation is known to be induced or maintained by local concentrations of diffusible molecules. In many cases these patterns are dictated by localized determinants, which are themselves created by complex antecedent processes, so that it is difficult to pinpoint the process in which the pattern actually arises. In other cases, however, supracellular order appears effectively de novo. Reaction-diffusion mechanisms are one class of process capable of generating order de novo. A signature of reaction-diffusion processes is that the spatial structure has a characteristic length which is determined by the ratio of diffusion and breakdown constants and is thus independent of the system size.

It is far from clear, however, that all biological structures which arise de novo show this signature. *Dictyostelium discoideum* is a relatively simple example of an organism which successfully regulates its development over a wide range of sizes.

In *Dictyostelium*, a multicellular mound is formed by the aggregation of 10^2–10^5 cells. Upon mound formation, cAMP levels rise, triggering a signalling cascade that eventually initiates cell type differentiation. Recent studies suggest that in the early mound, prespore cells differentiate at random positions, while prestalk O (pstO) and prestalk A (pstA) cells arise independently,

pstO also in a random manner and pstA in the skirt of the mound [9]. Then prestalk cells sort out to the tip, and the mound elongates to become a cylindrical finger that falls onto the substratum and forms the migrating slug. Development culminates with the slug tip moving into itself to produce a stalk, whereas most of remaining cells turn into a spore mass on top of this stalk.

Cell type pattern in the slug is highly organised along an anterior-posterior axis. The anterior is composed of three different subtypes of prestalk cells, with pstA cells occupying the ~5–10% foremost tip of the slug and pstO cells the following ~5–15% crown. At the core of this prestalk or anterior region lies a short segment of prestalk AB cells (pstAB). The remaining ~70–90% of the slug, which forms the posterior region, is mainly composed of prespore cells. However, scattered among them, there is a population of so-called anterior-like cells (ALC) that display most of the properties of prestalk cells. Cell-differentiation is known to be plastic up to the last stages of development. Transdifferentiation in normal conditions is believed to occur between pairs of cell types only within a specified pathway: prespore ⇔ ALC ⇔ pstO ⇔ pstA [6].

This pattern of cell types is qualitatively the same for slugs of all different sizes, from ~100 cells to more than $\sim 10^5$ cells [3]. If either the anterior or the posterior region are removed, some of the remaining cells transdifferentiate into the other cell type until a new normally patterned slug is formed [24]. These observations led Bonner [3] to the formulation that "differentiation remains proportionate irrespective of size". The idea of essentially perfect proportioning has been embraced by the *Dictyostelium* field with an enthusiasm that is perhaps unjustified by the quality of the original data; this shows a huge scatter: the prestalk proportion spans a range from ~15% to more than ~40% [3]. Several studies [4, 10, 21, 28] have shown clear departures from true proportionality in fruiting bodies, with the proportion of stalk and basal disk decreasing with increasing overall size. The existence of massive transdifferentiation after removal of prespore cells has been amply confirmed, [1, 23, 25, 26], but none of these studies established that the cell type proportion is exactly the same in original and regenerated slugs.

When considering theoretical models, the difference between an exact proportion regulation and a looser cell type regulation is important. Turing-type reaction-diffusion models yield characteristic lengths but not constant proportions (except under very demanding requirements [22]). Common global interaction models of bistable cell states (prespore/prestalk) can easily scale with size but produce a bandwidth of proportions rather than an exact value [20] and require an independent mechanism of cell sorting. It is an open question whether a global interaction model can explain the change in proportion after removing part of the slug. In order to establish the mechanism of cell type regulation at the multicellular level, it is fundamental to have an accurate quantification of the cell type proportion.

We have thus revisited the classical amputation experiments by Raper [24] and conducted a new study of proportioning in slugs using a recently developed labile β-galactosidase as a reporter of the prestalk cell specific gene *ecmA* carried on a novel vector (V18Tn5) that is supported by the wild strain NC4. The temporal and spatial resolutions of this reporter have made it possible to examine accurately the proportion of cell types both during regeneration and for slugs of different sizes.

Finally, upon examining the morphology of slugs we have come across another problem of *Dictyostelium* development: how a complex interaction of cell motion, cell adhesion and sheath tension regulates the shape of the slug. Previous qualitative observations established that during regeneration slug shape is progressively recovered [16, 24]. Here we present the first detailed analysis of how this shape regeneration occurs.

Methods

Cell strains and growth conditions

NC-4 cells were transformed [31] with a *ecmAO*-iα-gal vector. This vector has a V18Tn5 selection cassette (V18 promoter driving neomycin phosphotransferase) and a second cassette containing an *ecmAO* promoter driving a labile β-galactosidase. *ecmAO* is the whole promoter of the *ecmA* gene. The labile β-galactosidase is an improved version of "ile-gal" which has been estimated to have a protein half-life of less than one hour [8] and is appropriate for the observation of cell type transdifferentiation. In the improved version the alpha peptide has been fully restored [17] resulting in a 100× increase in specific activity (MacWilliams, unpublished).

NC-4-*ecmAO*-iα-gal cells were grown on *Klebsiella aerogenes* lawns on SM agar plates (10.0 g/l peptone (DIFCO), 1.0 g/l yeast extract (DIFCO), 7.5 g/l D-glucose, 4.4 g/l KH_2PO_4, 5.0 g/l $Na_2HPO_4 \cdot 12H_2O$, 1.0 g/l $MgSO_4 \cdot 7H_2O$, 15.0 g/l agar (Nakalai, Japan)) for 36–48 hours, then harvested and washed twice in BSS (0.6 g/l NaCl, 0.75 g/l KCl, 0.4 g/l $CaCl_2 \cdot 2H_2O$). Cells were then suspended for 5 minutes at 4°C in Mg-free LPS (20 mM KNa_2 phosphate buffer, pH 6.5, 1.5 g/l KCl) with 0.001–0.002% of the vital stain neutral red. After staining, cells were washed 3 or 4 times in BSS and plated in 10–20 µl drops at appropriate cell density on Millipore nitrocellulose filters of 0.45 µm pores on top of 1.5% agar plates. Slugs of different sizes were created by varying the inoculation cell density from 1.0×10^7 to 1.0×10^9 cells/ml. Slugs were incubated in dark for 36–48 hours at 22°C before manipulation.

Slug manipulations and histochemical detection of β-gal activity

To characterise cell type proportion and shape of intact slugs of different size, slugs were taken directly from the incubator, fixed, and quickly stained with standard gal-staining techniques [2]. Fixation time had to be increased up to 10-fold in big slugs so as to obtain the same intensity of staining as in small slugs. Photographs were taken from the top and the side.

In amputation experiments, slugs were cut with the 10–50 µm tip of a flame-drawn glass capillary (capillary drawn using the Model-P87 of a Flaming/Brown micropipette puller, Sutter Instrument Co.). The cut was made at the boundary between the anterior

and posterior regions as revealed by neutral red staining. Photographs were taken before and after the amputation. Amputated slugs were isolated and kept in the incubator at 22°C for a specified period, then fixed and gal stained. Photographs were taken before and after the gal staining.

Determining aspect ratio and cell type proportion

Photographs were scanned, stored in tif format, and processed using NIH Image. For each slug, the length and area of the whole body and the anterior region were measured from both top and lateral views. The volume of the whole slug or its anterior region were estimated as, $V = A_{lateral} \times A_{top}/L$, where V stands for volume, $A_{lateral}$ and A_{top} for the areas of slug as measured from the lateral view and top view pictures respectively. L stands for the length as measured from the view (either top or lateral) that better reflected the real axial length of the slug. During fixation, slugs shrink between 20–40% in volume. The magnitudes reported here should be consequently modified for comparison with in vivo measurements of slugs. Volumes are given in cubic microns, $(\mu m)^3$ which will be written as μm^3 to simplify notation.

Prestalk Proportion is defined as the proportion in volume of the anterior region in the whole slug, i.e. $100 \times V_{prestalk}/V_{slug}$, where $V_{prestalk}$ (prestalk volume) is the volume of the anterior region. This prestalk proportion only represents the proportion in volume of the pstA and pstO cell populations.

Slug Aspect Ratio is defined as the length/width ratio of the slug, where the width, W, is estimated as the top view area divided by the top view slug length (W = A/L).

Prestalk Aspect Ratio is defined similarly as the length/width ratio of the anterior region. The width W of the anterior is estimated as the top view area of the anterior divided by the top view anterior length.

Error associated with volume estimate

The method used to estimate the volume does not take into account the roughly hemispherical shape of the anterior region, nor the cylindrical shape of the posterior region. This introduces a systematic error that should be considered.

Assuming that the anterior region has the shape of half a spheroid (an ellipsoid of revolution), its real volume is $V_{ant} = 4\pi r^2 l/6$ where r stands for the radius of the spheroid and l for its length. For such a spheroid, the present estimation method gives an anterior volume of is $VE_{ant} = 4\pi r^2 l/4$. On the other hand, the volume estimate for the whole slug is $VE_{slug} = 4r^2 L$, where L is the length of the slug, while its "real" (cylindrical approximation) volume is $V_{slug} = \pi r^2 L$. From these results, one can easily compare the more realistic proportion $p = V_{ant}/V_{slug}$ with the prestalk proportion obtained with the estimate $pE = VE_{ant}/VE_{slug}$. It is found that the prestalk proportion estimate is an 8% smaller than the "real" prestalk proportion (p = 1.08/pE).

Imprecisions in the measurement of lengths and areas can yield between a 5% and a 15% error in the calculation of prestalk proportion of a single slug. Since the systematic error due to the volume estimate method is smaller than the error and standard deviations (see tables), it has not been corrected for in this study.

Results

ecmA expression in NC4 and neutral red staining overlap

Fig. 1 shows slugs formed by NC4 cells transformed with *ecmAO*-iα-gal after gal staining. The expression pattern differs from that described in axenic strains: instead of staining the tip most intensely or the entire prestalk zone uniformly [9], ecmAO expression in NC4 [31] is highest in the rear part of the anterior region and weak in the tip. This pattern defines a very sharp boundary between the anterior and posterior regions of the slug. This pattern is not explained by the use of a short-lived reporter, as was previously seen in NC4 using a stable β-galactosidase [31], while the labile gal reporter produces anterior or uniform staining in axenic strains (MacWilliams, unpublished). Instead, it appears to result from a failure of NC4 to express the pstA element of the *ecmAO* promoter (MacWilliams, unpublished).

Neutral red staining in the same NC4-*ecmAO*-iα-gal slugs does not always draw a clear line between the anterior and posterior region, in particular for young slugs. However, whenever the boundary is sharp, it nicely overlaps with the one defined by the gal staining. This overlap has been observed even during regeneration experiments (data not shown), thus confirming that neutral red can be used as an *in vivo* reporter even in transdifferentiation processes. The only other *in vivo* reporters with good temporal resolution, short-lived GFP reporters [7], require strong blue irradiation, which is the normal signal for slug culmination.

Prestalk proportion decreases as slug size increases for intact slugs

As has been known since early studies on *Dictyostelium*, the pattern of differentiated cells is qualitatively unchanged over 3 orders of magnitude in slug volume (see Fig. 1) [3]. Here the slug volume has been varied from $\sim 10^5$ to 5×10^7 μm^3. Fig. 2A shows the progressive increase of prestalk volume as the slug volume increases. Although at a first glance it might seem from Fig. 2A that the prestalk proportion is "roughly constant", one must bear in mind that this is a log-log plot. As shown in Fig. 2B, the prestalk proportion (as defined in Methods) is found to steadily decrease from an average value of 23.7 ± 4.5% for small slugs, to an average value of 9.8 ± 2.8% for the biggest slugs, almost a 2.5-fold change.

Proportion regulation after amputations or transplantations

After posterior amputation, the prestalk proportion remains above the initial value. Mature NC4-*ecmAO*-iα-gal slugs were cut at the boundary between the anterior and the posterior region using the neutral red staining patterns as a guide. After the cut, the posterior region of the slug was removed, leaving an anterior fragment (containing close to 100% prestalk cells) that kept migrating. Fig. 5a shows the change in prestalk proportion over time. Three hours after completely removing the pos-

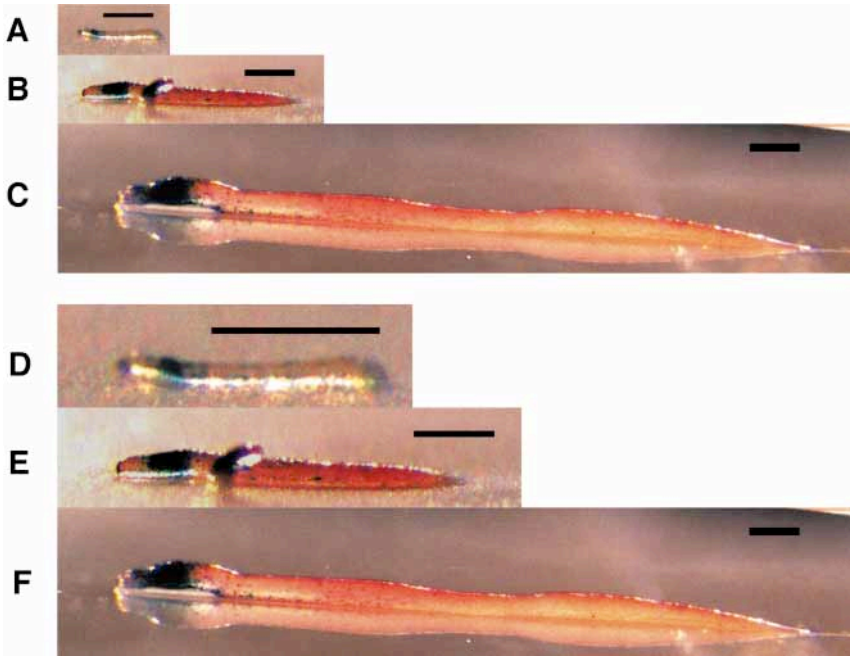
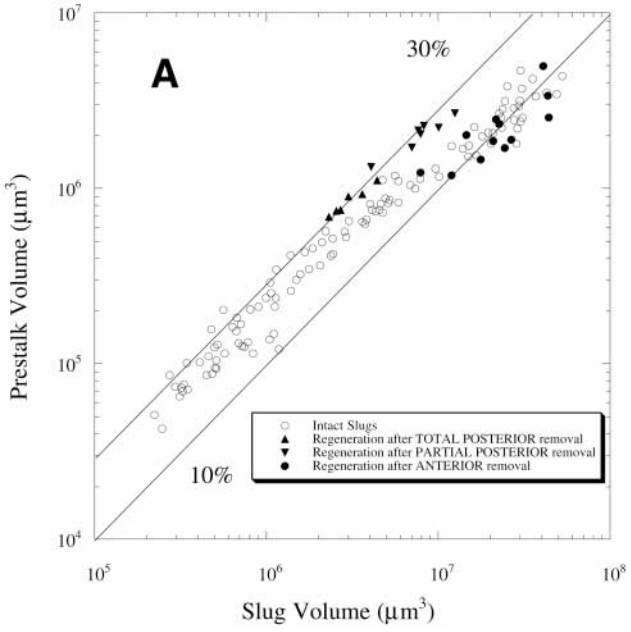


Fig. 1 NC4-*ecmAO*-iα-gal transformant slugs of different sizes. The same slugs are shown at the same magnification in (**A, B**), and (**C**) and after appropriate scaling in (**D, E**), and (**F**), respectively. All *scale bars* represent 0.2 mm. It can be observed that prestalk proportion decreases with size, yet prestalk aspect ratio (Length/Width) is similar for all sizes. The volumes of slugs (**A, B**), and (**C**) are 3.3×10^5 μm^3 , 4.3×10^6 μm^3, and 3.7×10^6 μm^3. Prestalk proportions are 21.5 %, 17.1%, and 9.0%.

terior region, the posterior $24 \pm 12\%$ of the slug was free of *ecmAO* staining.

The proportion stabilised between 12 and 18 hours after the amputation of the posterior region. However, the final value of $28.0 \pm 2.1\%$ is almost 3-fold the initial one, $10.5 \pm 3.3\%$. Intact slugs of the same size as the regenerated slugs display a prestalk proportion of $17.2 \pm 2.7\%$, still significantly below that of the regenerated slugs (see Fig. 2A). Former results by Sakai [25] also show a low percentage of prespore cells (~ 60%) in re-



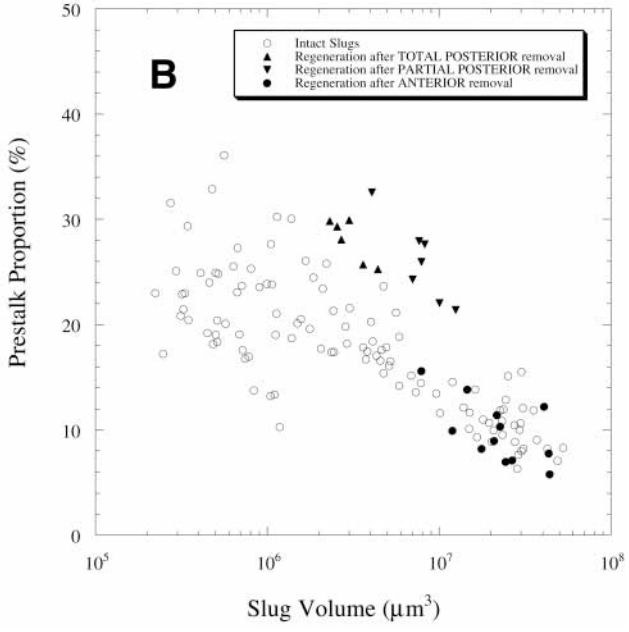


Fig. 2 (**A**) Prestalk volume as a function of slug volume. (**B**) Prestalk proportion vs. slug volume. For intact slugs, there is a clear decrease in prestalk proportion from $23.7 \pm 4.5\%$ to $9.8 \pm 2.8\%$ as slug volume increases. Slugs regenerated for 18 and 24 hours after total amputation of posterior region display a prestalk proportion at the upper threshold of proportions observed in intact slugs. Slugs regenerated for 12 hours after partial amputation (removal of about 2/3 of initial volume) of posterior region) also present prestalk proportions well above those found in intact slugs. However, slugs regenerated for 6 hours after amputation of the anterior region have proportions similar to intact slugs.

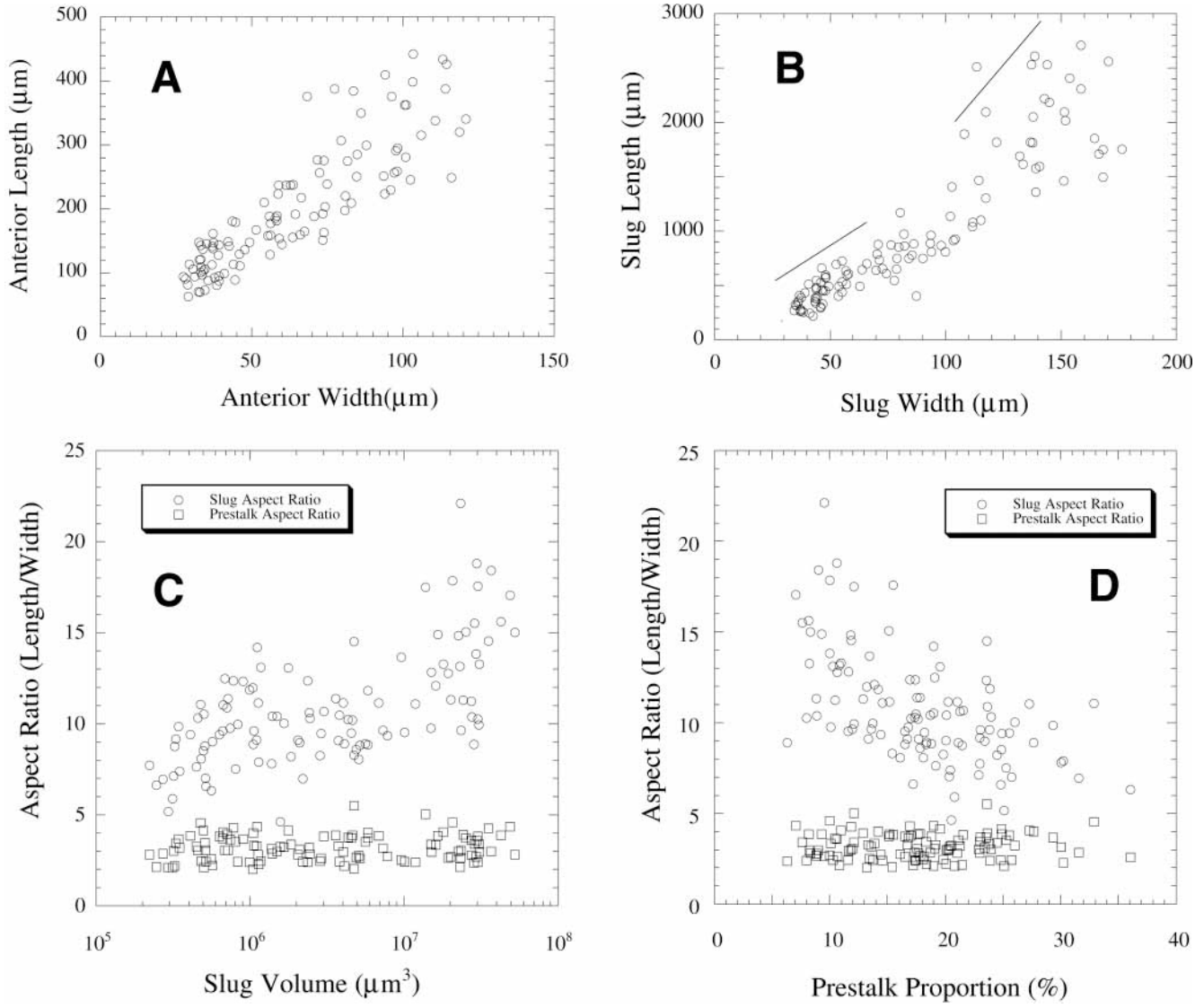


Fig. 3 Geometric correlations in whole slugs and anterior regions. (**A**) Anterior region length vs. anterior region width. A linear relation means that the prestalk aspect ratio (defined as the length/width ratio of the anterior region) is size independent. (**B**) Slug length vs. slug width. The two slopes shown in the graph represent different values of the slug aspect ratio for different slug sizes. (**C**) Slug aspect ratio and prestalk aspect ratio vs. slug volume for intact slugs. A correlation is found between slug aspect ratio and slug volume: big slugs tend to be relatively slimmer than small slugs. However, the prestalk aspect ratio remains constant. (**D**) Aspect ratio of slug and anterior region vs. prestalk proportion. Prestalk aspect ratio is independent of prestalk proportion, while slug aspect ratio increases as prestalk proportion is decreased.

generation after posterior amputation, although in the latter experiment, measurements ended 9 hours after amputation, and it was not clear whether or not the steady state had been reached.

Partial amputation of posterior region does not trigger transdifferentiation. Slug posteriors were partially amputated to see whether a limited increase of the prestalk proportion triggers transdifferentiation among the remaining cells. Between 1/2 and 2/3 of the posterior region was removed in each experiment, so that just after the cut, the prestalk proportion was slightly below 30%. Fig. 6 shows one such experiment and Fig. 7 the corresponding data. Twelve hours after the amputation the proportion remained unchanged.

Proportion regulation after amputation of the anterior region: Classic studies showed that after removal of the slug tip, the isolated posterior cannot keep migrating. Instead, it gathers to form a mound [24] which, after several hours, gives rise to a new slug. In our experiments, isolated posteriors developed up to the tipped mound or first finger stage by 3 hours after the amputation, with strong ecmAO expression in the newly

Table 1 Prestalk proportions, slug aspect ratio, and prestalk aspect ratio for slugs of different sizes. Mean value with standard deviations is given for each range of sizes. All measurements were performed on gal-stained slugs. *N* stands for the number of measured samples

Slug Volume (10^6 μm^3)	N	Prestalk Proportion (%)	Slug Aspect Ratio	Prestalk Aspect Ratio
0.2–0.5	16	23.7 ± 4.5	8.1 ± 1.6	3.1 ± 0.8
0.5–1.0	18	21.9 ± 5.1	9.8 ± 2.0	3.2 ± 0.7
1.0–3.0	23	20.8 ± 5.1	9.8 ± 2.1	3.0 ± 0.6
3.0–10.0	21	17.3 ± 2.7	10.1 ± 1.7	3.3 ± 0.8
10.0–30.0	23	10.8 ± 2.1	13.4 ± 3.4	3.2 ± 0.7
30.0–60.0	9	9.8 ± 2.8	14.6 ± 3.0	3.4 ± 0.7

formed tips (data not shown), in agreement with reports by Detterbeck et al. [8] of *pspA* negative tips 2 hours after amputation.

Previous experiments by Sakai [25] had shown that transdifferentiation from prespore to prestalk is accomplished within 3–4 hours. In our experiments, 6 hours after the amputation, most of the posterior isolates had formed regenerated slugs with a prestalk proportion typical of intact slugs (see filled circles in Fig. 2B).

Aspect ratio of anterior region is size independent

We now turn our attention to the issue of slug shape. Figure 1, in which slugs of various sizes have been magnified to the same apparent length, suggests that large slugs are proportionately thinner than small ones. Quantitatively, this can be characterised by the aspect ratio (Length/Width). Figure 3 shows that slug aspect ratio indeed increases with size, but that the aspect ratio of the prestalk zone is size-independent. These tendencies are statistically meaningful (see Table 1); the wide scatter may be related to the contractions and elongations that the slug displays during migration as its tip repeatedly falls on the substratum and rises again. Further analysis reveals that prestalk aspect ratio is also constant in relation to prestalk proportion (see Fig. 3D).

In Fig. 4, which shows gal stained slugs at different times after posterior amputation, it can be seen that slugs progressively elongate as transdifferentiation proceeds; Fig. 5 shows that the aspect ratio stabilises in 12–18 hours, at about the same time the proportions do. After partial posterior amputation, where no regulation occurs and there is thus no shift in proportions, the aspect ratio also remains constant.

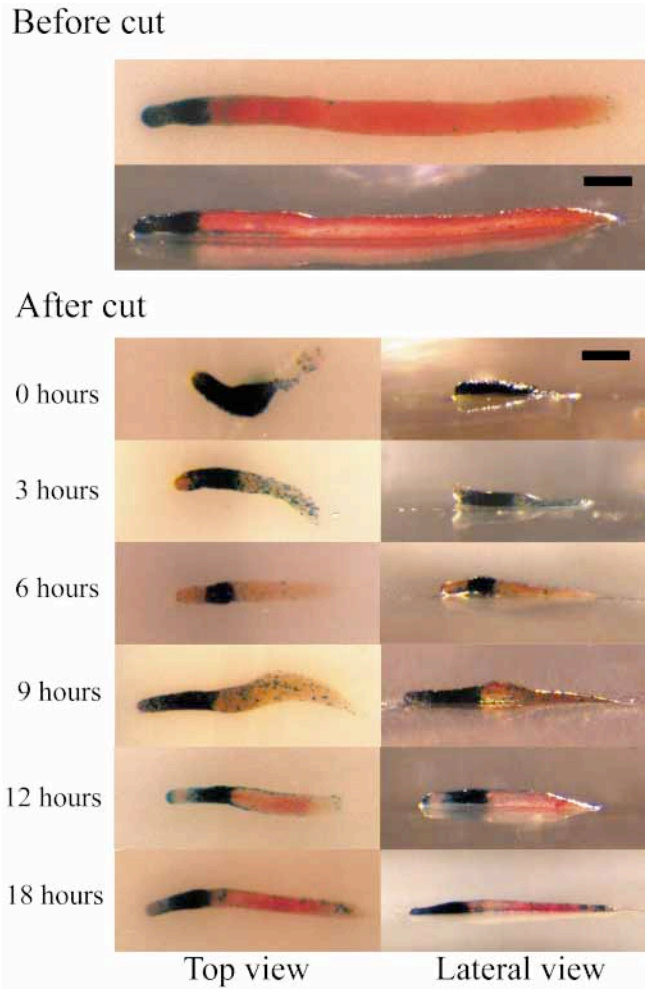


Fig. 4 Regeneration of slug after total removal of the posterior region. The top panel shows a typical slug before amputation. The lower display slugs fixed and gal-stained at different times after the amputation. The same slugs are presented from a top view *(left)* and a lateral view *(right)*. *Scale bar*: 0.2 mm. Note that the "tail" that slugs have during regeneration might introduce a large error in the estimated prestalk proportion if only the top view were considered. The beginning of regeneration is clearly apparent by 3 hours after amputation. As transdifferentiation proceeds and a new posterior region appears, prestalk proportion and initial aspect ratio are progressively, but only partially, recovered. The average prestalk aspect ratio remains constant during the whole regeneration process although there are large variations.

Discussion

Allometry in *Dictyostelium* slugs

Our results suggest that in normal, unmanipulated slugs, the proportion of prestalk cells varies with overall slug size. The variation is substantial; with a 2.5-fold shift

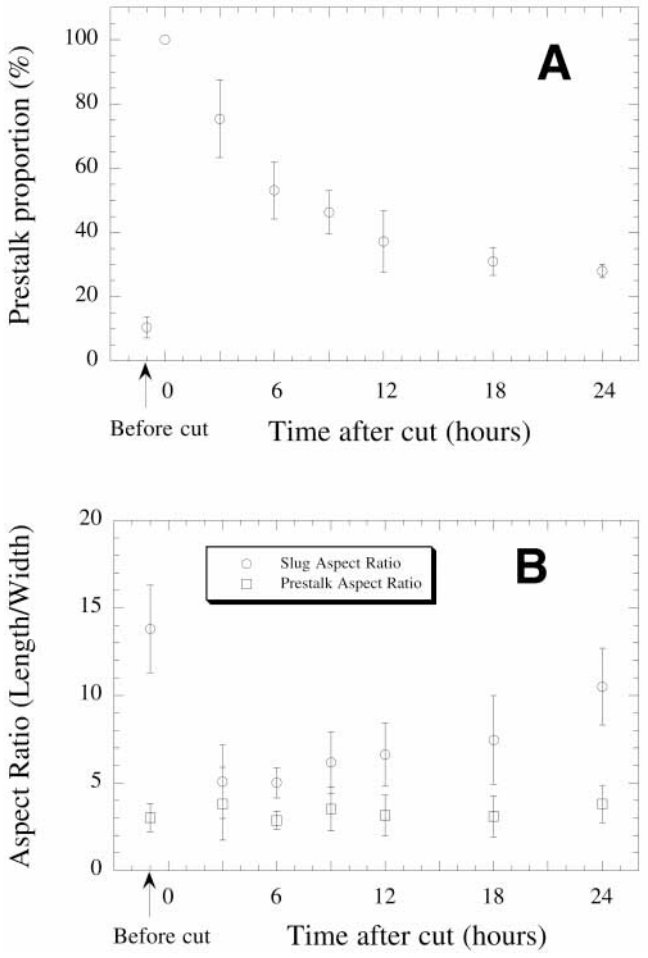


Fig. 5 Quantitative analysis of the regeneration process after total removal of posterior region. (**A**) The prestalk proportion decays with time in an asymptotic fashion until it stabilises at 28.0 ± 2.1 %, well above the initial value of 10.5 ± 3.3%. (**B**) The slug aspect ratio increases as regeneration proceeds, while prestalk aspect ratio remains roughly constant at a value slightly above that of intact slugs. Both in (**A**) and (**B**), each point is the mean of several experiments (see details in Table 2). Bars display standard deviations.

from 25% prestalk in the smallest slugs to 10% in the largest. This effect is similar to observations reported in small fruiting bodies [10, 21, 28], but conflicts, however, with two previous studies which describe shifts in the opposite direction for big fruiting bodies and slugs respectively [28, 29]. Our data contain about twice as many individual measurements as either of the earlier studies and cover a 3 to 10-fold greater range of sizes. In addition, the use of the ecmAO-iα-gal reporter allowed us to pinpoint the prestalk/prespore boundary with particular accuracy. Finally, we were able to use a more sophisticated procedure for estimating slug volumes. A direct comparison with previous results is difficult to assess due to the different methods used to measure the proportion and the diversity of culture conditions. We are, however, fully confident in our findings.

Our results necessarily reflect the balance between the prespore + ALC ⇔ pstO + pstA populations. One may wonder whether there is a more exact regulation between other groups of cell types (e.g. prespore ⇔ ALC + pstO + pstA) [21]. Including the ALC with the prestalk population would certainly narrow the range of prestalk proportions. However, after extrapolating Sternfeld and David [30] data for ALC to our results, one finds that the newly defined ALC + pstO + pstA prestalk proportion still varies between a ~20% and ~35%. Similarly, it can be argued that proportion regulation will not be significantly more exact in fruiting bodies than it is in slugs by extrapolating former results [4, 10, 21, 28].

A dependence of proportioning on size, or *allometry*, is widely observed in biological systems at all levels [11] and the explanations are diverse. In another "simple system", the fresh-water coelenterate *Hydra*, allometry has been proposed to be a by-product of a reaction-diffusion patterning system: head differentiation is thought to be specified by a diffusible "activator" [19], whose diffusion range is a small fraction of the size of a large animal, but a large fraction of the size of a small one. A similar idea was advanced many years ago to explain allometry in cellular slime moulds [18]. A second way to explain allometry in *Dictyostelium* would be to invoke, in one way or another, the surface-to-volume ratio. An important prestalk marker is in fact found only on the surface in early development [9]. An effect of oxygen concentration also seems conceivable; in large slugs, average available oxygen would be less, which could lower the proportion of prestalk cells [29].

A zone of tolerance in prestalk/prespore proportioning

We have used the same methods to study pattern regulation: the re-proportioning that occurs after partial or complete removal of one of the slug's cell types. Surprisingly, these experiments show that the proportions depend on slug history as well as slug size. This effect is easy to see when the prestalk zones are isolated from very large slugs and allowed to regulate. In such cases, the beginning slugs had about 10% prestalk, although even allowing for the decrease in mass, 15–20% prestalk would be expected in the regenerate. Regeneration concludes, however, at about 30% prestalk (see Fig. 5). We also saw this effect when only part of the prespore zone was removed; here the expectation was about 12% prestalk, but the actual values were around 25%. If, instead, the prespore zones are isolated, regeneration produces a slug with about 10% prestalk (see Fig. 2B, Fig. 8 for a schematic diagram).

The range of proportions as a fingerprint of cell type bistability

Neither of the explanations offered for slug allometry suffices to explain the dependence of proportions on his-

Table 2 Prestalk proportion, slug aspect ratio, and prestalk aspect ratios for regeneration experiments. Mean value is given with standard deviation. Measurements were carried on gal-stained slugs except for the following cases, in which neutral red staining was taken as reference: (i) slugs before and immediately after amputation; (ii) aspect ratio after total removal of posterior. The reason for using neutral red staining was to avoid the shape distortions produced by fixation, which were sometimes severe for regenerating slugs

Time after cut	*n*	Prestalk Proportion (%)	Slug Aspect Ratio	Prestalk Aspect Ratio
Regeneration after TOTAL REMOVAL OF POSTERIOR region				
Before cut	28	10.5 ± 3.3	13.8 ± 2.5	3.0 ± 0.8
0 hours	9	100.0 ± 0.0	3.2 ± 0.7	3.2 ± 0.7
3 hours	14	75.4 ± 12.1	5.4 ± 0.8	3.9 ± 0.9
6 hours	14	53.1 ± 8.9	6.4 ± 1.2	3.7 ± 0.6
9 hours	15	46.3 ± 6.7	7.3 ± 1.6	3.7 ± 0.6
12 hours	15	37.2 ± 9.6	8.9 ± 1.2	3.7 ± 0.7
18 hours	10	31.0 ± 4.3	8.5 ± 2.1	3.4 ± 0.8
24 hours	6	28.0 ± 2.1	11.4 ± 2.9	3.9 ± 0.8
Regeneration after PARTIAL REMOVAL OF POSTERIOR region				
Before cut	7	12.7 ± 1.9	16.0 ± 1.5	3.5 ± 0.7
0 hours	7	27.6 ± 7.0	7.8 ± 1.3	3.6 ± 0.5
12 hours	7	26.0 ± 3.9	6.8 ± 1.1	3.2 ± 0.6
Regeneration after REMOVAL OF ANTERIOR region				
6 hours	6	9.0 ± 2.5	7.7 ± 1.6	2.3 ± 0.5

tory. The history effect suggests that there is a "tolerance zone" from 10% to 25% prestalk, within which proportions can vary without activating regulatory mechanisms. Our results also suggest that when regulation does occur, cell type interconversion ceases as soon as the border of this zone is attained.

Several lines of evidence suggest that proportioning in *Dictyostelium* involves a "prespore inhibitor" (assumed to be DIF-1) secreted by prespore cells and degraded by prestalk cells [12, 14, 23]. As has been pointed out many times, negative feedback alone would lead to pattern instability after the prestalk and prespore cells sort out [14, 16], and a compensating positive feedback in some form is expected. This positive feedback can either be extracellular [18] or intracellular. The latter case is particularly interesting as it could lead to *bistability*, in effect a different sensitivity of prespore and prestalk cells to the prespore inhibitor [14, 15] (Ràfols et al., in preparation). Our finding that prestalk proportion may shift from ~10% to ~30% without triggering transdifferen-

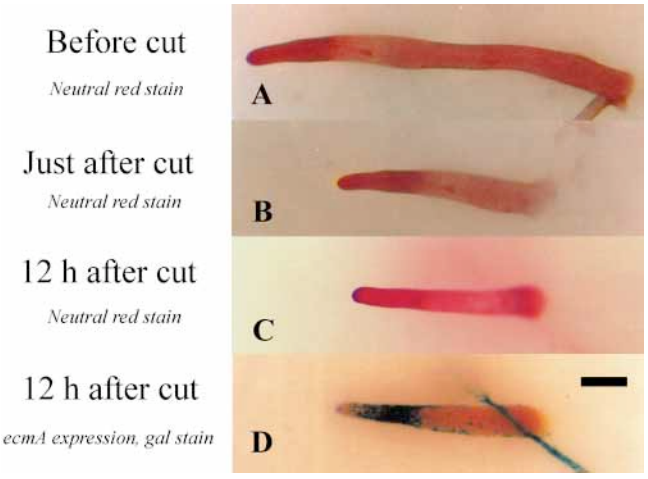


Fig. 6 Partial removal of posterior region does not trigger transdifferentiation. (**A**) Neutral red staining before amputation. (**B**) Neutral red staining immediately after partial amputation. The cut was placed so that the prestalk proportion would take a value slightly below the 30% threshold. (**C**) 12 hours after amputation, no transdifferentiation is observed. (**D**) Gal staining 12 hours after amputation. *Scale bar*: 0.2 mm. The pattern of *ecmA* expression confirms that cell type proportions have remained unchanged since the partial amputation was performed. This result is the most straightforward evidence that slug can be stable under different prestalk proportions.

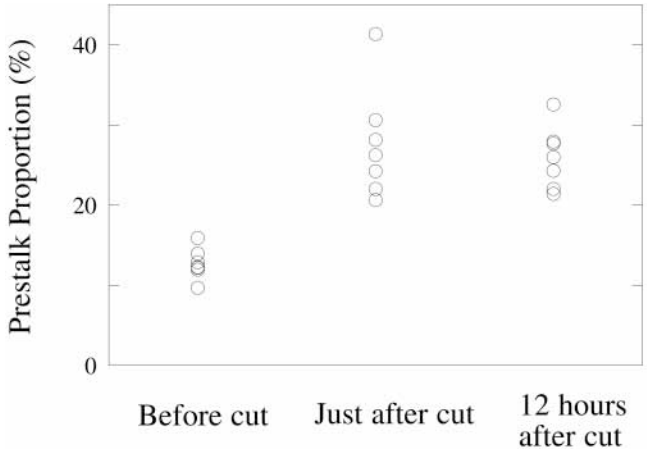


Fig. 7 Prestalk proportion before, immediately after, and 12 hours after partial removal of posterior region. Immediately after the amputation, the prestalk proportion is more or less twice that found in intact slugs. However, 12 hours later no significant change in proportion is detected, suggesting that slugs with prestalk proportions within and below this range are stable (see details in Table 2).

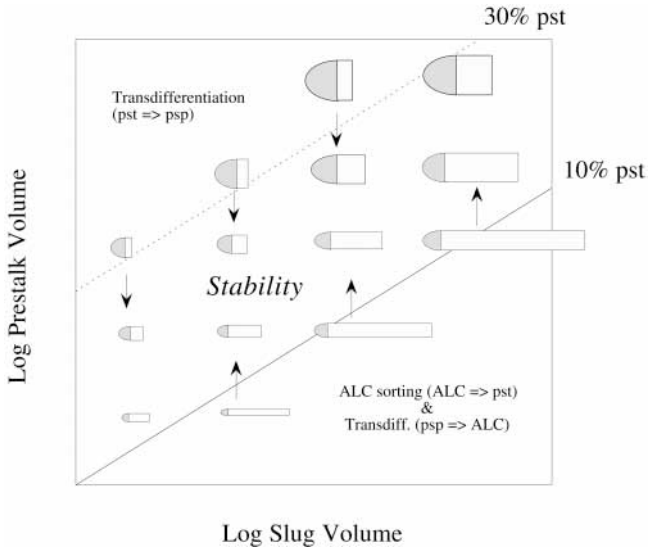


Fig. 8 Schematic description of the stable range of prestalk proportions and slug aspect ratios. In accordance with the experimental results, the anterior region of slugs is plotted with a constant aspect ratio, whatever the proportion of cell types and the size of the slug. Keeping prestalk aspect ratio constant means that for a given slug volume, the slug becomes progressively fatter as the prestalk volume and prestalk proportion increase (fix a point in the x-axis and move upwards). Contrarily, for a given prestalk volume the anterior region will always have the same width. Therefore, an increase in the total slug volume only results in an elongation of the slug (fix a point in the y-axis and move laterally). Within this framework of possible slugs, we propose that only slugs with prestalk proportions between lower and upper thresholds are stable. Experimental results presented in the text show that above the maximum prestalk proportion (~30%), some prestalk cells transdifferentiate until the proportion decreases below the threshold. Preliminary experiments show that below a minimum prestalk proportion (~10%), some cells (probably ALC) sort out to the anterior region until the prestalk proportion is recovered. To compensate for the sorted ALC, some prespore cells may be expected to transdifferentiate into ALC.

tiation can be interpreted as evidence for such cell type bistability.

It should be clear that even if the bistability accounts for the tolerance zone, it does not itself explain why, in the absence of any manipulation, small slugs have prestalk proportions at the upper limit of this zone, while large slugs tend toward the lower limit. This must be explained by characteristics of the initial pattern-formation process. As far as we can see, a sensitivity to the surface-to-volume ratio during early development would suffice.

Slug shape regulation

Raper [24] and MacWilliams [16] had previously noted that after posterior amputation, the prestalk isolate elongates; Fig. 5B and Table 2 are, to our knowledge, the first quantitative data on this shape regeneration process. We find that the slug aspect ratio, dramatically lowered by the amputation, increases its value progressively, in parallel with the recovery of proportion, but does not recover completely. We have also found that the prestalk aspect does not change substantially at any time during this process. The latter is in concord with the observation that in intact slugs the prestalk aspect ratio is constant at any size and at any proportion of cell types (see Fig. 3).

At present we cannot explain this. In principle one expects the slug shape to be determined by the cell motion, which depends on differential chemotaxis (or motive force) between cell types and differential cell adhesion [16]. These same mechanisms have been invoked in models of slug movement [5, 13, 27], but none of the models has yet been developed to account for shape regulation.

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References

1. Akiyama Y, Inouye K (1987) Cell-type conversion in normally proportioned and prestalk-enriched populations of slug cells in *Dictyostelium discoideum*. Differentiation 35:83–87
2. Araki T, Nakao H, Takeuchi I, Maeda Y (1994) Cell-cycle-dependent sorting in the development of *Dictyostelium* cells. Dev Biol 162:221–228
3. Bonner JT (1957) A theory of the control of differentiation in the cellular slime molds. Quart Rev Biol 32:232–246
4. Bonner JT, Slifkin MK (1949) A study of the control of differentiation: The proportions of stalk and spore cells in the slime mold *Dictyostelium discoideum*. Am J Bot 36:727–734
5. Bretschneider T, Vasiev B, Weijer CJ (1999) A model for *Dictyostelium* slug movement. J Theor Biol 199:125–136
6. Brown JM, Firtel RA (1999) Regulation of cell-fate determination in *Dictyostelium*. Dev Biol 216:426–441
7. Deichsel H, Friedel S, Detterbeck A, Coyne C, Hamker U, MacWilliams HK (1999) Green fluorescent proteins with short half-lives as reporters in *Dictyostelium discoideum*. Dev Genes Evol 209:63–68
8. Detterbeck S, Morandini P, Wetterauer B, Bachmair A, Fischer K, MacWilliams HK (1994) The 'prespore-like cells' of *Dictyostelium* have ceased to express a prespore gene: Analysis using short-lived beta-galactosidases as reporters. Development 120:2847–2855
9. Early A, Abe T, Williams J (1995) Evidence for positional differentiation of prestalk cells and for a morphogenetic gradient in *Dictyostelium*. Cell 83:91–99
10. Hashimoto Y, Nakamura R, Muroyama T, Yamada T (1988) Studies on tiny fruiting bodies of the cellular slime mold, *Dictyostelium discoideum*. Cytologia 53:337–340
11. Huxley JS (1932) Problems in relative growth. Methuen: London
12. Inouye K (1989) Control of cell type proportions by a secreted factor in *Dictyostelium discoideum*. Development 107:605–610
13. Inouye K, Takeuchi I (1979) Analytical studies on migrating, movement of the pseudoplasmodium of *Dictyostelium discoideum*. Protoplasma 99:289–304
14. Kay RR, Flatman P, Thompson CRL (1999) DIF signalling and cell fate. Sem Cell Dev Biol 10:577–585

15. Lewis J, Slack JMW, Wolpert L (1977) Thresholds in development. J Theor Biol 65:579–590
16. MacWilliams HK (1984) Cell-type ratio and shape in slugs of the cellular slime molds. In: Malacinski GM, Bryant SV (Eds) Pattern Formation: A Primer in Developmental Biology. MacMillan, New York, pp 127–162
17. MacWilliams HK, Gaudet P, Deichsel H, Bonfils C, Tsang A (2001) Biphasic expression of *rnB* in *Dictyostelium discoideum* suggests a direct relationship between cell cycle control and cell differentiation. Differentiation 67:12–24
18. MacWilliams HK, Bonner JT (1979) The prestalk-prespore pattern in cellular slime molds. Differentiation 14:1–22
19. Meinhardt H (1982) Models of biological pattern formation. Academic Press: London
20. Mizuguchi T, Sano M (1995) Proportion regulation of biological cells in globally coupled nonlinear oscillators. Phys Rev Lett 75:966–969
21. Nanjundiah V, Bhogle AS (1995) The precision of regulation in *Dictyostelium discoideum*: Implications for cell-type proportioning in the absence of spatial pattern. Indian J Biochem Biophys 32:404–416
22. Othmer H, Pate E (1981) Scale-invariance in reaction-diffusion models of spatial pattern formation. Proc Natl Acad Sci USA 77:4180–4184
23. Oyama M, Okamoto K, Takeuchi I (1983) Proportion regulation without pattern formation in *Dictyostelium discoideum*. J Embryol Exp Morphol 75:293–301
24. Raper KB (1940) Pseudoplasmodium formation and organization in *Dictyostelium discoideum*. J Elisha Mitchell Sci Soc 56:241–282
25. Sakai Y (1973) Cell type conversion in isolated prestalk and prespore fragments of the cellular slime mold *Dictyostelium discoideum*. Devel Growth Differ 15:11–19
26. Sampson J (1976) Cell patterning in migrating slugs of Dictyostelium discoideum. J Embryol Exp Morphol 36:663–668
27. Savill NJ, Hogeweg P (1997) Modelling morphogenesis: From single cells to crawling slugs. J Theor Biol 184:229–235
28. Stenhouse FO, Williams KL (1977) Patterning in *Dictyostelium discoideum*: the propertions of the three differentiated cell types (spore, stalk, and basal disk) in the fruiting body. Dev Biol 59:140–152
29. Sternfeld J (1988) Proportion regulation in *Dictyostelium* is altered by oxygen. Differentiation 37:173–179
30. Sternfeld J, David CN (1982) Fate and regulation of anterior-like cells in *Dictyostelium* slugs. Dev Biol 93:111–118
31. Wetterauer B, Morandini P, Hribar I, Murgia-Morandini I, Hamker U, Singleton C, MacWilliams HK (1996) Wild-type strains of *Dictyostelium discoideum* can be transformed using a novel selection cassette driven by the promoter of the ribosomal V18 gene. Plasmid 36:169–181