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Evolution of irreversible differentiation under stage-dependent cell differentiation

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The specialization of cells is a hallmark of complex multicellularity. Cell differentiation enables the emergence of specialized cell types that carry out separate functions previously executed by a multifunctional ancestor cell. One view about the origin of cell differentiation is that it first occurred randomly in genetically identical cells exposed to the same life history environment. Under these conditions, differentiation trajectories producing more offspring could be favored by natural selection; yet, how dynamic variation in differentiation probabilities can affect the evolution of differentiation patterns is unclear. We develop a theoretical model to investigate the effect of dynamic—stage-dependent—cell differentiation on the evolution of optimal differentiation patterns. Concretely, we model trajectories in which cells can randomly differentiate into germ or soma cell types at each cell division. After comparing many of these trajectories, we found that irreversible differentiation, where cells gradually lose their ability to produce the other cell type, is more favored in small organisms under dynamic than under constant (stage-independent) cell differentiation. Furthermore, we found that the irreversible differentiation of germ cells, where germ cells gradually lose their ability to produce soma cells, is prominent among irreversible patterns. Only large variations in the differentiation probabilities prohibit irreversible trajectories from being the optimal pattern.

The evolution of multicellularity has been viewed as a major transition for the evolution of life on earth[1–5]. One important aspect of this transition is that cells differentiate into different cell types. Cooperation and division of labor between cell types have been widely investigated in the evolution of multicellularity[6–10]. Cell types mentioned here refer to the cells that differ in either their morphology or function, or both[5,11]. Although unicellular organisms, which are believed to be the ancestors of multicellular life[12,13], display heterogeneity of what can be thought of as differentiated cell types, they do not have specialized cell types coexistent spatially. For example, *Naegleria gruberi* can switch between a flagellated swimmer phenotype and a deformable crawler ("ameboid") phenotype[14]. However, compared with unicellular organisms, multicellular organisms with multiple cells allow individuals to possess distinct specialized cell types to perform diverse functions[15–17]. In turn, differentiated cells of multicellular organisms perform distinct functions under varying conditions and can, thus, increase an organism's reproductive fitness. For example, cell differentiation under adverse environmental conditions can increase an organism's survival chance, such as cyanobacteria differentiating nitrogen-fixing heterocysts to use N_2 when combined-nitrogen is insufficient[18], and *Myxococcus xanthus* producing a new cell type under starvation[13].

Several mechanisms could lead to cell differentiation and phenotypic variation, including gene expression, mutations, epigenetics, and the environment[5,12,14,17,19–22]. These mechanisms are complementary, and thus, more than one could act during the evolution of cell differentiation[14,20]. These mechanisms usually assume that multifunctional unicellular ancestors differentiate into specialized cells to carry out segregated functions, even when the cells are genetically identical and have been exposed to the same environment. Cell differentiation can depend on the developmental state of an organism. For example, one out of successive 10 to 15 vegetative cells differentiate into a new cell type, heterocyst, in filamentous cyanobacteria *Anabaena sp.*PCC 7120[23]; *Volvox* differentiates into two cell types at its 6th round of division in its whole 11~12 rounds of cell divisions[24]. Moreover, it has been found that the differentiation patterns of fully differentiated multicellular *Volvox* species likely originated from the partially differentiated species *V. aureus* and *V. gigas* which further originated from the undifferentiated unicellular genus *Gonium*. Partial differentiation refers to cells performing the same cellular functions but with a different emphasis. While smaller *Gonium* species have identical cells without cell

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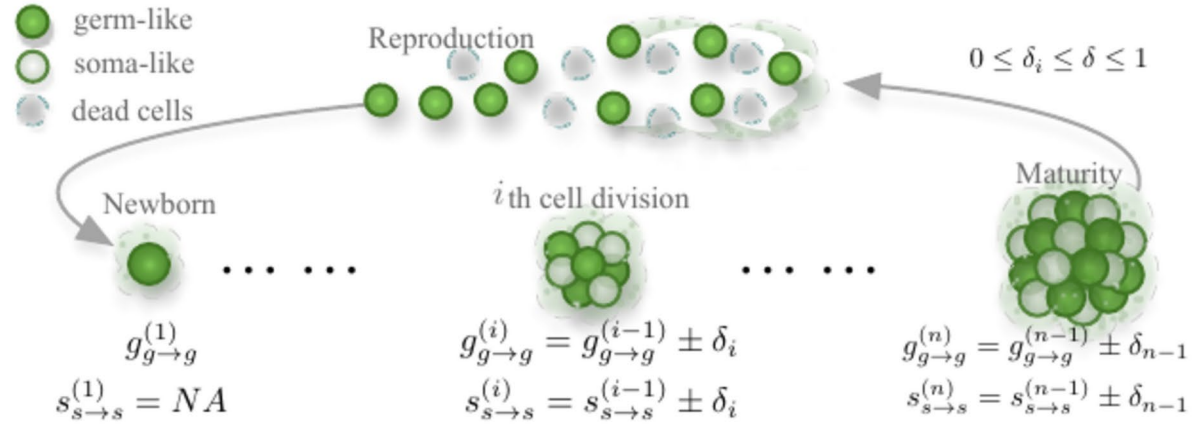
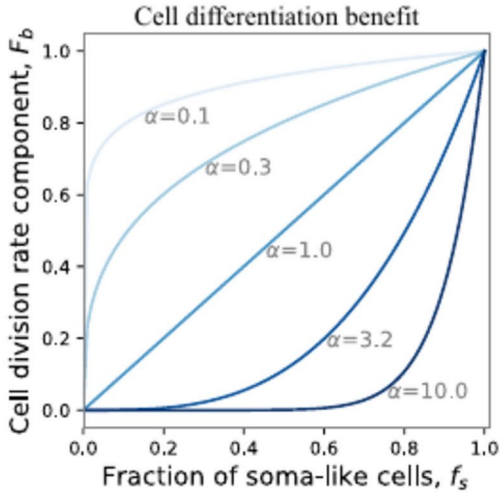
A. Dynamic developmental trajectories and cell differentiation categories

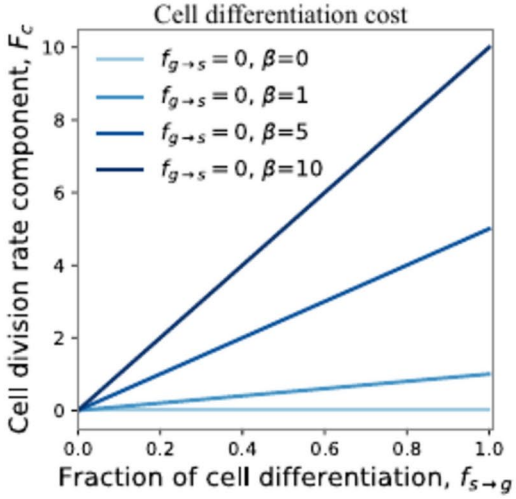


B. Cell differentiation strategies

Differentiation probability	Differentiation category	
$g^{(i)}_{g\to s} = g^{(i-1)}_{g\to s} \pm \delta_i, s^{(i)}_{s\to g} = s^{(i-1)}_{s\to g} \pm \delta_i$	Stage-independent $\delta = 0$	Stage-dependent $\delta \neq 0$
$g^{(i)}_{g\to s} \equiv 0, i = 1, 2, \ldots, n$	ND^i	ND^i
$g^{(i)}_{g\to s} \not\equiv 0, i = 1, 2, \ldots, n-1.$ $g^{(n)}_{g\to s} = 0$ *or* $s^{(n)}_{s\to g} = 0$	ID^i	ID
others	RD^i	RD

C. Cell division rate components





differentiation, the intermediate-sized species *V. aureus* and *V. gigas* have partial germ-soma differentiation (i.e. the beginning of cell differentiation), and *V. carteri* and *V. obversus* have complete germ-soma differentiation[24]. How state-dependent cell differentiation shaped the evolution of cell differentiation patterns is still unclear.

Research on cell differentiation has studied the optimal condition where mature cells allocate their resources to different tasks[25–36]. However, despite the focus on investigating the proportions of cell types, the stochastic switching to other cell types during cell divisions remains largely unknown. Some authors have considered cell differentiation of only one cell type while other cell types were terminally differentiated (without division ability)[26,28,31]. Rodrigues et al. considered the ability to differentiate as an evolving trait, but the trait was coupled with varying cell division rates and cells constrained to a filament form[37]. More recently, Cooper et al. introduced a random specialization model where the random process impacted the final fractions of different cell types but not the internal organization of tasks allocated to cells during the cell division process[36]. Gavrilets et al. and Gao et al. considered cell differentiation between cell types, but the differentiation probabilities were assumed to be

◀**Fig. 1**. Illustration of the stage-dependent cell differentiation, differentiation strategies, and cell division rate components. **A** Schematic of an organism's life cycles. Organisms start from single germ-like cells and undergo *n* synchronous cell divisions before reproduction. For newborn organisms, the cell differentiation probability for soma-like cells is irrelevant as there are no soma-like cells. Cell differentiation probabilities can change from the $(i-1)$th cell division to the *i*th cell division by a small quantity δ_i ($0 \leq \delta_i \leq 1$ and $i = 1, 2, \ldots, n$). δ is the maximum change between successive cell differentiation probabilities i.e. $0 \leq \delta_i \leq \delta \leq 1, i = 1, \ldots, n$.**B** Cell differentiation strategy classification. Based on the cell differentiation probabilities at the last cell division, we classify cell differentiation into three categories: non-differentiation *ND*, reversible differentiation *RD*, and irreversible differentiation *ID*. The upper script *i* means the strategy is stage-independent i.e. $\delta = 0$.For *ND*, since $g^{(i)}_{g\to s} = 0, i = 1, \ldots, n$, thus *ND* equals ND^i.**C** Cell division rate components. The left panel shows the benefits of cell differentiation. We assume that the cell division rate increases with the fraction of soma-like cells f_s. For the associated benefit, we assume the function $F_b = b(f_s)^\alpha$, where the shape is controlled by α. The right panel shows the costs of cell differentiation. We assume that the cell division rate decreases with the fraction of cell divisions that turn a soma-like cell into a germ-like cell and vice versa. For the associated cost, we assume the function $F_c = c(f_{g\to s} + \beta f_{s\to g})$. Here, we show the values of F_c with varying $f_{s\to g}$ and β by setting $f_{g\to s} = 0$ (Parameters: $b = 1$ in the left panel and $c = 1$ in the right panel).

fixed rather than stochastic[27,38]. Thus far, little is known about the effect of stage-dependent differentiation on the evolution of reversible and irreversible differentiation patterns.

In this study, based on our previous work[38], we develop a theoretical model to investigate the effect of stage-dependent cell differentiation on the evolution of optimal differentiation patterns in multicellular organisms. Stage-dependent refers to the ability of cells to have different differentiation probabilities between any two successive cell divisions. Comparatively, stage-independent differentiation refers to cell types having a fixed differentiation probability across all cell divisions[38]. Inspired by the division of labor in *Volvox*, where germ cells reproduce, and somatic cells are for viability[24], we consider two cell types in the organism: germ-like and soma-like cells. We use the population growth rate (expected offspring number of the organism) as the simplest direct proxy for its fitness[39]. We assume that organisms grow by cell divisions which further depend on the fraction of soma-like cells and the differentiation probabilities. By calculating the population growth rates numerically under different parameters, we identify the evolutionary optimal strategies that maximize the fitness of stage-dependent and stage-independent strategies. We observe that, with low differentiation cost, stage-dependent differentiation favors reversible strategies as they "recycle" soma-type cells for reproduction. However, even without costs, irreversible differentiation is favored more by stage-dependent than stage-independent differentiation in small organisms.

Model and methods

We designed a life cycle model for organisms with stage-dependent cell differentiation. We compare this to our previous work on stage-independent differentiation[38]. As we are interested in the origin of differentiation patterns, we consider an organism, in which cells take either of two temporary roles: germ-like or soma-like. These roles can change in either direction during the life cycle and do not necessarily create an inheritable cell type. This life cycle is inspired by *V. aureus*, a species with partial germ-soma differentiation (the beginning of germ-soma differentiation) of the genus *Volvox*[24]. In the model, the two cell types can differentiate into each other, and we investigate the possible differentiation steps along the cell divisions, which we refer to as differentiation strategies. Different strategies lead organisms to different developmental trajectories and fitness. In the model, the population growth rate (the expected offspring number) is a fitness proxy as it is the simplest direct way to measure organisms' fitness[39]. We assume that each organism starts with a single germ-like cell, see Fig 1A. Cells divide synchronously, each cell producing two daughter cells at a time. After the *i*th cell division, organisms have 2^i cells in total. Organisms grow and mature until they reach a maturity size 2^n, where *n* is the total number of cell divisions. After the final division, each germ-like cell is released as offspring to start a new organism (and life cycle). Meanwhile, all soma-like cells die after the last division. There are three possible outcomes of a cell division: two germ-like cells, one germ-like cell and one soma-like cell, and two soma-like cells. At each division, cells have a set of probabilities to produce daughter cells of a certain type. Here, $g^{(i)}_{gg}$ is the probability of a germ-like cell producing two germ-like cells at the *i*th cell division. The probabilities $g^{(i)}_{gs}$, $g^{(i)}_{ss}$, $s^{(i)}_{gg}$, $s^{(i)}_{gs}$ and $s^{(i)}_{ss}$ are defined in a similar manner, where we have $g^{(i)}_{gg} + g^{(i)}_{gs} + g^{(i)}_{ss} = 1$ and $s^{(i)}_{gg} + s^{(i)}_{gs} + s^{(i)}_{ss} = 1$ for each growth stage *i*, $i = 1, 2, \ldots, n$. We denote $d_i = [g^{(i)}_{gg}, g^{(i)}_{gs}, g^{(i)}_{ss}, s^{(i)}_{gg}, s^{(i)}_{gs}, s^{(i)}_{ss}]$ as the cell differentiation probabilities in the *i*th cell division. In addition, $g^{(i)}_{g\to s} = \left(g^{(i)}_{ss} + \frac{g^{(i)}_{gs}}{2}\right)$ and $s^{(i)}_{s\to g} = \left(s^{(i)}_{gg} + \frac{s^{(i)}_{gs}}{2}\right)$ are referred to as transition probabilities, $i = 1, 2, \ldots, n$. The cell differentiation probabilities across the successive *n* cell divisions of an organism can be expressed in matrix form as

$$\mathrm{D} = \begin{pmatrix} d^{(1)} \\ \vdots \\ d^{(i)} \\ \vdots \\ d^{(n)} \end{pmatrix} = \begin{pmatrix} g^{(1)}_{gg} & g^{(1)}_{gs} & g^{(1)}_{ss} & s^{(1)}_{gg} & s^{(1)}_{gs} & s^{(1)}_{ss} \\ \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\ g^{(i)}_{gg} & g^{(i)}_{gs} & g^{(i)}_{ss} & s^{(i)}_{gg} & s^{(i)}_{gs} & s^{(i)}_{ss} \\ \vdots & \vdots & \vdots & \vdots & \vdots & \vdots \\ g^{(n)}_{gg} & g^{(n)}_{gs} & g^{(n)}_{ss} & s^{(n)}_{gg} & s^{(n)}_{gs} & s^{(n)}_{ss} \end{pmatrix}, \tag{1}$$

where the *i*th row of the matrix contains the cell differentiation probabilities in the *i*th cell division. We call D "stage-dependent" cell differentiation, as the probabilities can change between different division steps (rows of the matrix). We assume that this change cannot be larger than δ, e.g. $g^{(i)}_{g\to s} = g^{(i)}_{ss} + \frac{g^{(i)}_{gs}}{2} = g^{(i-1)}_{g\to s} \pm \delta_i$ with $\delta_i \leq \delta$ sufficiently small such that all probabilities remain well defined. If $\delta = 0$ for $i = 1, 2, \ldots, n$, then D is a "stage-independent" cell differentiation strategy, where a cell type maintains a fixed set of probabilities to produce daughter cells at each division. We should note that this stage-independent cell differentiation is defined by the values of $g^{(i)}_{g\to s}$ and $s^{(i)}_{s\to g}$ which do not change across *i*.

Alternative cell differentiation strategies lead to various differentiation degrees. For example, if a strategy has $g^{(i)}_{g\to s} \equiv 0$ for $i = 1, 2, \ldots, n$, then organisms do not differentiate. Stage-dependent differentiation allows many different trajectories. To distinguish them we only consider the probabilities in the last division (Fig 1B). If $g^{(i)}_{g\to s} \equiv 0$ for $i = 1, 2, \ldots, n$, then we call it non-differentiation *ND*. Otherwise, if germ-like cells produce soma-like cells at least one time before the final cell division, i.e. $g^{(i)}_{g\to s} \not\equiv 0, i = 1, 2, \ldots, n-1$, but at least one cell type abstains from differentiation at the last division i.e. $g^{(n)}_{g\to s} = 0$ or $s^{(n)}_{s\to g} = 0$, we call it irreversible differentiation *ID*. Thus, strategy *ID* captures the strategies, where differentiation initially occurs but cells gradually lose their differentiation capabilities throughout the life cycle. The remaining differentiation trajectories belong to reversible differentiation *RD*. We stress the limitation of this classification, as different strategies could lead to a similar development trajectory, especially in large organisms. Nevertheless, this classification is a simple way that distinguish between differentiation patterns. For convenience, we use the upper script *i* to name the stage-independent strategies (differentiation probabilities are fixed). From its definition, we know that *ND* is a stage-independent strategy. Thus, we use ND^i to denote it afterward. For strategy ID^i, only soma-like cells can possess irreversibility i.e. $s_{s\to g} = 0$[38]. In the following, the acronyms of differentiation strategies are stage-dependent unless otherwise stated.

We assume that cell differentiation impacts the cell division rates within an organism[38]. The effects are further decomposed into cell differentiation benefits and costs. While differentiation decreases the number of offspring as resources are converted to somatic cells rather than reproductive germ cells; we assume that soma-like cells are beneficial and increase the cell division rate of an organism. This assumption is inspired by the partial division of labor in *Volvox*, where somatic cells are responsible for viability and germ cells are responsible for reproduction[24]. We also assume that cell differentiation between germ-like cells and soma-like cells is costly and decreases cell division rates.

Organisms grow faster with higher cell division rates. Specifically, $r^{(i)}$ represents the cell division rate in the *i*th cell division and is determined by two components

$$r^{(i)} = \frac{1 + F^{(i)}_b(f_s)}{1 + F^{(i)}_c(f_{g\to s}, f_{s\to g})}, \tag{2}$$

where $F^{(i)}_b(f_s)$ and $F^{(i)}_c(f_{g\to s}, f_{s\to g})$ are the effects of cell differentiation benefit and cell differentiation cost in the *i*th cell division, $i = 1, 2, \ldots, n$. F_b is a function of the fraction of soma-like cells f_s,

$$F_b(f_s) = b f_s^{\alpha}, \tag{3}$$

where the *b* is the benefit scale, $b \geq 0$ and α controls the shape of the function, see Fig. 1C.

Here, $F_c(f_{g\to s}, f_{s\to g})$ is a function of the fraction of cell differentiation between germ-like $f_{g\to s}$ and soma-like cells $f_{s\to g}$,

$$F_c(f_{g\to s}, f_{s\to g}) = c(f_{g\to s} + \beta f_{s\to g}), \tag{4}$$

where *c* is the cost scale, $c \geq 0$. Here, β measures the relative cost of differentiation from a soma-like to a germ-like cell, see Fig. 1C. The fractions of cell differentiation in the *i*th cell division are

$$\begin{aligned} f^{(i)}_{g\to s} &= f^{(i-1)}_g g^{(i)}_{g\to s} \\ f^{(i)}_{s\to g} &= f^{(i-1)}_s s^{(i)}_{s\to g}, \end{aligned} \tag{5}$$

where $f^{(i-1)}_g$ and $f^{(i-1)}_s$ are the fraction of germ-like cell and soma-like cell after the $(i-1)$th cell division, respectively. Note that $f^{(i-1)}_g + f^{(i-1)}_s = 1$, $g^{(i)}_{g\to g} + g^{(i)}_{g\to s} = 1$, and $s^{(i)}_{s\to s} + s^{(i)}_{s\to g} = 1$, $i = 1, 2, \ldots, n$. Specifically, $f^{(i)}_g$ and $f^{(i)}_s$ are calculated by using transition probabilities, see Eq. (3) in S1 Online Appendix. Taking Eqs. (2), (3), (4) and (5) together, we have

$$r^{(i)} = \frac{1 + b(f^{(i-1)}_s)^{\alpha}}{1 + c(f^{(i)}_{g\to s} + \beta f^{(i)}_{s\to g})}. \tag{6}$$

After the $(i-1)$th cell division, the waiting time before the *i*th cell division occurring $t^{(i)}$ follows the exponential distribution $f(t^{(i)}) = r^{(i)} e^{-r^{(i)} t^{(i)}}$, $i = 1, 2, \ldots, n$. Thus the expected waiting time from the $(i-1)$th to the *i*th cell division is $t^{(i)} = \frac{1}{r^{(i)}}$[40]. The expected lifespan i.e. the period of cell division time of organisms with *n* rounds of cell divisions is

$$t = \sum_{i=1}^{n} t^{(i)} = \sum_{i=1}^{n} \frac{1}{r^{(i)}} = \sum_{i=1}^{n} \frac{1 + c\left(f_g^{(i-1)} g_{g\to s}^{(i)} + \beta f_s^{(i-1)} s_{s\to g}^{(i)}\right)}{1 + b\left(f_s^{(i-1)}\right)^{\alpha}}. \tag{7}$$

We consider a density-independent population, see[41] for a discussion when density dependence is relevant in a related model. The population growth rate only depends on the number of offspring and the lifespan of an organism. The number of offspring that an organism produces at the end of its life is $2^n f_g^{(n)}$. Therefore, organisms grow exponentially and a population's growth rate can be approximated by

$$\lambda = \frac{\ln N}{t} = \frac{\ln(2^n f_g^{(n)})}{\sum_{i=1}^{n} \frac{1}{r^{(i)}}} = \frac{\ln\left(2^n f_g^{(n)}\right)}{\sum_{i=1}^{n} \frac{1 + c\left(f_g^{(i-1)} g_{g\to s}^{(i)} + \beta f_s^{(i-1)} s_{s\to g}^{(i)}\right)}{1 + b\left(f_s^{(i-1)}\right)^{\alpha}}}, \tag{8}$$

where n is the number of cell divisions an organism undergoes before maturity. $f_g^{(i)}$ and $f_s^{(i)}$ are fractions of germ-like cell and soma-like cell after the ith cell division and $f_g^{(0)} = 1$ and $f_s^{(0)} = 0$. Here, $g_{g\to s}^{(i)}$ and $s_{s\to g}^{(i)}$ are the transition probabilities between germ-like and soma-like cells in the ith cell division ($1 \le i \le n$), see the S1 Online Appendix. We provide the calculation details of the population growth rate in S1 Online Appendix and S2 Online Appendix.

The expected number of offspring per unit time of an organism captures the effect of its differentiation strategy. It should be noted, however, that the population growth rate calculated here is not the exact population growth rate for each realization. As each strategy in the model is stochastic, each one has different potential developmental trajectories. Therefore the population growth rate of a strategy is a random variable depending on the probability of each trajectory that an organism can develop. The population growth rate calculated via Eq. (8) is an approximation of the mean population growth rate. Here, we compute the population growth rate of an average trajectory, rather than the average population growth rates across trajectories which is different from the previous population growth rate calculation method[9], where the accurate algorithm of the calculation of the population growth rates in stochastic life cycles of heterogeneous organisms is presented. We test the robustness of the approximation in S3 Online Appendix. Our results show that the approximation is consistent with the mean population growth rate of an organism.

This model generalizes our previous study of stage-independent trajectories that used individual-based simulations[38]. Here, however, we investigate the mean developmental trajectory numerically, which is more efficient, especially for the complex developmental trajectories of the stage-dependent scenario. In addition, a power function of cell differentiation benefit instead of a piecewise function is used in this new model, which contains fewer parameters but still captures the contribution of somatic cells[38]. We show that the current model setting is robust to the previous conclusion, see the S4 Online Appendix for more details. Finally, we can connect our model to classic population growth. Concretely, because each organism starts with a single germ-like cell, in ND^i cells only produce germ-like cells, thus $g_{g\to g}^{(i)} = 1$ for all i, leading to $f_g^{(i)} \equiv 1$, $f_s^{(i)} \equiv 0$. Thus from Eq. (8), with the ND^i strategy, an organism doubles its size per unit of time ($\lambda_{ND^i} = \ln 2$).

Results

Stage-dependent cell differentiation promotes irreversible cell differentiation in small organisms

Theoretically, differentiation probabilities at different cell divisions could be arbitrary values in the range 0 to 1. However, drastic changes in cell differentiation probabilities during an organism's development should be rare, changing slowly during development instead. For instance, in a series of closely related *Volvox* species the degrees of germ-soma differentiation differ gradually[24]. It is natural to assume then, that the maximum value of the change of two successive differentiation probabilities is small. Therefore, we restrict our attention to small changes in the range $0 \le \delta_i \le \delta = 0.1, i = 1, \ldots, n$ in this section and investigate the effect of larger δ in the third section. We found that stage-dependent differentiation promotes the evolution of irreversible strategies *ID* more often compared with stage-independent differentiation ID^i in small organisms, see blue and green areas in Fig. 2. Specifically, stage-dependent differentiation *ID* evolves for more values of the benefits and cost parameters. However, this *ID* gradually loses its advantage when organismal size increases. Instead, stage-independent differentiation ID^i is more likely to evolve in large organisms, see ID^i changing under different n in Fig. 2, consistent with our previous findings[38].

With a constrained δ, we proceeded to investigate the effect of the benefit (b) and cost (c) parameters on the population growth rate of stage-dependent and stage-independent differentiation strategies. We first focus on the parameter space where both evolve the same strategies. Non-differentiation, ND^i, dominates under both stage-independent and stage-dependent differentiation at high costs c, as this cost largely decreases the population growth rate during differentiation (strategies RD^i, ID^i, *RD* and *ID*). We show that in the absence of differentiation benefits, $b = 0$ and $c > 0$, ND^i is optimal analytically in S5 Online Appendix. Additionally, if there is only a single cell division ($n = 1$) and no cost ($c = 0$ and $b > 0$), ND^i is still the optimal strategy, S5 Online Appendix. This is because the benefits $F_b^{(i)}$ at the ith division are based on the fraction of soma-like

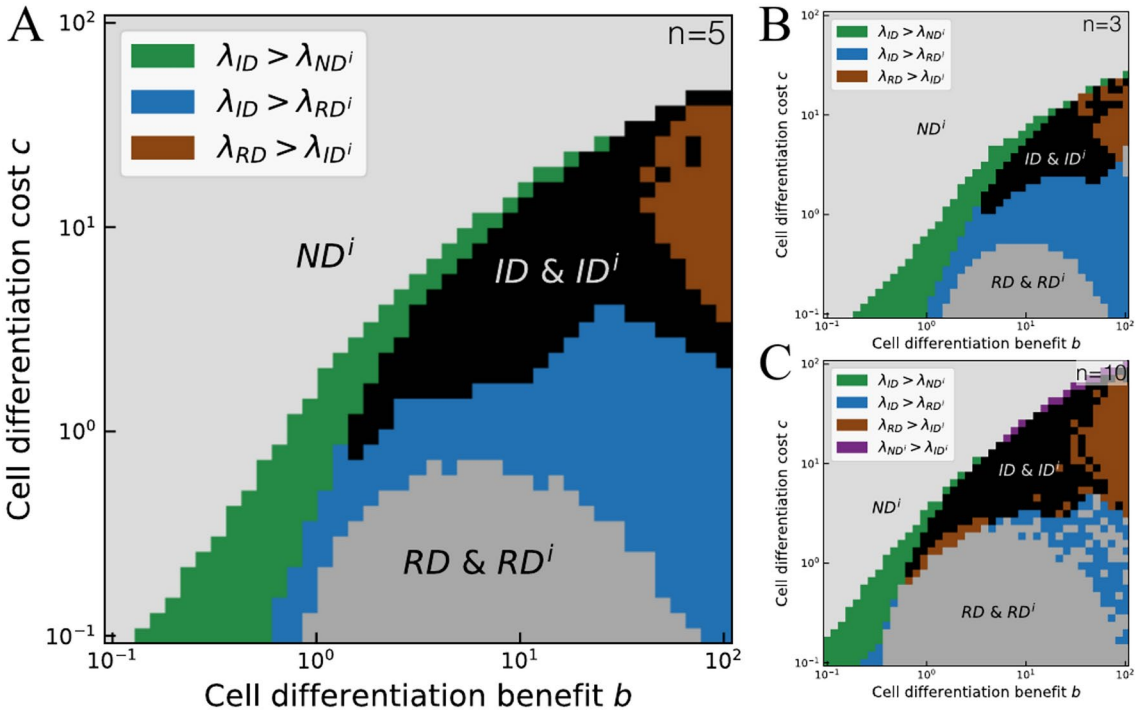


Fig. 2. Comparison of optimal strategies between stage-independent and stage-dependent differentiation. Comparison of the parameter space of the optimal strategy between stage-independent and stage-dependent cell differentiation under a maximal number of cell division rounds $n = 5$ (panel **A**), $n = 3$ (panel **B**) and $n = 10$ (panel **C**). The grey, dark grey, and black areas represent the parameter space where the optimal strategies are the same under both stage-independent and stage-dependent cell differentiation. The green strip represents stage-dependent irreversible differentiation *ID* leading to a larger population growth rate than stage-independent non-differentiation ND^i. Similarly, the blue area and the brown area represent *ID* and reversible differentiation *RD* leading to higher population growth rates than stage-independent RD^i and ID^i, respectively. Purple color represents ND^i leading to a higher population growth rate than ID^i in panel **C**. In addition, we should note that the strategies that appeared here are optimal, thus if $\lambda_{ID} > \lambda_{RD^i}$, it means λ_{ID} is greater than all other strategies. As the optimal stage-dependent strategies are investigated by their likelihood, thus if $\lambda_{ID} > \lambda_{RD^i}$, it means over 50% of the optimal strategy is *ID* in the condition where RD^i is optimal in stage-independent strategy. Parameters of all panels: $0 \leq \delta_i \leq 0.1$, and $\alpha = \beta = 1$. Parameters of calculating optimal strategy: the number of initial sampling $d^{(1)}$,$M = 1000$, the number of stage-dependent strategies starting with a given $d^{(1)}$,$R = 100$, for more detail, see S2 Online Appendix. At each pixel, the frequency of each optimal strategy was calculated across 100 replicates in panel **A** and 20 replicates in the rest panels.

cells after the $(i-1)$th division, which is zero. Similarly, under a scenario of high costs and low benefits, stage-dependent strategies bear huge costs, thus only ND^i is optimal. But, when benefits are much higher than costs, reversible strategies (RD^i, *RD*) are chosen under both stage-independent and stage-dependent differentiation. This is because differentiation benefits will cover the costs caused by cell differentiation during divisions, see S6 Online Appendix for the optimal strategy under larger scales of benefits and costs.

Next, we analyzed the effect of an organism's size on the occurrence of irreversible differentiation, *ID*. In our model, stage-dependent differentiation plays a dual role in population growth rate. It provides benefits, but also incurs costs. The best strategy is one that can maximally use those benefits and at the same time reduce the costs. We found that under the conditions of high benefits or high costs, only *ND* is selected. Due to the randomness of the differentiation probabilities, some stage-dependent strategies can adjust the fraction of germ-like cells to gain the benefits and avoid the costs of differentiation, especially in large organisms that undergo more cell divisions, see Fig. 2. In small organisms, due to the constraint on the change between two successive differentiation probabilities, stage-dependent *ID* strategies only accumulate limited differentiation benefits. Since *ID* needs to undergo germ-like to soma-like differentiation first and then become irreversible at the last cell division, higher differentiation costs, especially from germ-like to soma-like will prohibit it from being the optimal strategy. However, in large organisms needing more divisions to mature, *ID* can benefit from germ-like to soma-like differentiation in the first divisions, then remain irreversible to avoid costs in subsequent cell divisions. Overall, stage-dependent strategies (*RD* or *ID*) can lead to higher population growth rates than stage-independent ones (RD^i or ID^i) in small organisms due to their ability to flexibly adjust their differentiation probability patterns, see S7 Online Appendix for more detail.

Irreversible germ differentiation dominates among optimal stage-dependent irreversible strategies

To further analyze why irreversible strategies in small organisms are more favored in stage-dependent (*ID*) than in stage-independent (ID^i) cases, we study the strategies contained in *ID*. In the model, an organism can contain two cell types, thus irreversibility can occur on either of them. Therefore, stage-dependent irreversible differentiation (*ID*) contains three subcategories: irreversible germ differentiation *IGD* ($g_{g\to s}^{(n)}=0$ and $s_{s\to g}^{(n)}\neq 0$), irreversible soma differentiation *ISD* ($s_{s\to g}^{(n)}=0$ and $g_{g\to s}^{(n)}\neq 0$), and irreversible germ and soma differentiation *IGSD* ($g_{g\to s}^{(n)}=s_{s\to g}^{(n)}=0$). Next, we investigate the occurrence conditions of each of these sub-strategies.

Our results show that among optimal *ID* in small organisms, *IGD* evolves at most benefits and costs of the parameter space, see Fig. 3A and B. Compared to the stage-independent case, *IGD* replaces ND^i as the optimal strategy in small organisms for small differentiation costs, see Figs. 2A and 3B. Specifically, we found that *IGD* strategies replace ND^i when the benefits (b) are slightly larger than the costs (c). Under this scenario, the best strategy would be to produce a few soma-like cells to gain the benefits, but decrease the differentiation between cell types to avoid the costs brought about by a tradeoff between differentiation benefits and costs, Eq. (8). Thus, the *IGD* strategy producing few soma-like cells in the first few divisions and turning them into irreversible at the end becomes optimal (first panel in Fig. 3C). *IGD* can keep a high fraction of germ-like cells increasing the population growth rate by having more offspring, i.e. $2^n f_g^{(n)}$. Although the differentiation probabilities of soma-like cells $s_{s\to g}^{(n)}$ is not small, we should note that the low number of soma-like cells ($2^i(1-f_g^{(i)})$ after the ith cell division makes the differentiation costs small. We found that *IGD* is optimal when b is much larger than c in small organisms (second panel in Fig. 3C). Under this scenario, due to the tradeoff between benefits and costs, the *IGD* strategy with higher germ-like differentiation probabilities $g_{g\to s}$ at first several cell divisions becomes optimal. Taken together, we found that an *ID* sub-strategy, *IGD*, evolves at low cost c. Furthermore, we show in an analytical proof that except for one cell division, $n=1$ (S5 Online Appendix), either reversible (*RD*) or irreversible (*ID*) strategies are optimal in the absence of differentiation costs (S8 Online Appendix). The finding indicates that without the punishment of differentiation costs non-differentiation (*ND*) cannot be selected.

Meanwhile, we found that irreversible soma *ISD* and irreversible germ soma *IGSD* differentiation, the other subcategories of *ID*, evolve at intermediate values of benefits and costs, see the last two panels in Fig. 3B. We analytically proved that both benefits and costs are indispensable factors for the evolution of *ISD* and *IGSD*, see the proof in S9 Online Appendix. Specifically, germ soma irreversibility (*IGSD*) evolves at higher benefits (b) and costs (c), driven by their differences in cell irreversibility. Compared with *ISD* strategies, in *IGSD* both cell types are irreversible at last cell division bearing lower differentiation costs, thus evolving at higher c or lower b and c (last two panels of Fig. 3C). Meanwhile, *IGSD* has relatively higher fractions of germ-like cells than *ISD*, leading to more offspring, $2^n f_g^{(n)}$, and a higher population growth rate (Eq. 8). Additionally, it is noteworthy that the result of the evolution conditions of ID^i is consistent with our previous study, where ID^i is the stage-independent irreversible soma differentiation[38].

Compared with our investigation of stage-independent differentiation[38], stage-dependent strategies promote the evolution of irreversible differentiation under the effects of α and β, parameters that determine the functional form of the benefits and costs, see S10 Online Appendix. We found that α plays a similar role as in stage-independent differentiation, where irreversible strategies are optimal if the benefits component F_b accelerates with α (occurring when $\alpha<1$), S10 Online Appendix. Meanwhile, β, which measures the relative impact of transitioning between germ and soma, differs from stage-independent differentiation in that irreversible strategies evolve across all values of β. Concretely, irreversible germ differentiation (*IGD*) evolves when β is small, and irreversible soma (*ISD*) and irreversible germ soma (*IGSD*) evolve when β is large. This is because large β increases the differentiation costs from soma cell to germ cell, which inhibits the strategies *ISD* and *IGSD* being optimal.

Large changes in two successive differentiation probabilities prevent irreversible strategies from becoming optimal

Now, we consider the unconstrained change of the differentiation probabilities between successive divisions ($0\le\delta_i\le\delta=1$). An extreme strategy that optimally exploits the benefit of somatic cells would be one where both cell types produce soma-like cells in the first $(n-1)$ divisions, and then all produce germ-like cells in the last division. We refer to this case as "extreme differentiation" (*ED*). We will first take *ED* as a typical example to investigate the effects of stage-dependent differentiation without constraints. While *ED* fully uses cell differentiation benefits, ND^i is a strategy that does not benefit from producing soma cells but is not affected by differentiation costs. Naturally, we expect that when benefits are large ($b\gg 1$) and costs low ($c\ll 1$), *ED* is evolutionary optimal, while ND^i is optimal in the opposite conditions ($c\gg 1$ and $b\ll 1$,). Based on Eq. (8), the population growth rate of *ED* is $\lambda_{ED}=\frac{\ln 2}{\frac{n+b+(1+b+\beta)c}{n(1+b)}}$ and the population growth rate of ND^i is $\lambda_{ND^i}=\ln 2$. Thus, when $c<\frac{(n-1)b}{1+b+\beta}$, we have $\lambda_{ED}>\lambda_{ND^i}$ and when $c>\frac{(n-1)b}{1+b+\beta}$, we have $\lambda_{ED}<\lambda_{ND^i}$, Fig. 4 illustrates our expectations.

Next, we investigate the effect of δ on the population growth rates of the general stage-dependent strategies *RD* and *ID*, as well as on ND^i. We found that irreversible differentiation (*ID*) cannot be optimal under large δ, see Fig. 5. Because large δ allows more randomness between cell divisions, it intensifies the variation of organismal population growth rate, except for ND^i whose differentiation probabilities are constant. For instance, we found that the population growth rate of the optimal reversible (*RD*) and irreversible (*ID*) strategies all increase with δ, with a relatively greater increase of *RD* (compare panels A_1-A_3 and B_1-B_3 in Fig. 5). Furthermore, we found that *RD* outcompetes *ID* and becomes the optimal strategy when δ is 1. When $\delta=0.1$, we found that irreversible

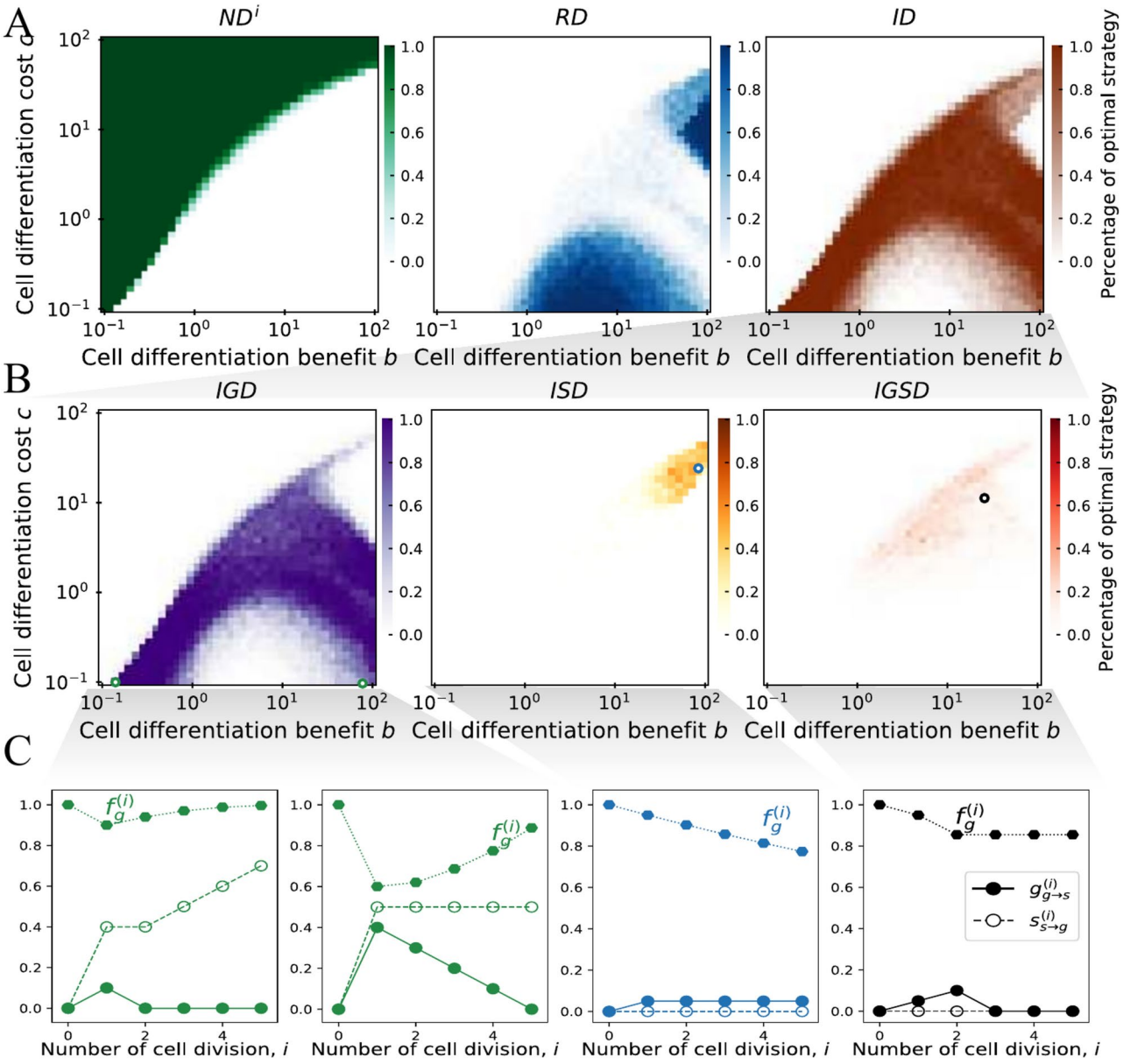


Fig. 3. Irreversible germ differentiation evolved mostly under stage-dependent differentiation. **A** Fractions of three stage-dependent cell differentiation strategies being optimal under differentiation benefits and costs. **B** Fractions of the three irreversible sub-strategies being optimal under differentiation benefits and costs. **C** Cell differentiation probabilities ($g^{(i)}_{g\to s}$, $s^{(i)}_{s\to g}$) and frequencies of germ-like cell ($f^{(i)}_g$) of the optimal irreversible sub-strategies at the parameter space indicated by circles in panel **B**. The circle color follows that in panel **B**. Parameters of all panels: maximal cell number of division rounds $n = 5, 0 \le \delta_i \le 0.1$, and $\alpha = \beta = 1$. At each pixel, the frequency of each optimal strategy was calculated across 100 replicates. Parameters of calculating optimal strategy: the number of initial sampling $d^{(1)}$, $M = 1000$, the number of stage-dependent strategies starting with a given $d^{(1)}$, $R = 100$, replicates for each pixel is 100, for more detail, see S2 Online Appendix.

germ strategies (*IGD*, sub-strategy of *ID*) lead to higher population growth rates than *RD*, whereas when $\delta = 1$, the population growth rate of *RD* outcompetes *IGD*, see the two groups between A_1-A_3, and B_1-B_3 in Fig. 5. Based on Eq. (8), we know that the population growth rate depends both on cell division rates and the number of offspring (the fraction of germ-like cells after the *n*th division). Furthermore, the cell division rate is proportional to the fraction of soma-like cells, but inversely proportional to the differentiation probabilities which cause differentiation costs. Taken together, the largest population growth rate is attained by the strategy with a higher fraction of soma-like cells all the time, a higher fraction of germ-like cells after the last cell division, and lower differentiation probabilities. Thus, reversible differentiation *RD* contains strategies to increase the fraction of soma-like cells in the middle stages of cell divisions and the number of offspring (germ-like cells) after the *n*th cell division, prohibiting the emergence of strategy *ID* as the optimal.

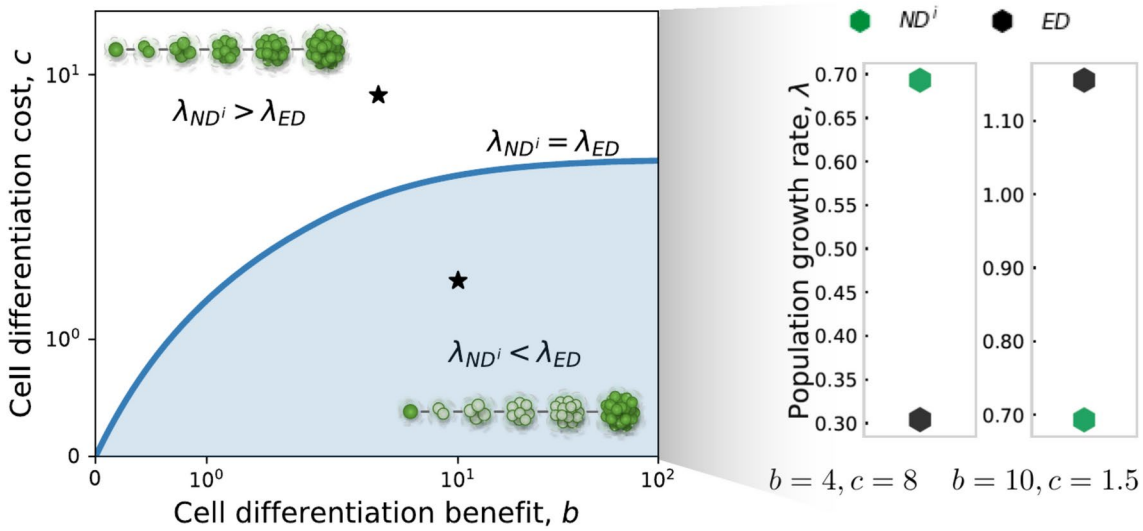


Fig. 4. Evolutionary conditions for non-differentiation ND^i and extreme differentiation *ED* , and their corresponding population growth rates. The blue line represents the condition for $\lambda_{ND^i} = \lambda_{ED}$. The shaded area represents where $\lambda_{ND^i} < \lambda_{ED}$. We found that ND^i is optimal under high cost (*c*) and *ED* is optimal under high benefit (*b*). The black stars correspond to the parameter combinations where population growth rates have been calculated in the right panel. Parameters: $n = 5$ and $\alpha = \beta = 1$.

Conclusion and discussion

We investigated the effect of stage-dependent differentiation on an organism's cell division process and compared it with stage-independent cell differentiation. Stage-independent differentiation only allows a fixed differentiation probability for a cell type. Stage-dependent differentiation, by contrast, refers to being capable of changing probabilities in consecutive cell divisions. The most extreme case would be an organism that consists entirely of soma-like cells until the last cell division when all cells turn into germ-like cells to produce as many offspring as possible. Stage-dependent differentiation intensifies the fluctuation of the germ-soma ratio during an organism's cell division process, which further increases the complexity of competition between different strategies. We used the population growth rate as a proxy to investigate the growth competition of different strategies under different benefits and costs. Based on the differentiation probabilities in the last division, we classify stage-dependent strategies into three categories: non-differentiation ND^i, reversible differentiation *RD*, and irreversible differentiation *ID*. The evolution of irreversible differentiation under stage-independent differentiation has been demonstrated by previous work to be challenging[38]. Contrary to our expectations, we found that stage-dependent differentiation favors *ID* (in the last division step) more than stage-independent irreversible differentiation ID^i in smaller organisms when $n = 5$ (Fig. 2). Specifically, *IGD*, a sub-strategy of *ID*, leads to a higher population growth rate than other strategies in small organisms. Additionally, *ISD* and *IGSD* evolved in the parameter space with intermediate benefits and costs, consistent with previous findings[38]. Finally, we found that large differentiation probability variation prohibits irreversible differentiation *ID* from becoming the optimal strategy. The findings indicate that stage-dependent strategies favor the evolution of irreversible differentiation in small organisms with limited variations between successive cell divisions.

That irreversible differentiation is favored in small organisms is contrary to the intuition provided by stage-independent differentiation, where irreversible differentiation is favored in large organisms[38]. This discrepancy arises because of the flexibility of the developmental trajectories under stage-dependent differentiation. These complex developmental trajectories intensified with cell divisions, increase the growth differences between different strategies. Thus, reversible differentiation outcompetes irreversible differentiation in large organisms. Our result implies irreversible differentiation in the organisms with two cell types is unlikely to occur in large organisms. In addition, stage-dependent irreversible differentiation evolves two more subcategories than the stage-independent case: irreversible germ differentiation *IGD* and irreversible germ and soma differentiation *IGSD*. The broad form of stage-dependent differentiation strategies can capture more cell differentiation patterns in reality. For example, the evolution of *IGSD* can help us to understand cell lineage segregation in nature[24]. Our model can screen the stage where irreversible differentiation emerges, in line with the question of early segregation of germ and soma in animals[19,42–45], but late in most plants[46]. To identify the segregation, we must investigate the irreversible developmental states of germ-like and soma-like cells in our model. Future work is necessary to seek and analyze the conditions where different segregation occurs.

Previous investigations of cell differentiation mostly focused on the state with a group of undifferentiated clonal cells[25,27,32–37,47] or cells with randomly chosen initial cell types (similar to aggregated organisms)[37]. The focus of these studies was on the final static conditions that lead to the division of labor rather than the dynamic process during an organism's cell division process. These models ignored the dynamic developmental trajectories of organisms from newborn to maturity. In our model, the developmental trajectories of each organism are recorded by stage-dependent differentiation probabilities, allowing us to know the dynamic fractions of each cell

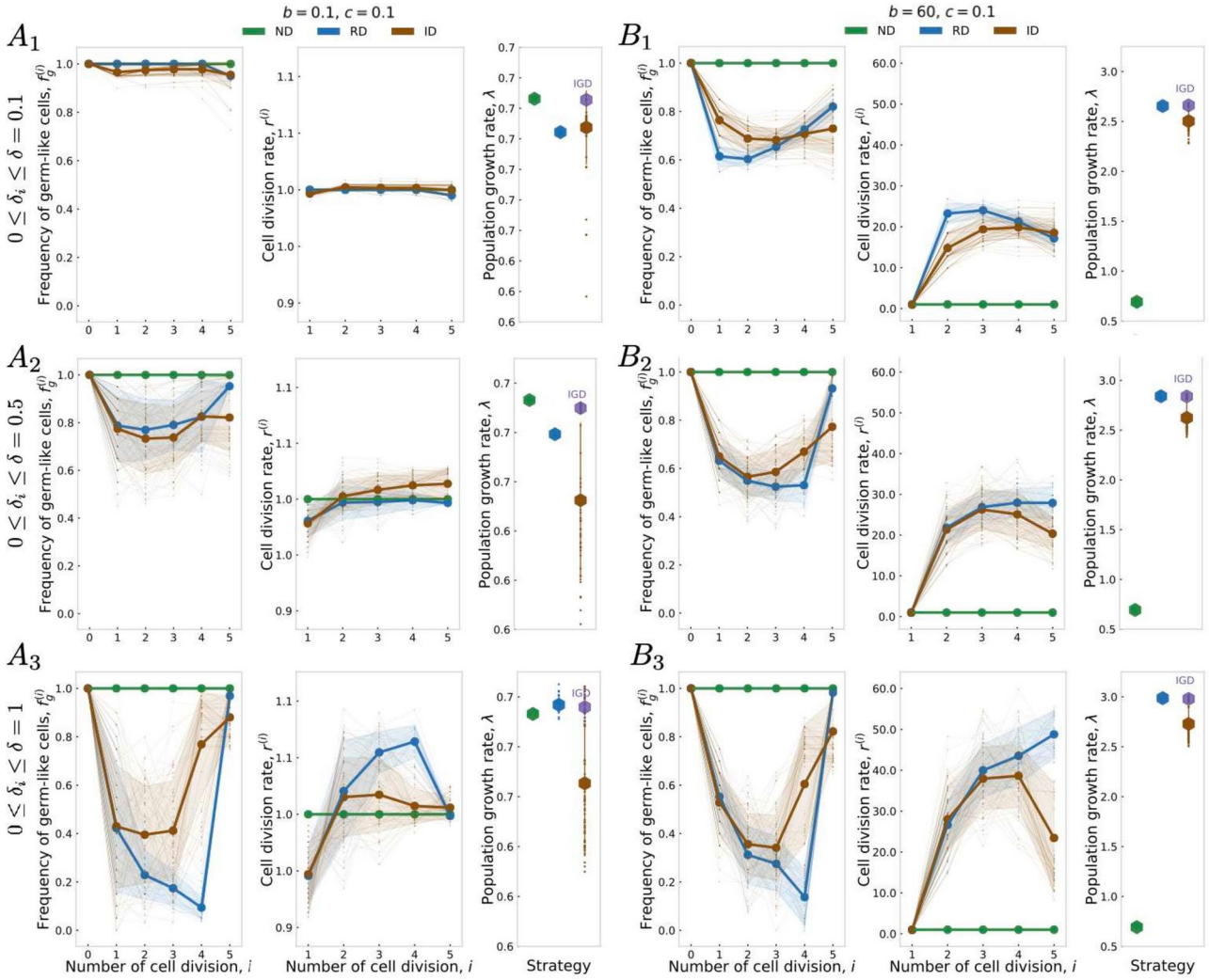


Fig. 5. Effect of the maximum change of two successive differentiation probabilities δ on the population growth rate of optimal strategies. Frequencies of germ-like cells, cell division rates, and population growth rate of the optimal stage-dependent strategy of each category under $\delta = 0.1$, $\delta = 0.5$ and $\delta = 1$ respectively, $i = 1, 2, \ldots, n$. Small dots are the values of the feature of interest at each cell division. Thick lines are the averaged values at each cell division. The shaded areas indicate the standard deviation. $\alpha = \beta = 1$ and colors correspond with those in Fig. 3. Parameters: $n = 5$, and $\alpha = \beta = 1$, we chose 20 duplicates for generating the optimal strategies in each category which includes the subcategories.

type during an organism's cell division process, which further allows us to investigate cell differentiation patterns. In addition, Rodrigues et al. have considered cell differentiation probability as an evolving trait to understand the evolution of differentiation[37]. They concluded that differentiation costs, compared with the difference in division rates between cell types, have less impact on the evolution of terminal and reversible differentiation. They also found that differentiation costs played a crucial role in the evolution of diversity differentiation strategies. Moreover,[37] investigated developmental strategies in filament multicellular organisms with two essential tasks, and they found that high differentiation costs can promote the evolution of symbioses. In our model, we employ functions to demonstrate differentiation benefits and costs (Eqs. (3), (4) as they can capture more general forms of benefits and costs by varying relevant parameters.

Since we focus on the evolution process before cells develop final specialized types, in our model, we assumed that differentiation occurs randomly and both cell types are capable of cell differentiation[38]. The assumption is based on the cell differentiation situation of species observed in the genus *Volvox*, which reveals that cell types undergo an intermediate and partial differentiation stage in some closely related species before eventually becoming specialized cell types[24]. We classify the stage-dependent differentiation strategy based on its differentiation probability at the last round of cell division. The classification is based on the idea that the differentiation strategy (reversible and irreversible) describes the changes in differentiation capability along the cell division process. Nevertheless, we stress that this classification is imperfect, especially for large organisms with more cell divisions, where a more refined classification criterion is needed. However, owing to the simple classification, the current classification can still largely reflect the evolving situation of the specific strategies of

interest. For instance, the strategy where cells all turn into specialized types after half the number of cell divisions is a subset strategy of *ID*, thus it can only evolve in the parameter space that *ID* emerged. Meanwhile, we assumed that organisms are clonal, born from a single founding cell. The reasons for our clonal assumption are that multicellularity is formed commonly by clonal division rather than cell aggregation[14,48–52] and clonal organisms with identical genes have advantages at purging deleterious mutations and reducing conflicts among cells[49,53]. Therefore, clonal multicellularity is predicted to be evolutionarily stable[12]. In the cell differentiation models of aggregated multicellularity, a relatedness parameter can be used to evaluate the level of cooperation between cell types[30,33,34,54]. Additionally, the maturity size is fixed in the model as previous work has shown that selection favors life cycles where all organisms grow to the same size and fragment into pieces with the same pattern[55]. The assumption is generally in line with the size observation in some species such as *Volvox*[24].

We assumed that cell differentiation costs influence an organism's cell division rates. In nature, cell differentiation and cell plasticity usually originally occur under severe environmental conditions, indicating a differentiation cost involved[13,18,56,57]. Differentiation cost has been considered in previous theoretical research via varying forms[27,30,37,58–60]. The modeling purpose of cell differentiation costs in the model is the same, i.e. reducing an organism's fitness. In our model, we are interested in the relative growth advantage between different differentiation strategies. Therefore, we assume that differentiation costs affect the population growth rate, reducing cell division rates. Finally, we suppose that cells undergo synchronous cell divisions. This is not true for large multicellularity with many more cell divisions[24,61]. Asynchronous cell division has been explored under stage-independent differentiation in previous studies, leading to the same predictions as the synchronous one[38]. Yet, it still needs to be investigated whether asynchronous cell division leads to the same conclusion as synchronous ones under stage-dependent differentiation in the future. Our model could be further extended by including cell death or differentiation costs related to the risk of organism death. Yet, our model gives first insights into understanding the effects of dynamic differentiation on the evolution of cell differentiation in multicellularity.

Data availability

Numerical data produced by this work has been deposited in GitHub: https://github.com/YuanxiaoGao/Stage-dependent_cell_differentiation.

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Author contributions

Yuanxiao Gao, Yuriy Pichugin, and Arne Traulsen conceived the presented idea. Yuanxiao Gao and Román Zapién-Campos developed the theory and performed the computations. Yuriy Pichugin and Arne Traulsen verified the analytical methods. Arne Traulsen helped supervise the project. Yuanxiao Gao took the lead in writing the manuscript. All authors provided critical feedback and helped shape the research, analysis, and manuscript.

Declarations

Competing interests

The authors declare no competing interests.

Additional information

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