Int. J. Dev. Biol. 63: 429-438 (2019)
https://doi.org/10.1387/ijdb.190192ts

THE INTERNATIONAL JOURNAL OF DEVELOPMENTAL BIOLOGY
www.intjdevbiol.com

Small molecules and cell differentiation in *Dictyostelium discoideum*

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ABSTRACT ***Dictyostelium*** **is a microorganism found in soils that are known as the battle fields of chemical warfare. Genome analysis of** ***Dictyostelium*** **revealed that it has great potential for the production of small molecules, including secondary metabolites such as polyketides and terpenes. Polyketides are a large family of secondary metabolites which have a variety of structures. In accordance with their structural variety, polyketides have a plethora of biological activities, including antimicrobial, antifungal, and antitumor activities. Unsurprisingly, they have exceptional medical importance. Polyketides in nature work as protective compounds and /or function in pheromonal communication. Terpenes belong to another family of structurally diverse secondary metabolites which play roles in ecological interactions, including defence against predators and formation of mutually beneficial alliance with other organisms. Polyketides and terpenes work as intra- or inter-species signalling compounds, i.e. they play the role of a chemical language. However, in** ***Dictyostelium*****, they work as paracrine signalling compounds which control the organism's multicellular morphogenesis. This review is primarily focused on the small molecules that regulate pattern formation in the slug stage of the organism and their biosynthetic pathways. Current** ***in vivo*** **understandings of polyketide DIF-1 induced cell differentiation and DIF-1-dependent/independent pathways are also discussed.**

KEY WORDS: *polyketide, terpene, transcriptional regulation, Type III PKS, bZIP transcriptional factor, STAT*

Complexity in a simple pattern

Cellular slime mold, *Dictyostelium*, lives in soil as isolated amoeba eating bacteria. Once surrounding bacterial food source is depleted, approximately 100,000 *Dictyostelium* cells start to aggregate by chemotaxis toward cyclic adenosine monophosphate cAMP, form a hemispherical multicellular structure, called mound, and further undergo cell differentiations and morphogenic transformations. Resulting terminal structure, fruiting body, consists of two major cells: spore and stalk. Spores are dormant cells protected against various environmental stresses, and stalk is formed by vacuolated dead cells lifting up the spore mass on top of the structure.

This simple-looking final structure conceals their complex and sophisticated developmental program. In the mound, cells commence cell differentiation in "Salt and Pepper" fashion (Thompson *et al.*, 2004; Kay and Thompson, 2009). Cell sorting and three-dimensional morphogenetic movement make a clear pattern along anterior-posterior axis in following slug-shaped structure; anterior one-fourth of cells are prestalk cells which will terminally differentiate into stalk cells in fruiting body, and posterior three-fourth are prespore cells which will be spores (Bonner and Slifkin, 1949). This prestalk and prespore ratio is tightly controlled in the slug. Formed slug behaves like a multicellular organism; slug migrates towards light and heat to find suitable place for the fruiting formation (Raper, 1940) and is even equipped with immune system-like defence mechanism (Chen *et al.*, 2007).

The most well-studied intracellular signalling molecule in *Dictyostelium* is cAMP (3',5'-cyclic adenosine monophosphate). Extracellular cAMP signal is tightly regulated by the combinations of three adenylyl cyclases (ACA, ACB, and ACG: Kriebel and Parent, 2004), four cAMP receptors (CAR1-4; Kim *et al.*, 1998; Verkerke-Van Wijk *et al.*, 1998), and three phosphodiesterases (PdsA/PDE1, PDE4, and 7: Barder *et al.*, 2007), and controls vari-

Abbreviations used in this paper: ALC, anterior like cell; cAMP, 3'5'-cyclic adenosine monophosphate; DIF, differentiation inducing factor, 1-[(3,5-dichloro-2,6- dihydroxy-4-methoxy)phenyl]hexan-1-one; DQ, dictyoquinone, 2-hydroxy-5-methyl-6-pentylbenzoquinone; ecm, extracellular matrix; MPBD, 4-methyl-5-pentylbenzen-1,3-diol; PKS, polyketide synthase; Psp, prespore; Pst, prestalk; THPH, (2,4,6-trihydroxyphenyl)-1-hexan-1-one.

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Submitted: 1 June, 2019; *Accepted:* 5 July, 2019.

ISSN: Online 1696-3547, Print 0214-6282

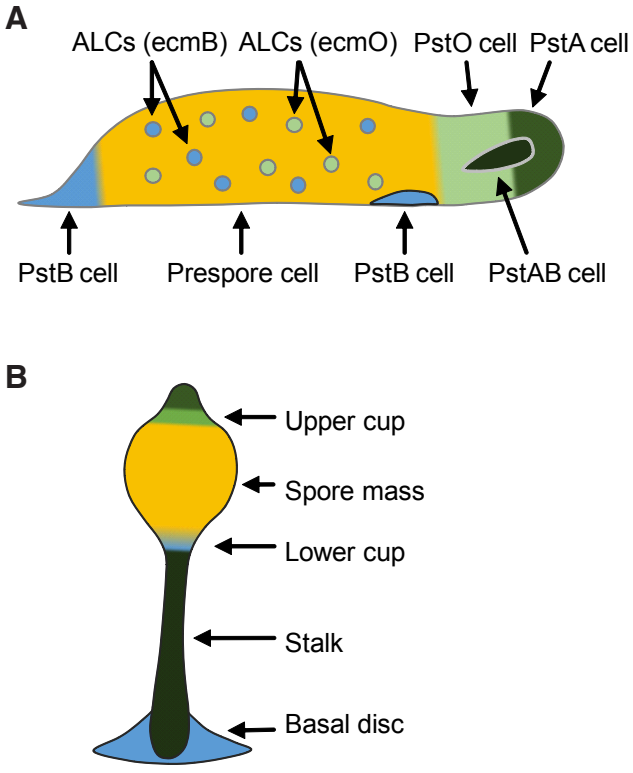


Fig. 1. Schematic drawings represent major cell sub-types at two multicellular stages in *Dictyostelium* development: migrating slug (A) and fruiting body (B). *Each cell sub-type is indicated with different colour. At the fruiting body formation, stalk formation occurs at the core of prestalk region (PstAB cell) with reverse fountain manner and spore mass is lifted up on top of the structure. ALCs (ecmO) move upward and ALCs (ecmB) downward, forming supporting structures of Spore mass, Upper cup and Lower cup respectively. Two PstB cell populations in slug differentiate into Basal disc in fruiting body. Recently-identified two prestalk cell sub-types, PstU and PstV*^{A} *are not included in these drawings and are described in main text and in Figures 4 and 5.*

ous developmental processes. While nanomolar cAMP oscillatory signal works as chemotaxis signal in aggregation, extracellular cAMP concentration is elevated in multicellular structure and micromolar cAMP regulates morphogenesis and cell differentiation (Schaap *et al.*, 1986; Pitt *et al.*, 1993). Prestalk and prespore cells at mound stage have different response to cAMP, resulting in cell sorting to form antero-posterior prestalk/prespore pattern in slug (Sternfeld and David, 1981; Traynor *et al.*, 1992; Dormann *et al.*, 2000). *In vitro* monolayer assays revealed that cAMP signal induces a number of prespore genes (Mehdy *et al.*, 1983; Schaap and van Driel, 1985), and cAMP at first induces the competency for stalk cell differentiation and later inhibits the stalk cell formation (Berks and Kay, 1988; Soede *et al.*, 1996). Series of *in vitro* monolayer results indicate that the stalk cells are induced by the combination of cAMP and a low molecular weight, developmentally regulated molecule, differentiation inducing factor (DIF) (Kay, 1981). Further details about cAMP signaling in *Dictyostelium* are discussed in other reviews of this special issue.

Since the identification of polyketide DIF-1 {1-[(3,5-dichloro-2,6-dihydroxy-4-methoxy)phenyl]hexan-1-one} as stalk cell inducer, the molecular mechanism of cell differentiation has been studied. *EcmA* and *ecmB* genes, both encode extracellular matrix proteins, are identified as DIF-1 inducible and prestalk specific genes. *EcmA* promoter drives strong gene expression in virtually entire prestalk region (PstAO cells; Williams *et al.*, 1989) and, therefore, has been widely used as prestalk marker. Promoter analysis revealed that *ecmA* promoter (referred to as ecmAO promoter) can be divided into two, cap-site proximal and distal, regions (Early *et al.*, 1993). Cap-site proximal half (ecmA promoter) regulates the expression in cells at anterior half of prestalk (PstA cells). Distal half region (ecmO promoter) directs expression in cells at posterior half of prestalk region (PstO cells) and also in cells at scattered position in prespore region (Anterior-Like-Cells: ALCs). In the process of fruiting body formation, ALCs move upward to make cup-shaped structure (upper cup) which supports and also pushes the spore mass up. *EcmB* expressing cells are observed at scattered in prespore region (ALCs) and also at the ventral surface (PstB cells) and the rear end (Rear-guard cells) of slug (Ceccarelli *et al.*, 1991). Contrary to ecmO expressing ALCs, *ecmB* positive ALCs form lower cup, supporting structure at the bottom of the spore mass in the fruiting body. Ventral *ecmB*-positive cells (PstB cells and Rear-guard cells) differentiate into basal disc, supporting structure of whole fruiting body at the bottom of stalk. Both *ecmA* and *ecmB* genes are strongly expressed in the cells at the cone-shaped core (PstAB cells) of the prestalk region, where stalk formation occurs with reverse fountain manner at the onset of fruiting body formation (Ceccarelli *et al.*, 1991). On the other hand, prespore cells at the posterior of slugs are repressed by DIF-1 signal (Early and Williams, 1988) and are thought to be uniform, although gradient prespore specific SP60 gene expression is reported in its anterior-posterior axis (Haberstroh and Firtel, 1990). Fig. 1 shows a schematic representation of cell sub-types in migrating slugs (A) and fruiting body (B).

Analysis of just two DIF-1 inducible and prestalk cell specific genes tells us the complexity of cell population in *Dictyostelium*. Microarray and subsequent whole-mount *in situ* hybridization analysis confirmed these prestalk patterns and cell sub-types in the migrating slug (Maeda *et al.*, 2003). At the same time, this work shows the existence of DIF-1-independent mechanism in prestalk cell differentiation and also the dynamic nature of cell-type specific gene expression during the developmental process. Understanding diffusible signalling molecules, e.g. DIF-1, MPBD, and Dictyoquinone etc., and their sensing mechanisms can be the keys to understand cell differentiation and pattern formation in *Dictyostelium* development. A summary of small molecules discussed in this review is shown in Table 1.

DIF-1: biosynthesis and inactivation

Polyketides comprise a highly diverse class of secondary metabolites found in bacteria, fungi, plants, and animals, and are known to have diverse biological activities and pharmacological properties. Polyketides are synthesized through a decarboxylative condensation (Claisen condensation) using malonyl-CoA as an extension unit. The enzymes that catalyse this condensation reactions are polyketide synthases (PKSs). Genome analysis revealed that *D. discoideum* has 45 putative PKS genes in its genome, of which five appear to be pseudogenes (Eichinger *et al.*, 2005; Zucko *et al.*, 2007). The related species *D. purpureum* is also reported to have a similar number of PKS genes (Sucgang *et al.*, 2011).

Interestingly, Dictyostelia seem to have a novel type of PKS, the fusion of type I and type III PKSs (Austin *et al*., 2006). To the best of our knowledge, these fusion-type PKSs are found exclusively in the cellular slime molds. This enzyme is known as Steely (Austin *et al*., 2006). Steely is basically an iterative type I PKS that has a type III PKS domain in the C-terminal region instead of a thioesterase domain of the enzyme (Figs. 2A and 3A).

DIF-1 is one of the most well-characterized differentiation-inducing molecules in *Dictyostelium*. There are many reviews concerning experiments of DIF-1, including some on various biomedical activities in mammalian cell lines (Williams, 2006; Kay and Thompson, 2009; Fukuzawa, 2011; Kubohara and Kikuchi, 2018). Therefore, here we focus on the biosynthetic pathway of DIF-1 and the function of DIF-1, which was revealed by the knockout mutants of biosynthetic enzyme genes (Fig. 2B).

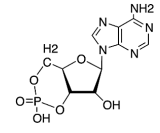
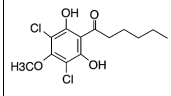
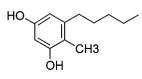
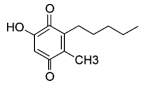
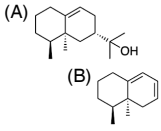
In 1998, three steps of the DIF-1 biosynthetic pathway were proposed and DIF-1 was suggested to be a polyketide (Kay, 1998). Since then, step by step, each biosynthetic enzyme has been reported until a dedicated biosynthetic pathway was completely elucidated (Yellow box in Fig. 2B). In 2000, DmtA, an O-methyltransferase that regulates the final step of DIF-1 biosynthesis, was reported (Thompson and Kay, 2000). A *dmtA*- mutant is the first example of DIF-1-less mutant that has a defect specifically in DIF-1 biosynthesis. The analysis of this *dmtA*- mutant showed that DIF-1 induced PstO cells instead of PstA cells to form a major part of the prestalk. Early development of the *dmtA*- mutant is similar to that of a wild type strain Ax2. The striking feature of DIF-1-less mutant phenotype was found in the slug stage. The *dmtA*- mutant slugs break up and lay down the stalk behind them like related Dictyostelia *D. mucoroides*, which produces a stalk continuously during slug migration and fruiting body formation. The *dmtA*- mutant fruiting bodies also show a clear defect in fruiting body morphology, i.e. slipping down sorus from top of the stalk.

PKS gene producing DIF-1 was identified in 2006 (Austin *et al*., 2006). SteelyB, one of two hybrid type PKSs, produces (2,4,6-trihydroxyphenyl)-1-hexan-1-one (THPH), which forms a polyketide backbone of DIF-1. *In vitro* analysis of purified SteelyB type III PKS domain revealed the process of formation of THPH from hexanoyl-CoA and malonyl-CoA. ^{36}Cl labelling showed that *stlB*- mutant lost the ability to produce DIF-1 and the addition of THPH to these mutant cells restored the production of DIF-1 *in vivo*. *StlB*- mutants produced break-up slugs like *dmtA*- mutants. In the fruiting body stage, *stlB*- mutant showed clear defects, in that there was a lack of the basal disc which supports the stalk to make an upright fruiting body (Saito *et al*., 2008). As a result, a *stlB*- mutant made a slanting fruiting body on the substratum with slipping down sorus, due to the lack of a lower cup that supports the spore head. These phenotypes indicated that *stlB*- mutants were lacking a subset of anterior like cells (ALCs) and pstB cells. Analysis on a traction force microscope suggested the mechanism of *stlB*- mutant slug disintegration. *StlB*- mutants lack PstB cells in the posterior part of the slug and hence produce little motive force in the rear region and disintegrate immediately (Rieu *et al*., 2009). The phenotypic analysis of *dmtA*- and *stlB*- mutants defined the DIF-1-less phenotype.

The enzyme responsible for dichlorination of THPH was identified in 2010 (Neumann *et al*., 2010). The gene coding this enzyme, *chlA,* was identified by genome mining with the clue from the prokaryotic flavin-dependent halogenase. At first, chlorination of THPH was supposed to be catalysed by chloroperoxidase because the enzymatic activity was stimulated by hydrogen peroxide (Kay, 1998).

TABLE 1

LIST OF SMALL MOLECULES DISCUSSED IN THIS REVIEW

Name	Structure	Dev. Stage	Proposed functions in *Dictyostelium* development
cAMP		Early	• Chemoattractant (at nM order, pulse)
		Mid + Late	• Prespore cell induction • Prestalk cell competency induction (at mM order, continuous/pulse)
DIF-1		Early	• Regulation of chemotactic response
		Mid + Late	• PstB cell and PstO cell regulations • Prespore cell inhibition • Autophagic cell death induction
MPBD		Early	• Regulation of chemotactic response
		Late	• Spore maturation (encapsulation)
Dictyoquinone (DQ)		Early + Mid	• Regulation of chemotactic response • Prespore cell induction • Cell differentiation competency
Discoidol (A)/ Discodiene (B)		Late	• Promotion of fruiting body formation

In *Dictyostelium* development, cAMP (3',5'-cyclic adenosine monophosphate) works not only as intracellular second messenger but also as extracellular signalling molecule in various events with different formats (concentration, signal pattern, and combination with other small molecules). Two hybrid type polyketide synthase (PKS) enzymes, SteelyA (StlA) and SteelyB (StlB), are responsible for the production of DIF-1 {1-[(3,5-dichloro-2,6-dihydroxy-4-methoxy) phenyl]hexan-l-one} and MPBD (4-methyl-5-pentylbenzen-1,3,diol), respectively. Dictyoquinone (DQ; 2-hydroxy-5-methyl-6- pentylbenzoquinone), originally identified from its prespore-cell-promoting activity, is a putative MPBD metabolite. Discoidol, a sesquiterpene alcohol, is produced by a terpene synthase, DdTPS8, and a cytochrome P450 enzyme, CYP521A1, catalyzes oxidative degradation of dicoidol to discodiene, a trisnorsesquiterpene.

The first flavin-dependent halogenase from *Pseudomonas* was described in 2000 (Keller *et al*., 2000). Since then, a number of flavin-dependent halogenases were found in prokaryotes and fungi (van Pée and Patallo, 2006; Zeng and Zhan, 2010). The *chlA* gene is located adjacent to the *stlB* gene in a head-to-head manner on chromosome 5. The developmental expression profile of *chlA* was reported to be identical to that of *stlB* and these two genes share a common promoter (Neumann *et al*., 2010). Therefore, they might be under the same transcriptional regulation.

DIF-1 is made in the prespore region in the slug stage and induces the formation of the prestalk. In this prestalk region, DIF-1 is decomposed and inactivated (Kay and Thompson, 2009). The first step of this inactivation is dechlorination (Kay *et al*., 1999; Green box in Fig. 2B). The enzyme DIF dechlorinase, DrcA, was identified in 2011(Velazquez *et al*., 2011). This enzyme was a member of glutathione-S-transferase and catalyses the reductive dechlorination using glutathione. The authors suggest the possibility of the use of these enzymes in bioremediation of halogenated contaminants in the environment.

DIF-1 was first identified in the 1970's and since then it has been

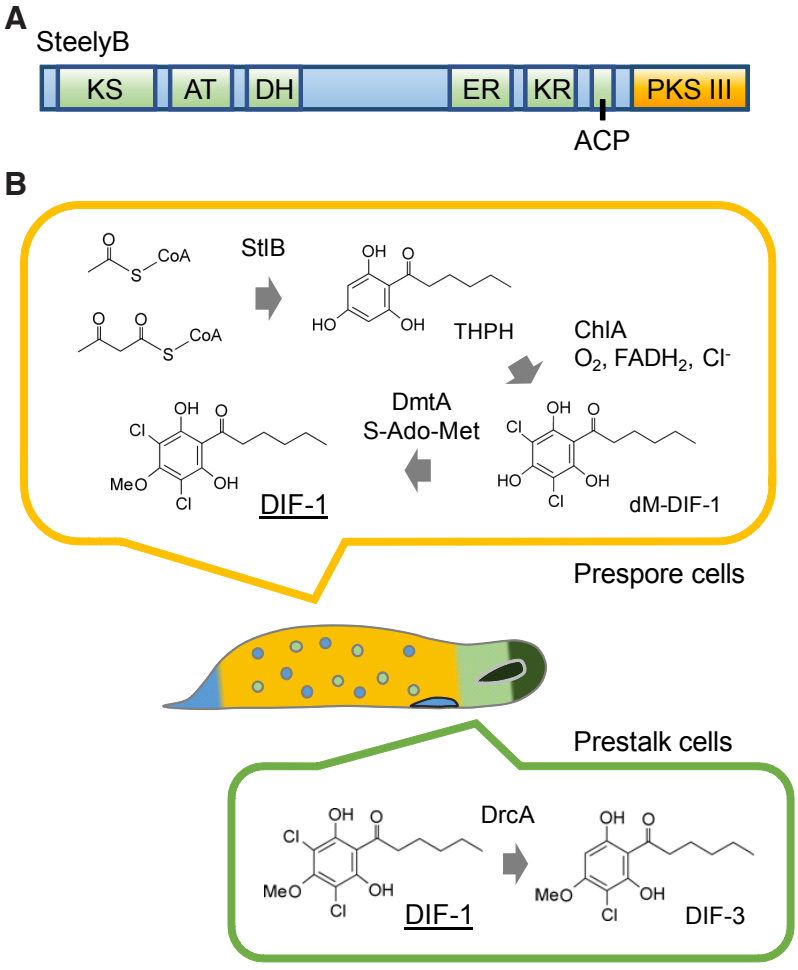


Fig. 2. Structure of SteelyB protein (A) and DIF-1 biosynthesis and degradation (B). (A) *Domain structure of SteelyB enzyme consisting of N-terminal type I PKS and C-terminal type III PKS. N-terminal type I PKS constitutes a β-ketoacyl synthase (KS), an acyltransferase (AT), a dehydratase (DH), an enol-reductase (ER), a keto-reductase (KR), and acyl carrier protein (ACP). C-terminal thioesterase domain of type I PKS is replaced by type III PKS domain in Steely enzyme.* **(B)** *The structure and DIF-1 biosynthesis and degradation. DIF-1 biosynthetic pathway consists of three steps. The first step is SteelyB mediated THPH polyketide formation. Second step is ChlA mediated dichlorination and final step is DmtA mediated O-methylation. The first step of DIF-1 degradation pathway is dechlorination mediated by DrcA.*

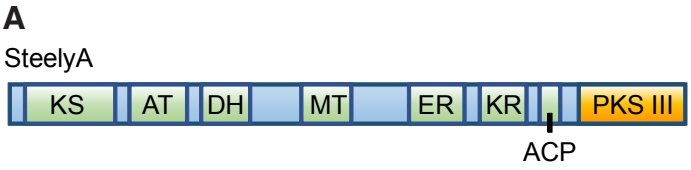
at the centre of developmental biology of *Dictyostelium* (Kay *et al.*, 1978). Identification of biosynthetic enzymes led us to understand the function of DIF-1 in development. On analysis of biosynthetic enzyme null mutants, to our surprise, it was found that DIF-1 does not induce the differentiation of the major part of the prestalk and stalk cells. This might be due to differences in experimental conditions. In one set of experiments, the DIF activity was analysed by a submerged monolayer assay, which is an *in vitro* assay (Brookman *et al.*,1982). However, the developmental morphology was examined with gene knockout mutants, which is an *in vivo* assay. For example, in submerged monolayer conditions, the cells remained as amoebae and moved in the presence of a polyketide synthase inhibitor, cerulenin (Kay, 1998); while on the agar containing cerulenin, treated cells display normal appearance of fruiting body but with very fragile and single layered stalk tube (Sato *et al.*, 2016). This means that cerulenin completely inhibited stalk differentiation in submerged monolayer conditions but not in the agar medium containing cerulenin. These differences reflect the differences in experimental conditions. These cerulenin-treated cells developed on cerulenin-containing agar still had *ecmA*-LacZ-stained cells in the very tip region of the slug (Sato *et al.*, 2016). This indicates that there must be a non-polyketide prestalk inducer in the slug stage. It may be noteworthy to mention early observation that is indicating the existence of additional factor(s) to induce stalk cell in monolayer condition. There shows a difference in stalk cell induction rate between two *D. discoideium* wild-type strains, V12M2 and NC4, in *in vitro* monolayer condition (Berks and Kay, 1988). NC4 strain, the parent of widely used axenic strains (Ax2, Ax3 and KAX3), requires extra factor(s) (in conditioned medium) in addition to cAMP and DIF-1 to achieve efficient stalk cell differentiation, compared to V12M2 strain. This still unidentified factor(s) is possibly the one to induce ecmA gene expression in the tip region of the slug.

MPBD: biosynthesis and function

The compound 4-methyl-5-pentylbenzen-1,3-diol (MPBD) was first identified as a new differentiation-inducing factor from the conditioned medium of HM1030, a *dmtA*- mutant (Saito *et al.*, 2006). The structure of this new differentiation-inducing factor was confirmed by chemical synthesis. The biological function of MPBD was mysterious. It induced stalk cell differentiation in submerged monolayer assays, but the maximal induction rate was about 20% and dose response curves were erratic. The analysis of biological function of MPBD had to wait till the identification of the biosynthetic enzyme gene.

When MPBD was identified, it was speculated to be a polyketide based on its structural features, and later another fusion type PKS, SteelyA, was identified to be responsible for the production of MPBD (Narita *et al.*, 2011; Fig. 3A). The phenotypical defects of *stlA*- mutants were examined to understand the function of MPBD in development. Clear defect was detected in the fruiting body stage. The *stlA*- mutant cells made a normal stalk and glassy spore head. When examined under a microscope, most of the "spores" remained in the amoebae-like form. Calcofluor staining showed that amoebae-like cells in *stlA*-mutant lacked encapsulation of spores, due to which few cells were stained. The encapsulation rate was examined by heating at 37°C in the presence of EDTA, and about 22% cells in sorus were encapsulated without MPBD (Narita *et al.*, 2011). Although *stlA*- mutant showed spore encapsulation defect, expressions of prestalk and prespore marker genes were normal.

StlA- mutant showed another phenotypic defect in the early developmental stage. The development of the mutant was delayed by about 3 h from the aggregation stage and showed abnormally small aggregation territories. In the *stlA*- mutant, chemotactic cAMP response was impaired and the cAMP signalling genes were down-regulated. The addition of MPBD or endogenous cAMP pulses lacking MPBD rescued the aggregation defect of the *stlA*- mutant. This indicates that MPBD may act on the same pathway as cAMP



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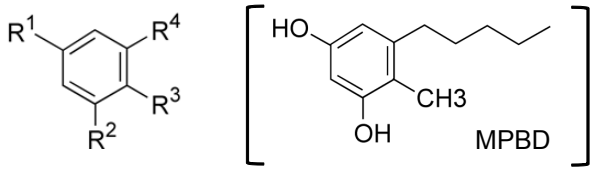

	R^1	R^2	R^3	R^4	Sporulation	Aggregation
MPBD	OH	OH	Me	Pe	++	++
EPBD	OH	OH	Et	Pe	-	++
PPBD	OH	OH	Pr	Pe	-	-
diMe-MPBD	OMe	OMe	Me	Pe	-	+
MPP	OH	H	Me	Pe	-	+
Olivetol	OH	OH	H	Pe	-	-
Orcinol	OH	OH	H	Me	-	-

Fig. 3. Structure of SteelyA protein (A) and effects of MPBD-derivatives on sporulation and aggregation (B). (A) *Domain structure of SteelyA enzyme consisting of N-terminal type I PKS and C-terminal type III PKS. N-terminal type I PKS constitutes a β-ketoacyl synthase (KS), an acyltransferase (AT), a dehydratase (DH), a methyltransferase (MT), an enol-reductase (ER), a keto-reductase (KR), and acyl carrier protein (ACP). C-terminal thioesterase domain of type I PKS is replaced by type III PKS domain.* **(B)** *Structure of MPBD and its derivatives and effects on sporulation efficiency and on chemotaxis defects in* stlA- *mutant are shown in the table: OH, hydroxyl group; Me, methyl group; OMe, methoxy group; Et, ethyl group; Pr, propyl group; Pe, pentyl group. The sporulation efficiency of* stlA- *mutant decreased to about 30% compared to that of Ax2. The addition of 200 nM MPBD recovered the sporulation efficiency of* stlA- *mutant to >70%. Meanwhile all MPBD derivatives, olivetol and orcinol did not affect the sporulation efficiency. Two hundred nanomolar of each chemical compound was added from the initiation of starvation to examine the recovery of cell aggregation of* stlA- *mutant. The recovery of chemotaxis after treatment with EPBD, PPBD, diMe-MPBD or MPP were confirmed. diMe-MPBD or MMP recovered the chemotaxis of* stlA- *mutant to ~70% of that for MPBD.*

pulse (Narita *et al.*, 2014). The defect of spore encapsulation in *stlA*- mutants was not rescued by MPBD addition only in the aggregation stage, indicating that the defect of spore encapsulation is not due to the defect of cAMP signalling in the aggregation stage. In fact, the structure-activity relationship study revealed that the functional groups essential for the induction of spore maturation and induction of cell aggregation were different in MPBD molecules (Kondo *et al.*, 2019; Fig. 3B).

The signalling pathway of MPBD for cell aggregation is unknown, but for spore encapsulation was evaluated and G-protein coupled receptor, CrlA, was reported as its receptor (Anjard *et al.*, 2011). In this case, the authors used *crlA*- mutants with an Ax3 strain background. In order to confirm the signalling pathway of MPBD, the new *crlA*- mutant with the Ax2 background, the same parental strain with that of *stlA*- mutant was created. *CrlA*- mutant with the Ax2 background showed normal cell aggregation, unlike the *stlA*- mutant. This indicates that MPBD activity in the early development of Ax2 does not require CrlA (Narita *et al.*, 2017).

Psi-factor and dictyoquinone

Low molecular weight compounds also regulate the differentiation of prespore cells. Psi-factor is a glycoprotein encoded by *psiA* gene that induces differentiation of prespore cells (Oohata 1995; Nakagawa *et al.*, 1999; Kawata *et al.*, 2004). The other molecule that induces prespore cell differentiation is Polyketide like factor (PLF), also known as Dictyoquinone (DQ). Interestingly, DQ was reported to have D-factor activity, which can induce cell aggregation in *Polysphondylium violaceum* aggregation defect mutant *aggA*. DQ seems to be a putative MPBD metabolite, because it was reported that synthetic MPBD treated with Frémy's salt gave a good yield of DQ (Takaya *et al.*, 2014). Although there is no report of conversion of MPBD to DQ *in vivo*, cell aggregation was restored in *stlA*- cells by addition of DQ but not spore encapsulation. Psi-2 was reported as a prespore cell-inducing factor and it seemed to be a polyketide (Serafimidis and Kay, 2005). Chemical identity of Psi-2 is yet to be elucidated.

Terpene

Terpenes are also structurally diverse secondary metabolites and are involved in ecological interactions, including defence against predators and formation of mutually beneficial alliance with other organisms. Terpenes are hydrocarbons, constructed from the branched five-carbon skeleton of isoprene which are assembled to each other by various ways. The key enzymes for terpene synthesis are terpene synthases (TPS) and are found only in plants and fungi among eukaryotes. Recent findings indicate a wider distribution of TPS genes in social amoeba (Chen *et al.*, 2016). For example, *D. discoideum* has 11 putative TPS genes in its genome, of which 9 are full-length sequences, while *D. purpureum* genome contains 12 functional TPS genes (Chen *et al.*, 2018). Based on the profiling of volatile compounds by solid-phase microextraction followed by gas chromatography-mass spectrometry, no evidence of volatile terpenes was detected at t0 stage of *D. discoideum* development. The production of terpenes gradually increased during development. This suggests that terpenes are indeed involved in *Dictyostelium* development.

In 2019, a novel sesquiterpene alcohol discoidol was identified as a product of *DdTPS8* (Chen *et al.*, 2019). A cytochrome P450 enzyme encoding gene, *CYP521A1*, is located 685 bp away from *DdTPS8* in a head-to-head manner on chromosome 6. It turned out that these two genes form a biosynthetic cluster. CYP521A1 catalysed the oxidative degradation of discoidol and formed discodiene, a novel trisnorsesquiterpene. *DdTPS8*- mutants showed developmental delay from t16 after starvation when the pattern formation of prestalk and prespore occurred. These compounds might, therefore, be involved in the morphogenesis of *Dictyostelium*.

DIF-1 induced cell differentiation

Since its biochemical isolation and identification, DIF-1 signal-

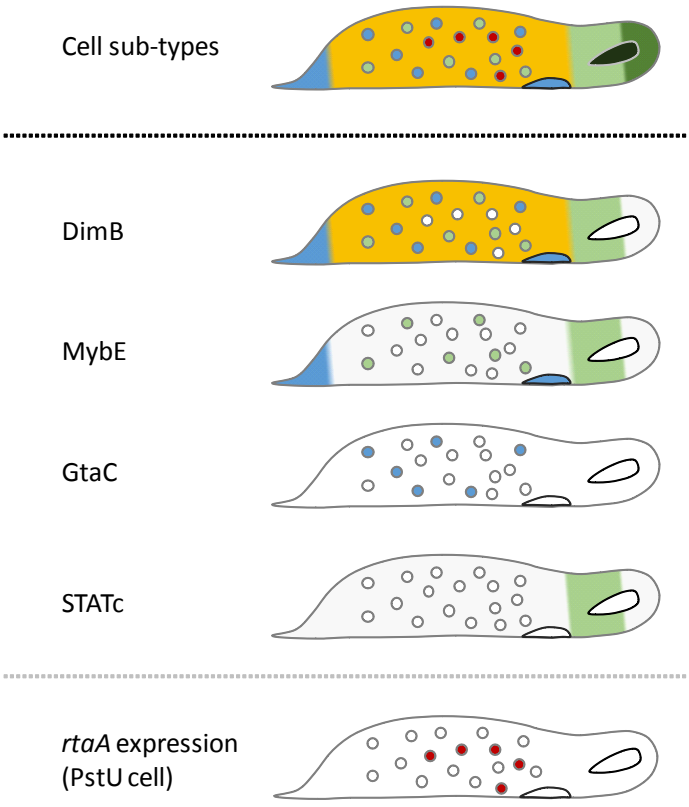

Fig. 4. Complex activation patterns of DIF-1 induced transcriptional activities. *Schematic drawings of cell sub-types at which each DIF-1 inducible transcriptional factor (DimB, MybE, GtaC and STATc) and expression of target gene (*rtaA *gene: PstU cell) are activated. DIF-1 induced activations are observed in multiple cell-types with different combinations throughout migrating slug, except in PstA cells which occupy the anterior half of prestalk region.*

ling pathway have been analysed extensively because of its strong stalk cell inducing activity in *in vitro* monolayer assay. A number of transcriptional factors has been identified and analysed. Fig. 4 shows a schematic representation of the cell-types at where each transcriptional factor is activated by DIF-1.

Two basic leucine zipper (bZIP) family transcriptional factors, DimA and DimB, are involved in DIF-1 signalling pathway (Thompson *et al.*, 2003; Zhukovskaya *et al.*, 2006; Huang *et al.*, 2006). DimB forms heterodimer with DimA and nuclear localises upon the exposure of DIF-1, and *dimA*- and *dimB*- mutants show similar morphological phenotypes to "DIF less" mutants. In the development, DimB first shows nuclear localization at the mound stage and then becomes highly nuclear enriched in the PstB cells of slug which will form the lower cup and the basal disc of fruiting body (Yamada *et al.*, 2011). DimB directly binds to the *ecmB* promoter region in response to DIF-1 signal. In addition, these two bZIP transcriptional factors regulate PstO cell differentiation and directly regulate the repression of prespore specific genes, *pspA* and *cotB* (Nuñez-Corcuera *et al.*, 2012; Huang *et al.*, 2006).

A single myb domain containing SHAQKY family transcriptional factor, MybE, binds to distal region fragment of *ecmA* promoter (ecmO promoter) (Fukuzawa *et al.*, 2006). In *mybE*- mutant, ecmAO expression is tightly restricted in the PstA cells, there are almost no expressions in the regions where is normally occupied by PstO cells and ALCs. *EcmB* gene is expressed in scattered cells (ALCs) throughout the slug but there is no cone of PstAB cells in anterior prestalk region. Thus, MybE protein is essential for ecmO expressing ALCs formation but not for *ecmB* expressing ALCs, supporting the previous observations of the heterologous ALCs population (Jermyn and Williams, 1991; Jermyn *et al.*, 1996).

GtaC gene encodes DIF-1 inducible GATA family transcriptional factor. Not only *gtaC* gene expression is directly regulated by DIF-1, but also GtaC protein accumulate rapidly in response to DIF-1 (Keller and Thompson, 2008). *gtaC*- mutant is a phenocopy of other DIF-1 signalling mutants: longer and often break-up slugs and no basal disc formation in fruiting body. In *gtaC*- mutant slug, an increased number of scattered *ecmB* positive cells (ALCs) exists but clustered *ecmB* expressing cells at the ventral surface of slug (PstB cells) are not observed. It is suggested that this difference in these two *ecmB* expressing population observed in *gtaC*- mutant is due to the impaired cell sorting of PstB cells.

Differential genome-wide microarray analysis of *dimB*- and *mybE*- mutants let us know the depth of DIF-1 signalling pathway (Yamada *et al.*, 2010). *RtaA* gene is identified as one of the DIF-1 inducible genes in *dimB*- or *mybE*- background, meaning that DimB and MybE both negatively control *rtaA* and some other DIF-1-inducible genes. *RtaA* gene is expressed in scattered ALCs at slug stage and preferentially in upper cup at fruiting body. *RtaA*-positive ALCs are distinct from *ecmA*-positive and *ecmB*-postive ALCs. This defines new prestalk cell sub-type, PstU cells.

All these prestalk cell sub-types in slug undergo terminal differentiation to vacuolated and dead stalk cells during fruiting body formation. This process is regulated by starvation induced manifestation of autophagosome and following autophagic cell death (ACD) (Giusti *et al.*, 2009). When developing *Dictyostelium* cells in *in vitro* monolayer condition are exposed to DIF-1, these cells start forming polarized paddle cells, followed by F-actin depolymerization, vacuolization, and cellulose wall formation; resulting in having similar morphological and physiological features to stalk cells in fruiting body. DIF-1 induced ACD depends on the activation of bZIP transcriptional factors, DimA and DimB (Thompson *et al.*, 2003; Zhukovskaya *et al.*, 2006; Huang *et al.*, 2006), and also autophagy-related protein kinase Atg1 (Luciani *et al.*, 2011), endoplasmic reticulum IP_3 gated Ca^2 channel IplA (Lam *et al.*, 2008), and cytoskeletal protein TalB (Giusti *et al.*, 2009).

Taken together, DIF-1 signalling has important roles in PstB, PstO and ALCs differentiation, proportion regulation, and terminal stalk cell differentiation in *Dictyostelium*. As can be seen from the heterogeneity of ALCs and the new finding of PstU cells, the complex network of transcriptional factors and their upstream regulators does exist in DIF-1 induced cell differentiation.

DIF-1 induced signalling pathways

How is DIF-1 signal transduced in the cell? Although DIF-1 receptor has not been identified, DIF-1 induced intracellular calcium increase, cytoskeletal changes has been long discussed (Kubohara and Okamoto, 1994; Schaap *et al.*, 1996; Wurster and Kay, 1990). Recent analysis shows that DIF-1 induces significant protein phosphorylation changes.

Phospho-SILAC (Stable Isotope Labelling by Amino acids in Cell culture) approach was used to perform quantitative analysis

of protein phosphorylation changes in the early response to DIF-1 stimulation (Sugden *et al.*, 2015). The result shows that DIF-1 induces global phosphorylation changes and triggers a major shift toward dephosphorylation. Gene Ontology (GO) analysis revealed that many phosphorylation changes are detected in "Signal transduction cellular response to stimulus", "Cell communications", and "A specific role in GTPase regulator". This analysis provides the evidence that Ca^{2+}/Calmodulin-dependent phosphatase Calcineurin plays a role in DIF-1 signalling to the bZIP transcriptional factor, DimB (Yamada *et al.*, 2013; Sugden *et al.*, 2015).

One of *Dictyostelium* STAT (Signal Transducers and Activators of Transcription) protein is also regulated by DIF-1 induced phosphorylation changes. STAT proteins are well-conserved in multicellular organisms and have major roles as a fast-path signalling in response to extracellular stimuli. STATc protein, one of four STAT proteins encoded in *Dictyostelium* genome, is rapidly activated by tyrosine phosphorylation and nuclear localised upon the stimulation of DIF-1 (Fukuzawa *et al.*, 2001). DIF-1 induces the phosphorylation and inhibition of Protein Tyrosine Phosphatase-3 (PTP3), resulting tyrosine phosphorylation and activation of STATc (Araki *et al.*, 2008). STATc protein is selectively activated in the PstO cells in the slug, and PTP3 overexpressing cells exhibit no STATc nuclear localization in PstO cells and also long and break-up slug phenotype as other "DIF-less" mutants.

DIF-1 induced global de-phosphorylation unveiled new level of DIF-1 signalling network. Function and regulatory mechanism of GTPases have been extensively analysed in the study of *Dictyostelium* chemotaxis (Charest and Firtel, 2007; Kortholt *et al.*, 2013; Nichols *et al.*, 2019). It is also reported that DIF-1 negatively modulates chemotactic cell movement towards cAMP through the regulation of cGMP phosphodiesterase, GbpB (Kuwayama and Kubohara, 2009). Understanding GTPases and other chemotaxis components may give us a new insight of DIF-1 signalling pathways and, possibly, clues to find DIF-1 receptor.

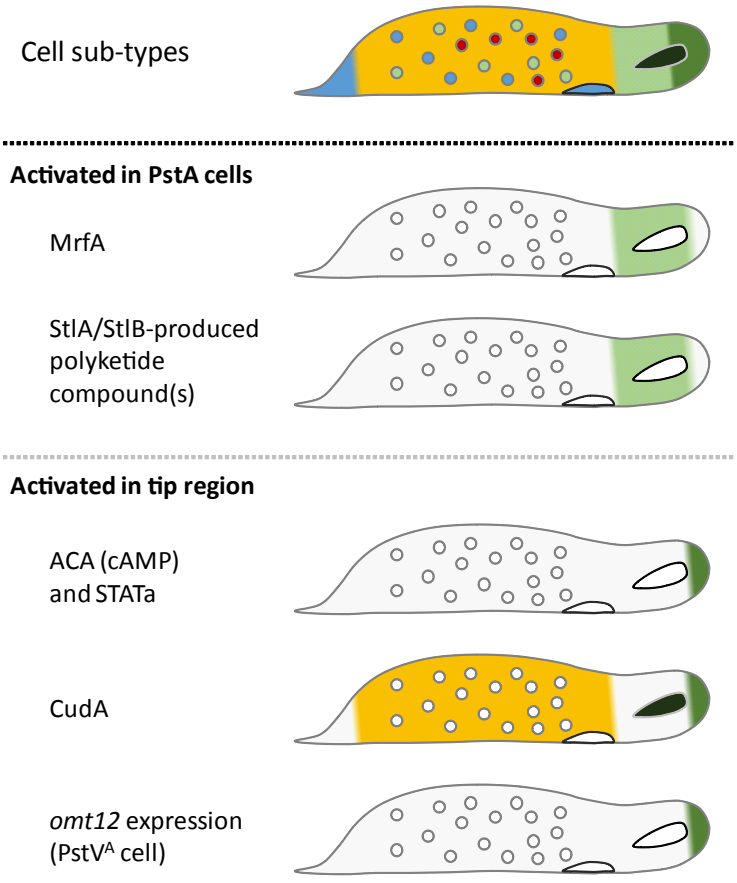

Fig. 5. Activated genes, proteins, and molecules in PstA cells and at the tip region of migrating slug. *Although PstA cell inducer(s) and Tip organizer have not been identified, some genes, proteins and molecules have been proposed to be involved. MrfA and unknown polyketide compound(s) produced by two PKSs, StlA and StlB, have roles in most of PstA cell differentiation, but not at the cells in the very tip region of the slug. In the tip region, it is shown that cAMP, produced by Adenylyl cyclase A (ACA), activates STATa protein and STATa protein induces CudA transcriptional factor. Lineage-primed PstV^A cells (*omt12 *expressing cells) also occupy the tip region.*

DIF-1 independent pathways

As described polyketide DIF-1 has important roles in PstB, PstO and ALCs differentiation, but PstA cell differentiation is not affected by DIF-1 (Thompson and Kay, 2000; Williams, 2006). So, what is the PstA cell inducing factor(s)?

Biochemical purification with PstA-specific (the CA-rich region in cap-site proximal) region of *ecmA* promoter isolated MrfA protein, *Dictyostelium* homologue of animal Myelin-gene Regulatory Factor (MRF)-like proteins (Senoo *et al.*, 2012). MrfA protein contains DNA binding domain with high similarity to Yeast Ndt80 sporulation-specific transcription factor. *MrfA*- mutants show almost no PstA cell differentiation, except at very tip region of the slug. This suggests that PstA cell differentiation is under at least two different regulations: MrfA transcriptional factor-driven mechanism and MrfA-independent tip specific mechanism.

Several lines of evidence suggest that polyketide(s) works on PstA cell differentiation (Sato *et al.*, 2013). By a PKS inhibitor, cerulenin, treatment during development, *Dictyostelium* cells can form fruiting body, although they look very fragile. PstA cell differentiation in resultant slug is largely suppressed and only limited to the very tip region of slug. Same phenotype in PstA cell differentiation is observed in double mutant of two PKS enzymes, StlA and StlB. As mentioned above, StlA and StlB produce MPBD and DIF-1 respectively. Interestingly, feeding of neither DIF-1 nor MPBD rescue this defect, while parental cell (Ax2)-released materials restore the PstA cell differentiation partially. These results strongly suggest that a novel polyketide compound(s) produced by two steely enzymes, StlA and StlB, regulates at least some part of PstA cell differentiation and, possibly, the activation of MrfA transcriptional factor.

Yet, we don't have much information about mechanism of PstA cell differentiation in tip region. It is known that slug tip region is functioning as organizer of various morphogenetic multicellular events. Pulsatile cAMP secretion from tip region organises three-dimensional cell movement in morphogenesis and slug movement and integrity (Weijer, 2004; Singer *et al.*, 2019). Removal of slug tip makes the halt of slug movement and initiates trans-differentiation and pattern re-formation (Raper, 1940; Sternfeld and David, 1981). Grafting slug tip cells to the rear part of other slug induce splitting

and formation of new slugs (Raper, 1940). Identity of tip cells is not yet known, thus so-called "Tip organizer" have been defined only as the cluster of cells at the very tip region of slug (Williams, 2006). It is shown that one of the STAT proteins, STATa, and its target gene product CudA, essential transcriptional factor for the initiation of fruiting body formation, are both activated in this region (Verkerke-Van Wijk *et al*., 2001; Araki *et al*., 1998; Fukuzawa *et al*., 1997; Fukuzawa and Williams, 2000). Recent work suggests that expression of O-methyl transferase-12 gene (*omt12*) shows the lineage-primed cell differentiation and defines a novel prestalk cell sub-type, PstV^{A} at the very tip of PstA cell region (Kuwana *et al*., 2016). The developmentally-specified PstA and the lineage-primed PstV^{A}, how are these two mechanisms regulated? Does PstV^{A} cells work as Tip organizer? Is there any link between STATa activation and PstV^{A} cells? Further detailed studies will be needed. Fig. 5 shows a brief summary of activated genes, proteins, and molecules in PstA cells and at the tip region of migrating slug.

Conclusion

1) The genome data and expression analysis revealed that all PKS and TPS genes are expressed during *Dictyostelium* development. It appears that *Dictyostelium* uses these secondary metabolites as signalling molecules to control (multicellular) development rather than chemical communication with its environment.

2) *In vivo* analysis of DIF-1 shows a glimpse of the depth in its signalling networks at multiple levels. Further investigation of functions and signalling mechanisms of *Dictyotelium* secondary metabolites will give us clear views of simple yet complex *Dictyostelium* development.

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