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 adenyl 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-
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 (Bersk 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. 

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 reaction are polyketide synthases (PKSs). Genome analysis revealed that Dictyostelium 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 (Sugang et al., 2011).

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 are not included in these drawings and are described in main text and in Figures 4 and 5.

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. EcmC 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 Dicyoquinone 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.
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 cytochrome P450 enzyme, CYP521A1, catalyzes oxidative degradation of dicoidol to discodiene, a cytochrome P450 enzyme, CYP521A1, catalyzes oxidative degradation of dicoidol to discodiene, a trinorsesquiterpene.

**TABLE 1**

| Name       | Structure | Dev. Stage | Proposed functions in *Dictyostelium* development |
|------------|-----------|------------|--------------------------------------------------|
| cAMP       | ![Structure of cAMP](image) | Early       | • Chemoattractant (at nM order, pulse) |
| DIF-1      | ![Structure of DIF-1](image) | Mid + Late  | • Prespore cell induction  
|            |           |            | • Prestalk cell competency induction (at mM order, continuous/pulse) |
| MPBD       | ![Structure of MPBD](image)  | Early       | • Regulation of chemotactic response |
|            |           |            | • Spore maturation (encapsulation) |
| Dicoyquinone (DQ) | ![Structure of Dicoyquinone (DQ)](image) | Early + Late | • Regulation of chemotactic response  
|            |           |            | • Prespore cell induction  
|            |           |            | • Cell differentiation competency |

**TABLE 1**

LIST OF SMALL MOLECULES DISCUSSED IN THIS REVIEW

In *Dictyostelium* development, *cAMP* (3',5'-cyclic adenosine monophosphate) works not only as an intracellular second messenger but also as an 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 1-(1',3',5',7'-tetramethyl-6'-hexyl)-1,3,5-tri-O-methyl-1H-indene-2,4-diol. In 2010, 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, DxtPS8, and a cytochrome P450 enzyme, CYP521A1, catalyzes oxidative degradation of dicoidol to discodiene, a trinorsesquiterpene.

Supposed to be catalysed by chloroperoxidase because the enzymatic activity was stimulated by hydrogen peroxide (Kay, 1998).

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 *chiA* gene is a member of glutathione-S-transferase and catalyses the reductive dechlorination of dicoidol to discodiene, a trinorsesquiterpene.
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. discoideum* 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-pentylen-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.
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 dictyquinone**

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 Dictyquinone (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 *DtTPS8* (Chen et al., 2019). A cytochrome P450 enzyme encoding gene, *CYP521A1*, is located 685 bp away from *DtTPS8* 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 trisnorresesquiterpene. *DtTPS8*- 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-
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 which 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 (ecmOP 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-positive 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 IP3 gated Ca2+ 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

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**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.
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"+/- 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.

**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-dimension 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; Sternefeld 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, PstVΔ at the very tip of PstA cell region (Kuwana et al., 2016). The developmentally-specified PstA and the lineage-primed PstVΔ, how are these two mechanisms regulated? Does PstVΔ cells work as Tip organizer? Is there any link between STATA activation and PstVΔ 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 Dictyostelium secondary metabolites will give us clear views of simple yet complex Dictyostelium development.

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