c-di-GMP induction of *Dictyostelium* cell death requires the polyketide DIF-1

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**ABSTRACT** Cell death in the model organism *Dictyostelium*, as studied in monolayers in vitro, can be induced by the polyketide DIF-1 or by the cyclical dinucleotide c-di-GMP. c-di-GMP, a universal bacterial second messenger, can trigger innate immunity in bacterially infected animal cells and is involved in developmental cell death in *Dictyostelium*. We show here that c-di-GMP was not sufficient to induce cell death in *Dictyostelium* cell monolayers. Unexpectedly, it also required the DIF-1 polyketide. The latter could be exogenous, as revealed by a telling synergy between c-di-GMP and DIF-1. The required DIF-1 polyketide could also be endogenous, as shown by the inability of c-di-GMP to induce cell death in *Dictyostelium* HMX44A cells and DH1 cells upon pharmacological or genetic inhibition of DIF-1 biosynthesis. In these cases, c-di-GMP-induced cell death was rescued by complementation with exogenous DIF-1. Taken together, these results demonstrated that c-di-GMP could trigger cell death in *Dictyostelium* only in the presence of the DIF-1 polyketide or its metabolites. This identified another element of control to this cell death and perhaps also to c-di-GMP effects in other situations and organisms.

**INTRODUCTION** A search for phylogenetically conserved elements in cell death mechanisms can benefit from the advantages of the model protist *Dictyostelium discoideum*. *Dictyostelium* belongs to a eukaryote supergroup distinct from but phylogenetically close to that comprising animals. *Dictyostelium* cells multiply vegetatively in rich medium and aggregate upon starvation. Within 24 h, this aggregate morphogenizes into a 1- to 2-mm-high fruiting body, which includes a stalk made of vacuolized, cellulose-walled dead cells. This *Dictyostelium* developmental cell death can be mimicked in vitro in monolayer conditions (Kay, 1987; Cornillon et al., 1994; Giusti et al., 2009). This death is neither apoptotic nor necrotic but vacuolar. Its induction requires two signals. The first signal, starvation, sensitizes the cells and also triggers autophagy. Autophagy protects cells from damage due to starvation and thus allows second signal-induced cell death (Luciani et al., 2011). Only cells that have undergone starvation can be induced to die by a second signal, classically the polyketide DIF-1 (Kay, 1987).

Recently not only DIF-1, but also the cyclic dinucleotide c-di-GMP was shown to induce cell death in *Dictyostelium* cell monolayers (Chen and Schaap, 2012) and to be required for the development of *Dictyostelium* (Chen and Schaap, 2012). c-di-GMP, a universal bacterial second messenger (Romling et al., 2013), has recently been shown to affect also animal cells, where it can trigger innate immunity upon bacterial infection (Danilchanka and Mekalanos, 2013) and regulate β-adrenergic stimulation (Lolicato et al., 2014). Studying c-di-GMP-induced *Dictyostelium* cell death may provide both an additional handle on mechanisms at play in this cell death and also more general information on how c-di-GMP acts in a eukaryotic cell. We unexpectedly found that c-di-GMP was not sufficient by itself to induce cell death in *Dictyostelium* cell monolayers. This induction of cell death by c-di-GMP required the synthesis of the polyketide DIF-1 or its metabolites.

**RESULTS** Exogenous DIF-1 and c-di-GMP trigger distinct pathways to cell death

Induction in vitro by DIF-1 or by c-di-GMP led to cell death with similar subcellular lesions, such as vacuolization and synthesis of...
This article, exogenous means experimentally added to the cells under test, and endogenous means produced by (some of) the cells under test and acting on the same or other cells. Similarly, cyclosporin A inhibited vacuolization and cell death induced by DIF-1 (Lam et al., 2008) but not by c-di-GMP (Supplemental Figure S1). In addition, as in other Dictyostelium strains (Huang et al., 2006; Zhukovskaya et al., 2006), in DH1 cells, the bZip transcription factor DimB translocated from the cytosol to the nucleus in a matter of minutes after addition of DIF-1 (with or without c-di-GMP; Figure 1C) but not after addition of c-di-GMP (Figure 1C). Taken together,
these differences indicated that at least parts of the pathways to cell death induced by exogenous DIF-1 and exogenous c-di-GMP were distinct.

**Exogenous DIF-1 and c-di-GMP synergize to induce cell death**

Unexpectedly, cells subjected to both exogenous DIF-1 and exogenous c-di-GMP together showed markedly more (Figure 1A, top, and Supplemental Figure S2A) and earlier (Supplemental Figure S3) vacuolization than cells subjected to either alone. Further, the synergy between DIF-1 and c-di-GMP occurred not only in parental DH1 cells, but also in the talinB and DhkMins mutant cells that did not vacuolate and did not die in the presence of DIF-1 alone (Figure 1A). Thus mutations interrupting the pathway used by DIF-1 alone (hereafter called the autonomous DIF-1 pathway) did not interrupt the DIF-1 pathway to synergistic vacuolization, indicating that these pathways were distinct.

Further, in iplA mutant cells, there was no more vacuolization upon addition of both DIF-1 and c-di-GMP than upon addition of c-di-GMP only (Figure 1A). Thus the IP3R was required, not only for the autonomous DIF-1 pathway to cell death (Lam et al., 2008), but also for the synergistic DIF-1– plus c-di-GMP–induced cell death, suggesting that the positive interaction between exogenous DIF-1– and c-di-GMP–induced pathways included an IP3R-dependent step.

When cell death induced by DIF-1 and c-di-GMP at low concentrations was tested by regrowth, synergy seemed much less than when tested by vacuolization (Figure 1B and Supplemental Figure S2B). Vacuolization was checked usually at 22–24 h postinduction, whereas “regrowth,” namely the number of cells present, was checked after a further 3 d of incubation in HLS. In these experiments, vacuolization leading to fewer regrowing cells could proceed even after addition of HLS (unpublished data). Thus the delay in vacuolization observed at 22 h for single compared with double induction could be compensated during the 3 d required for a regrowth test. In other words, single-induced vacuolization could catch up with double-induced vacuolization during the 22-h to 3-d incubation in HLS, leading to a lesser difference between single and double induction in terms of regrown cells at 3 d. As a corollary, vacuolization is a sign of cell death kinetically more accurate than non-regrowth. It will be often taken hereafter as equivalent to cell death, confirmed or not by regrowth tests.

Taking the results together, synergy increased the speed rather than the final extent of vacuolization and death. Most important, it indicated a positive interaction between pathways to cell death induced by exogenous DIF-1 and by c-di-GMP. Could this interaction between the effects of exogenous inducers reflect a required endogenous cooperation?

**HMX44A cells that produce little or no DIF-1 do not vacuolate or die upon addition of c-di-GMP**

We first used Dictyostelium HMX44A cells, which are known to make very little DIF-1 but to remain responsive to exogenous DIF-1 (Kopachik et al., 1983). Accordingly, these cells vacuolized well with exogenous DIF-1 (Figure 2A). In contrast, addition of exogenous c-di-GMP led to small, roundish, contrasted cells with almost no vacuolization (Figure 2A), no cellulose encasings (unpublished data), and no death when tested by regrowth (Figure 2B, right). In addition, in these HMX44A cells, where exogenous c-di-GMP alone did not induce cell death, there was synergy between exogenous DIF-1 and exogenous c-di-GMP (Figure 2C). Taken together, the results indicate that in HMX44A cells relative to DH1 cells, an unknown mutation, or mutations, prevented vacuolar cell death induced by c-di-GMP. Again, irrespective of their other possible defects, HMX44A cells produced no or little DIF-1. These results were in line with the possibility of a requirement of DIF-1 for c-di-GMP induction of cell death.

**Exogenous c-di-GMP requires polyketides to induce cell death**

To check in DH1 cells (for consistency with the aforementioned mutants) whether DIF-1 or other polyketides were required for c-di-GMP–induced cell death, we first used cerulenin, known to inhibit the biosynthesis of polyketides, including that of DIF-1 (Kay, 1998; Serafimidis and Kay, 2005). Cerulenin, as expected, did not impair vacuolization induced by exogenous DIF-1 (and thus did not impair vacuolization as such), but, remarkably, almost completely prevented induction of vacuolization by exogenous c-di-GMP (Figure 3A). This indicated that one or several cerulenin-inhibitable moieties were required together with c-di-GMP for induction of cell death. Cerulenin inhibits the β-keto-acyl domain of polyketide synthases, including in Dictyostelium not only the Stb polyketide synthase (Austin et al., 2006; Saito et al., 2008) required for the biosynthesis of the THPH precursor of DIF-1, but also the StApolyketide synthase, plus close to 40 other polyketide synthases, including two fatty acid synthases (Chance et al., 1976; Omura, 1976; Kridel et al., 2007; Zucko et al., 2007; Narita et al., 2014). What is the polyketide synthase catalyzing the synthesis of molecules required for c-di-GMP–induced cell death?

**DIF-1 is the main endogenous stb-dependent polyketide cooperating with c-di-GMP for induction of cell death**

Which stb-dependent polyketide(s) cooperate(s) with c-di-GMP? Within the stb-dependent biosynthetic cascade (Supplemental Figure S4), the DmtA methyltransferase catalyzed the last step to DIF-1 biosynthesis. We disrupted the DmtA gene in DH1 cells (Supplemental Figure S5), which was previously disrupted in strain AX2 (Saito et al., 2008). This stb mutation almost completely prevented c-di-GMP–induced vacuolization (Figure 3B) and cell death (unpublished data), thus accounting at least in part for the cerulenin results and showing that the stb-initiated biosynthetic cascade included at least one polyketide required for the major part of cell death induction by c-di-GMP.
DISCUSSION

Following the reports that the polyketide DIF-1 (Morris et al., 1987) or the cyclic-dinucleotide c-di-GMP (Chen and Schaap, 2012) in-duced Dictyostelium cell death in vitro, we demonstrated here that induction of cell death in vitro by c-di-GMP requires DIF-1, as sche-matized in Figure 5B. We first unexpectedly observed that exogenous DIF-1 and c-di-GMP acted in synergy, suggesting in particular that endogenous amounts were limiting. Through mutations of the autonomous DIF-1 pathway, we then disentangled a cooperative from an autonomous DIF-1 pathway. We then showed through the use of cerulenin that c-di-GMP induction of cell death requires the synthesis of polyketides. Through the use of stlB mutants, we showed that at least one of these polyketides was made in a stlB-dependent biosynthetic cascade and, through the use of DmtA mutants, that this polyketide was DIF-1 and/or its metabolites.

Together, present and previous results suggested the existence of several partially distinct DIF-1 pathways. An exogenous DIF-1-in-duced pathway autonomously led to vacuolar cell death and could be inhibited by the talinB, iplA, and DhkM mutations (Lam et al., 2008; Giusti et al., 2009, 2010). Another pathway required for c-di-GMP-induced cell death was the endogenous biosynthesis of DIF-1.
If DIF-1 were required, then DIF-1 biosynthesis mutants should not allow cell death induction in vivo—but they do (Thompson and Kay, 2000; Saito et al., 2008), except for basal disk cells. Thus DIF-1 cannot be the only cell death inducer in vivo. Is c-di-GMP this main cell death inducer? An argument in favor of this is the developmental phenotype of DgcA-null mutants (Chen and Schaap, 2012). These mutants, incapable of c-di-GMP biosynthesis, did not develop past the slug stage. They showed normal formation of slugs that could not form fruiting bodies but continued migration (Chen and Schaap, 2012). Moreover, exogenous c-di-GMP restored fruiting body formation (Chen and Schaap, 2012). This is consistent with a role of c-di-GMP for stalk cell formation but may also be interpreted as evidence for another role of c-di-GMP between slug migration and initiation of fruiting body formation. Experiments testing the expression of stalk-specific molecules in wild type, DgcA-null mutants, and DgcA-null mutants plus exogenous c-di-GMP might provide evidence either way.

An argument that at first sight is not in favor of a role for c-di-GMP in induction of cell death in vivo is the required cooperation with DIF-1 for c-di-GMP induction of cell death in vitro, as shown here. If DIF-1 were required, then DIF-1 biosynthesis mutants should not allow cell death induction in vivo—but they do (Thompson and Kay, 2000; Saito et al., 2008), except for basal disk cells. Thus DIF-1 cannot be the only cell death inducer in vivo. Is c-di-GMP this main cell death inducer? An argument in favor of this is the developmental phenotype of DgcA-null mutants (Chen and Schaap, 2012). These mutants, incapable of c-di-GMP biosynthesis, did not develop past the slug stage. They showed normal formation of slugs that could not form fruiting bodies but continued migration (Chen and Schaap, 2012). Moreover, exogenous c-di-GMP restored fruiting body formation (Chen and Schaap, 2012). This is consistent with a role of c-di-GMP for stalk cell formation but may also be interpreted as evidence for another role of c-di-GMP between slug migration and initiation of fruiting body formation. Experiments testing the expression of stalk-specific molecules in wild type, DgcA-null mutants, and DgcA-null mutants plus exogenous c-di-GMP might provide evidence either way.

In contrast, but still in vitro, c-di-GMP induced no detectable autonomous pathway to vacuolar cell death. Exogenous c-di-GMP induced cell death only when DIF-1 was exogenously or endogenously available (Figure 5). No mutation is known to inhibit this c-di-GMP–induced pathway, except those impairing DIF-1 synthesis. Of note, endogenously synthesized DIF-1, which is required and is in sufficient amount for c-di-GMP–induced cell death, is not sufficient to induce vacuolar or necrotic cell death autonomously, suggesting that quantitative and/or perhaps topological parameters are at play. Also in contrast to DIF-1, c-di-GMP did not induce necrotic cell death in atg1−mutant cells (unpublished data).

These results have implications for requirements for cell death in vivo, which is part of stalk cell differentiation within Dictyostelium multicellular development. Two candidate moieties could mediate induction of this cell death in vivo, namely the polyketide DIF-1 (Kay, 1987) and the cyclic dinucleotide c-di-GMP (Chen and Schaap, 2012). If DIF-1 were required, then DIF-1 biosynthesis mutants should not allow cell death induction in vivo—but they do (Thompson and Kay, 2000; Saito et al., 2008), except for basal disk cells. Thus DIF-1 cannot be the only cell death inducer in vivo. Is c-di-GMP this main cell death inducer? An argument in favor of this is the developmental phenotype of DgcA-null mutants (Chen and Schaap, 2012). These mutants, incapable of c-di-GMP biosynthesis, did not develop past the slug stage. They showed normal formation of slugs that could not form fruiting bodies but continued migration (Chen and Schaap, 2012). Moreover, exogenous c-di-GMP restored fruiting body formation (Chen and Schaap, 2012). This is consistent with a role of c-di-GMP for stalk cell formation but may also be interpreted as evidence for another role of c-di-GMP between slug migration and initiation of fruiting body formation. Experiments testing the expression of stalk-specific molecules in wild type, DgcA-null mutants, and DgcA-null mutants plus exogenous c-di-GMP might provide evidence either way.

An argument that at first sight is not in favor of a role for c-di-GMP in induction of cell death in vivo is the required cooperation with DIF-1 for c-di-GMP induction of cell death in vitro, as shown here. If c-di-GMP is the main cell death inducer in vivo, and if this requires DIF-1 cooperation, then mutants impairing DIF-1 biosynthesis should prevent most of cell death in vivo, but, again, they do not (Thompson and Kay, 2000; Saito et al., 2008). How is one to account for the persistence of cell death induction in vivo when there is no DIF-1 to cooperate with c-di-GMP, in contrast with the absence of cell death induction in vitro in the same circumstances? DIF-1 cooperation may be redundant in vivo. For instance, considering the considerable number of polyketide synthases encoded by the Dictyostelium genome (Eichinger et al., 2005; and its metabolites. This biosynthesis required the expression and activation of, in particular, the StlB and DmtA enzymes. Perhaps through cAMP-requiring activation of the transcription factor GbfA (Schnitzler et al., 1995; Gollop and Kimmel, 1997; Giusti et al., 2009), expression and activation of DmtA were induced by cAMP (Kay and Thompson, 2001), catalyzing the synthesis of DIF-1 and thus “sensitizing” the cells to subsequently added exogenous c-di-GMP. Under our experimental conditions, c-di-GMP induction of cell death required preincubation with cAMP (unpublished data). Yet another DIF-1–induced pathway, not sensitive to any of the mutations listed here, led to paddle cells (Levraud et al., 2003; Giusti et al., 2009), and a fourth pathway could lead in atg1−mutant cells to necrotic cell death (Kosta et al., 2004) due to particular DIF-1 functional groups (Luciani et al., 2009).
also affect cell death in vivo. More generally, our results have implications for the relationships (phylogenetic, mechanistic) between the induction by c-di-GMP of Dictyostelium developmental cell death and the induction by c-di-GMP of innate immunity and other effects in animal cells. In addition, c-di-GMP has been incriminated in animal cell death (Karakol et al., 2005; Chandra et al., 2014), and c-di-GMP can activate the inflammasome NLRP3 (Abdul-Sater et al., 2013), which can lead to cell death (Rillingham et al., 2007). The implication of c-di-GMP in Dictyostelium cell death makes it tempting to investigate further its possible role in the death of cells other than those of Dictyostelium. A requirement for polyketides may be worth investigating in these and other c-di-GMP effects in animal cells.

MATERIALS AND METHODS
Handling of Dictyostelium cells, induction of development, and general molecular biology techniques
These were as described previously (Giusti et al., 2008, 2009; Lam et al., 2008).

Induction and assessment of cell death as vacuolization
On the day before the experiment, cells were adjusted at 3 × 10^4 cells/10 ml of HL5 medium/Falcon T25 flask. On the day of experiment, these exponentially growing cells were washed once in phosphate-buffered saline (Sörensen buffer [SB]) and incubated in SB containing 3 mM cAMP (Sigma-Aldrich, St. Louis, MO) for 8 h at 22°C in Lab-Tek culture chambers (Nalge Nunc International, Rochester, NY) at a concentration of 3 × 10^5 cells/ml/chamber. Cells were then washed in SB and incubated at 22°C in either SB alone or SB containing the differentiation factor DIF-1 (DN1000; Affiniti Research Products, Exeter, United Kingdom) at a final concentration of 10⁻⁷ M, c-di-GMP sodium salt (C 057-01; Biolog, Bremen, Germany) at a final concentration of 10⁻⁵ M, or combinations thereof or at other concentrations as indicated. After the indicated period of incubation, cells in the Lab-Tek chambers were examined using an Axiovert 200 microscope (phase contrast, oil immersion, 100x; Carl Zeiss, Jena, Germany) and photographed using an AxioCam MRC camera controlled by AxioVision 4.7 (Carl Zeiss). Images were subsequently homogenously treated with Graphic Converter. Figures were assembled using Illustrator (Adobe Systems, San Jose, CA).

Assessment of cell death as non-regrowth
Wild-type or mutant cells were incubated in Lab-Tek chambers as described. After a variable incubation period at 22°C, to initiate regrowth, 0.5 ml of SB was removed from and 1 ml of HL-5 was added to each Lab-Tek chamber. After 48–72 h of additional incubation at 22°C, vegetative cells resulting from regrowth were counted in a hemocytometer, and the results were graphed using Prism 6 (GraphPad, La Jolla, CA).

Zucko et al., 2007), some of the corresponding non-stIB-dependent polyketides might cooperate with c-di-GMP in vivo at various stages and sites of development, such as the polyketide MPDB, which, of interest, is the product of the stIB polyketide synthase (Anjard et al., 2011). More generally, compared with cells in vitro, cells in slugs may be exposed to different cell contacts, be at a different developmental stages, or be subjected to other signals. This may allow a major role of c-di-GMP for cell death induction in vivo even in the absence of DIF-1 cooperation. Alternatively, it cannot be excluded that most cell death/vacuolization in vivo is governed neither by DIF-1 nor by c-di-GMP. There might be other, as-yet-unidentified inducers of Dictyostelium cell death. Multiple inducers of the same cell death mechanism have been described in other models—for instance, in apoptotic cell death of animal cells.

Taken together, our results contribute to the clarification of induction of Dictyostelium cell death in vitro and of the DIF-1/c-di-GMP relationship in this case. It identifies some constraints on Dictyostelium cell death in vivo. It also suggests modifications of our mutational approach to find molecules involved in cell death in vitro, since the DIF-1 and the c-di-GMP pathways should converge at some point, downstream of which mutations should block both pathways. Such mutations could be selected by inducing cell death by both DIF-1 and c-di-GMP simultaneously. Would such mutations (155380; Nalge Nunc International, Rochester, NY) at a concentration of 3 × 10^6 cells/ml/chamber. After a variable incubation period at 22°C, to initiate regrowth, 0.5 ml of SB was removed from and 1 ml of HL-5 was added to each Lab-Tek chamber. After 48–72 h of additional incubation at 22°C, vegetative cells resulting from regrowth were counted in a hemocytometer, and the results were graphed using Prism 6 (GraphPad, La Jolla, CA).

FIGURE 4: Mutation of the DmtA methylase prevented vacuolization by exogenous c-di-GMP. c-di-GMP-induced vacuolization was prevented by a DmtA mutation, which did not affect vacuolization induced by exogenous DIF-1 or by DIF-1 and c-di-GMP. Numbers are percentages of vacuolization as in the legend to Figure 1A. These results showed that DmtA-dependent polyketides, namely DIF-1 and/or its metabolites, were required for c-di-GMP-induced cell death. The differences in the extent of inhibition of vacuolization between DmtA⁻ cells in this figure and StlB⁻ cells in Figure 3 likely reflect the differences in the kinetics/extent of vacuolization of control DH1 parental cells between these experiments.

Wild-type or mutant cells were incubated in Lab-Tek chambers as described. After a variable incubation period at 22°C, to initiate regrowth, 0.5 ml of SB was removed from and 1 ml of HL-5 was added to each Lab-Tek chamber. After 48–72 h of additional incubation at 22°C, vegetative cells resulting from regrowth were counted in a hemocytometer, and the results were graphed using Prism 6 (GraphPad, La Jolla, CA).
GTTTGTTC), yielding a 0.35-kb product only in the wild type, and pUCf (ACGCAATTAATGTGAGTTAGCTCACTC) and DgcA-1549-AS (CAACAACATTTATTTGACTAATTCCTTTTTTC), yielding a 0.87-kb product only upon DgcA mutation.

Targeted mutagenesis of stlB

To create a stlB gene insertion construct, a stlB DNA fragment was amplified by PCR from genomic DNA nucleotide (nt) 6458 to nt 8295, using primer pairs stlB 6458/8295 bearing HhaI restriction sites, and then cloned into pGEMT-easy vector. After plasmid linearization by BsaBI (at nt 7416), a blasticidin resistance cassette removed from plasmid pLBLP was inserted into this fragment. The insertion plasmid was confirmed by sequencing and then digested with HhaI. The fragment was purified from agarose gel and introduced into DH1 cells by electroporation. Cells were distributed into 96-well plates immediately (7 × 10⁴ cells/well). After selection with blasticidin at 10 μg/ml and recloning, transformed clones were screened by PCR using primer pairs stlB-6370/Bsr5 and stlB-6370/Bsr8922. These primers should yield 1.2- and 4.1-kb products, respectively, in an insertion mutant, whereas in wt cells they should yield no product and a 2.5-kb product, respectively. Primer sequences were stlB-6458/HhaI, GCGCATCATCGTGGTTTTCG; stlB-8295/HhaI, GCGCATACCGCTGTGTGTTCATTTTGAC; and stlB-8922, GCGCAGCCAGGATGAGTAACAAAG; and Bsr5, Bsr cassette, CGCCAACCGGTTTGTGTTT.

Preparation of DH1.GFP-DimB cells

Twenty million DH1 cells were electroporated (3 μF; 1kV) with 10 μg of a GFP-DimB.BlastR vector expressing GFP-DimB controlled by the DimB promoter, kindly provided by Jeff Williams (University of Dundee, Dundee, United Kingdom), in 10 mM NaPO₄, pH 6.1, and 50 mM sucrose in Volvic water and then cultivated in HL5 medium at 22°C for 24 h. Bacitecin (10 μg/ml) was then directly added on cells. After 8 d of bacitracin selection, cells were starved for 4 h, and the "greenest" cells (0.1% of the total population) were cloned by distributing (FACSVantage; BD Biosciences, San Jose, CA) 1 cell/well in 96-well plates containing HL5 medium. Clones obtained after 7 d at 22°C were transferred to LabTek chambers, grown for 24 h, and then starved for 4 h in SB buffer plus 3 mM cAMP. Cells were washed, treated with 100 nM DIF-1 or 10⁻⁵ M c-di-GMP for 5–10 min, and screened for nuclear translocation.

Targeted mutagenesis of DgcA

The gene disruption vector pDgcA (Chen and Schaap, 2012) obtained from the Dicty Stock Center (www.dictybase.org/) was BamHI linearized and then electroporated in DH1 cells. These were selected by lacitacin at 5 μg/ml and cloned by limiting dilution in wells of microtiter plates. Screening for clones with a deletion in the DgcA gene through plasmid integration by homologous recombination was by PCR. Primers were dgcKO1rAS (GTAAATACCATCAATCCAGTTTTGAC) and dgcKO2f-AS (ATATTTATCAGATGTTGTTGTTTGTTT).

FIGURE 5: Recapitulation of some of the results on DIF-1 and c-di-GMP pathways inducing Dictyostelium cell death in monolayers. (A) TalinB− mutant cells were incubated or not with 50 μM cerulenin and with or without 100 nM DIF-1 and 10 μM c-di-GMP for 40 h. See the text for comments on the results. (B) Tentative representation of DIF-1 and c-di-GMP pathways inducing Dictyostelium cell death in vitro, with some of the mutations and drugs mentioned in this work. Bottom, a first signal—starvation plus cAMP—triggers autophagy and sensitizes cells to the second signal. Top, second signals would operate only on cells sensitized by the first signal. Exogenous DIF-1 triggers an autonomous pathway to cell death marked by several mutations. To induce cell death, exogenous c-di-GMP requires cooperation with endogenously synthesized (or exogenous, not represented) DIF-1. Middle, DIF-1 biosynthetic pathway, marked by other mutations, induced by cAMP, provides endogenous DIF-1 required for cooperation with exogenous c-di-GMP to induce cell death. We have not represented the iplA− mutation, which could inhibit both the exogenous autonomous DIF-1 pathway and exogenous DIF-1 cooperation.

Targeted mutagenesis of stlB

To create a stlB gene insertion construct, a stlB DNA fragment was amplified by PCR from genomic DNA nucleotide (nt) 6458 to nt 8295, using primer pairs stlB 6458/8295 bearing HhaI restriction sites, and then cloned into pGEMT-easy vector. After plasmid linearization by BsaBI (at nt 7416), a bacitracin resistance cassette removed from plasmid pLBLP was inserted into this fragment. The insertion plasmid was confirmed by sequencing and then digested with HhaI. The fragment was purified from agarose gel and introduced into DH1 cells by electroporation. Cells were distributed into 96-well plates immediately (7 × 10⁴ cells/well). After selection with bacitracin at 10 μg/ml and recloning, transformed clones were screened by PCR using primer pairs stlB-6370/Bsr5 and stlB-6370/Bsr8922. These primers should yield 1.2- and 4.1-kb products, respectively, in an insertion mutant, whereas in wt cells they should yield no product and a 2.5-kb product, respectively. Primer sequences were stlB-6458/HhaI, GCGCATATCCACAGTTGTTTTCG; stlB-8295/HhaI, GCGCATACCGCTGTGTGTTCATTTTGAC; and stlB-8922, GCGCAGCCAGGATGAGTAACAAAG; and Bsr5, Bsr cassette, CGCCAACCGGTTTGTGTTT.

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Targeted mutagenesis of DmtA
To create a DmtA gene insertion construct, a DmtA DNA fragment was amplified by PCR from genomic DNA nts 540–1168, using primer pairs DmtA A/AS bearing Hhal restriction sites, and then cloned into pGEMT-easy vector. After plasmid linearization by BsaBI (at nt 555), a blasticidin resistance cassette removed from plasmid pLBLP was inserted into fragment. The insertion plasmid was confirmed by sequencing and then digested with Hhal. The fragment was purified from agarose gel and named DmtAins construct. This construct was introduced into DH1 cells by electroporation. Cells were distributed into 96-well plates immediately (7 x 10^4 cells/well). After selection with blasticidin at 10 µg/ml and recloning, transformed clones were screened by PCR using primer pairs Bsr5/ DmtA1958 and DmtA323/DmtA1958. These primers should yield 1.6- and 3.2-kb products in an insertion mutant, respectively, whereas in wt cells they should yield no product and a 1.6-kb product, respectively. Primer sequences were DmtA-S, GCGCAGATGG-TACCAAAGTTGTTGCA (Hhal); DmtA-AS, GCGCAGACATCTTT-TACTATCTGGAGG (Hhal); DmtA-323, TGATGATCGGGTTAAAA-CAACTGTAA; DmtA-1958, TCAATTGTGCCAATCACTAAGGT; and Bsr5, GCGGAAAACGATTITTTTTTTAAAC.

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REFERENCES
Abdul-Sater AA, Tattoli I, Jin L, Grajkowski A, Levi A, Koller BH, Allen TC, Beaussale SL, Fitzgerald KA, Ting JP, et al. (2013). Cyclic-di-GMP and cyclic-di-AMP activate the NLRP3 inflammasome. EMBO Rep 14, 900–906.
Anjard C, Su Y, Loomis WF (2011). The polyketide MPBD initiates the SDF-1 signaling cascade that coordinates terminal differentiation in Dictyostelium. Eurkaryot Cell 10, 956–963.
Austen MB, Saito T, Bowman ME, Haydock S, Kato A, Moore BS, Kay RR, Noel JP (2006). Biosynthesis of Dictyostelium discoideum differentiation-inducing factor by a hybrid type I fatty acid-type III polyketide synthase. Nat Chem Biol 2, 494–502.
Chance K, Hemmingsen S, Weeks G (1976). Effect of cerulenin on the growth and differentiation of Dictyostelium. J Bacteriol 128, 21–27.
Chandra D, Quispe-Tintaya W, Jahangir A, Asafu-Adjei D, Ramos I, Sintim H, Willingham SB, Bergstralh DT, O'Connor W, Morrison AC, Taxman DJ, Traynor D, Kay RR (1991). The DIF-1 signaling system in Dictyostelium—new directions for oncology. Expert Opin Investig Drugs 16, 1817–1829.
Lam D, Kosta A, Luciani MF, Golstein P (2008). The IP3 receptor is required to signal autophagic cell death. Mol Cell Biol 19, 691–700.
Levrard J-P, Adam M, Luciani M-F; De Chastellerie C, Blanton RL, Golstein P (2003). Dictyostelium cell death: early emergence and demise of highly polarized paddle cells. J Cell Biol 160, 1105–1114.
Lolico A, Bucci A, Arrigonì C, Zucca S, Nardini M, Schroeder I, Simmons K, Aquila M, DiFrancesco D, Bolognesi M, et al. (2014). Cyclic dinucleotides bind the C-linker of HCN9 to control channel cAMP responsiveness. Nat Chem Biol 10, 457–462.
Luciani MF; Giusti C, Harms B, Oshima Y, Kubohara Y, Golstein P (2011). Atg1 allows second-signalized autophagic cell death in Dictyostelium. Autophagy 7, 501–508.
Reference text
Morris HR, Taylor GW, Masento MS, Jermyn KA, Kay RR (1987). Chemical structure of the morphogen differentiation inducing factor from Dictyostelium discoideum. Nature 328, 811–814.
Narita TB, Kikukawa TW, Sato YG, Miyazaki SH, Morita N, Saito T (2014). Role of fatty acid synthase in the development of Dictyostelium discoideum. J Oleo Sci 63, 281–289.
Omura S (1976). The antibiotic cerulenin, a novel tool for biochemistry as an inhibitor of fatty acid synthesis. Bacteriol Rev 40, 681–697.
Romling U, Galperin MY, Gomelsky M (2013). Cyclic di-GMP: the first 25 years of a universal bacterial second messenger. Microbiol Mol Biol Rev 77, 1–52.
Saito T, Kato A, Kay RR (2008). DIF-1 induces the basal disc of the Dictyostelium fruiting body. Dev Biol 317, 444–453.
Schnitzer GR, Briscoe C, Brown JM, Firtel RA (1995). Serpentine cAMP receptors may act through a G protein-independent pathway to induce postaggregative development in Dictyostelium. Cell 81, 737–745.
Serafimidis I, Kay RR (2005). New prestalk and prespore inducing signals in Dictyostelium. Dev Biol 282, 432–441.
Thompson CRL, Kay RR (2000). The role of DIF-1 signaling in Dictyostelium development. Mol Cell 6, 1509–1514.
Traynor D, Kay RR (1991). The DIF-1 signaling system in Dictyostelium—metabolism of the signal. J Biol Chem 266, 5291–5297.
Willingham SB, Bergstralh DT, O'Connor W, Morrison AC, Taxman DJ, Duncan JA, Barnoy S, Venkatesan MM, Flavell RA, Deshmukh M, et al. (2007). Microbial pathogen-induced necrotic cell death mediated by the inflammasome components CIAS1/cryopyrin/NLRP3 and ASC. Cell Host Microbe 2, 147–159.
Zhukovskaya NV, Fukuzawa M, Yamada Y, Araki T, Williams JG (2006). The role of fatty acid synthase in the development of Dictyostelium discoideum. J Oleo Sci 63, 281–289.
Reference text
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