Research article

*Gdt*2 regulates the transition of *Dictyostelium* cells from growth to differentiation

Margarita V Chibalina*[1,2], Christophe Anjard[3] and Robert H Insall[1]

Address: [1]School of Biosciences, University of Birmingham, Edgbaston, Birmingham B15 2TT, UK, [2]Cambridge Institute for Medical Research, University of Cambridge, Wellcome Trust/MRC Building, Addenbrooke's Hospital, Hills Road, Cambridge CB2 2XY, UK and [3]Department of Biology, University of California San Diego, 9500 Gilman Drive, La Jolla CA 92 093-0368, USA

Email: Margarita V Chibalina* - mvt24@cam.ac.uk; Christophe Anjard - canjard@biomail.ucsd.edu; Robert H Insall - R.H.Insall@bham.ac.uk

* Corresponding author

Abstract

Background: *Dictyostelium* life cycle consists of two distinct phases – growth and development. The control of growth-differentiation transition in *Dictyostelium* is not completely understood, and only few genes involved in this process are known.

Results: We have isolated a REMI (restriction enzyme-mediated integration) mutant, which prematurely initiates multicellular development. When grown on a bacterial lawn, these cells aggregate before the bacteria are completely cleared. In bacterial suspension, mutant cells express the developmental marker discoidin Iγ even at low cell densities and high concentrations of bacteria. In the absence of nutrients, mutant cells aggregate more rapidly than wild type, but the rest of development is unaffected and normal fruiting bodies are formed. The disrupted gene shows substantial homology to the recently described *gdt*1 gene, and therefore was named *gdt*2. While GDT1 and GDT2 are similar in many ways, there are intriguing differences. GDT2 contains a well conserved protein kinase domain, unlike GDT1, whose kinase domain is probably non-functional. The *gdt*2 and *gdt*1 mRNAs are regulated differently, with *gdt*2 but not *gdt*1 expressed throughout development. The phenotypes of *gdt*2⁻ and *gdt*1⁻ mutants are related but not identical. While both initiate development early, *gdt*2⁻ cells grow at a normal rate, unlike *gdt*1⁻ mutants. Protein kinase A levels and activity are essentially normal in growing *gdt*2⁻ mutants, implying that GDT2 regulates a pathway that acts separately from PKA. *Gdt*1 and *gdt*2 are the first identified members of a family containing at least eight closely related genes.

Conclusions: We have isolated and characterised a new gene, *gdt*2, which acts to restrain development until conditions are appropriate. We also described a family of related genes in the *Dictyostelium* genome. We hypothesise that different family members might control similar cellular processes, but respond to different environmental cues.

Background

Dictyostelium discoideum is a social amoeba whose life cycle consists of two distinct phases – growth and development. As long as nutrients are abundant, *Dictyostelium* cells grow as individual amoebae. When nutrients are depleted, starving cells aggregate to form a multicellular mound, and finally differentiate into fruiting bodies, consisting of several different cell types. The switch from growth to differentiation is controlled by a number of interacting factors, including the nutritional state of cells,

measures of the food supply, and a range of extracellular signals. Several of these signals are generated by nearby *Dictyostelium* cells. Some allow cells to judge the local density of competitors, while others allow populations of cells to cooperate and develop simultaneously. We are currently well short of an understanding of how all these signals are interpreted by cells and integrated to allow a switch in the genetic programme.

During growth *Dictyostelium* cells continuously synthesize and secrete autocrine factors which accumulate in proportion to cell density. At appropriate concentrations these factors cause changes in gene expression and prepare cells for the initiation of development. A glycoprotein called prestarvation factor (PSF) is secreted by growing cells [1]. The effect of this protein is counteracted by food bacteria. Cells can detect the level of PSF and thus estimate their own density relative to the abundance of the food source [2]. The "prestarvation response" occurs during increasing of PSF levels and decreasing of food source, and can be detected a few generations before actual starvation occurs. The discoidin I gene family is among the first genes to be activated in the prestarvation response. When food is depleted and cells stop growing, PSF production declines and a separate starvation response is activated. Starving cells secrete another glycoprotein, condition medium factor (CMF), which is essential for establishing of cAMP signalling and the initiation of aggregation [3,4]. Starvation causes a further increase in discoidin expression, after which discoidin continues to accumulate in early development until its transcription is inhibited by extracellular cAMP at the end of aggregation phase. Expression of discoidin is therefore an excellent reporter for the state of cells within the developmental programme.

Several genes have been found to regulate the growth-differentiation transition (GDT) in *Dictyostelium*. Protein kinase A (PKA) plays a critical role and has been implicated in multiple pathways involved in later development. Strains carrying disruptions in PKA exhibit arrest of development on early stage and fail to aggregate [5]. Similarly, the protein kinase YakA, homolog of yeast Yak1p growth regulating protein kinase, is also required for the turning off of growth stage genes and induction of developmental genes [6]. YakA- mutant cells are also unable to aggregate. Other genes regulating the growth-differentiation transition include *Dia*2, a gene of yet unknown function [7], and also *ami*A and *ami*B [8,9]. *Ami*A is homologous to a yeast gene of unknown function, while *ami*B does not show homology to any known genes. Both genes positively regulate the growth-differentiation transition possibly via regulation of adenylyl cyclase expression.

Three negative regulators of the growth-differentiation transition have been described. *Puf*A encodes a member of

Puf protein family of translational regulators [10]. PufA is a translational inhibitor of PKA mRNA, and its levels are downregulated by the YakA kinase. *Dia*1, a gene with no homology to any known genes, appears to be negatively coupled to both the cAMP receptor cAR1 and the adenylyl cyclase ACA [11]. Antisense-mediated inactivation of *dia*1 enhances the progression of differentiation. Finally, *gdt*1 is a gene which encodes a transmembrane protein with a kinase-homology domain [12], although interestingly the kinase domain is unlikely to be functionally active. *Gdt*1 null cells display accelerated aggregation. Examination of double PKA-/*gdt*1- mutants and *in vitro* phosphorylation data show that *gdt*1 is likely to be a downstream target of PKA [13]. In this study we identify a new gene related to *gdt*1. Like *gdt*1, *gdt*2 acts as a negative regulator of the growth-differentiation transition in *Dictyostelium*. We demonstrate that GDT1 and GDT2 exemplify a family of related proteins which together may serve to mediate the full complexity of the transition between growth and differentiation.

Results

Identification of REMI mutant IR32

The mutant described in this study was found during a screen of REMI-mutagenised library for mutants resistant to propranolol, a phospholipase D (PLD) pathway inhibitor. The original goal of the screen was to discover new genes involved in PLD function. We isolated a REMI mutant, IR32, which was able to aggregate on *Klebsiella* lawns in the presence of 1 mM propranolol. Under these conditions the parental Ax2 cells are totally unable to initiate aggregation (data not shown). However, both growth and development of IR32 cells were inhibited to the same extent as wild type cells by butan-1-ol, another PLD inhibitor (data not shown). It therefore appeared that the gene disrupted by REMI was involved in the initiation of development, rather than being part of a PLD signalling pathway.

A fragment of the disrupted gene was isolated from mutant IR32 by plasmid rescue using genomic DNA digested with BclI. The sequence of this fragment was used to search *Dictyostelium* genome project databases. Several partial cDNA clones and genomic contigs were found, allowing us to identify the disrupted gene as a homologue of the *Dictyostelium gdt*1 gene [12]. This new gene was therefore named *gdt*2. The *gdt*2 coding region was interrupted by REMI about 4 kb downstream of predicted start of the ORF (Fig. 1A). To prove that this disruption caused the phenotype, and that the phenotype was the result of a simple loss of function, we generated fresh mutants disrupted in the first half of the *gdt*2 gene. Several clones were identified by colony phenotype and shown by Southern blot to carry an insertion in *gdt*2 gene (Fig. 1B). All *gdt*2- clones displayed the same phenotype (data not shown)

A

BcII   BSR   BcII   BcII   BcII   BcII

probe for Southern ⎯⎯⎯⎯

probe for Northern ⎯⎯⎯⎯

0.5 kb

B

kb 5 —

4 —

3 —

2 —

Ax2   g14

C

```
    1  MNYILYILLILIIFSINNTFSIGSFVYTPDGYYNLYRINKFENFSKIPTKANSVNVEPHELYYPDICDSA
   71  LKNKNSNEFSIDNFPGSPFLKANIKITKGSNMTLDPLLSIHPSYINILCIEGSFTAGSQQTLTLGGIIVL
  141  PGGNFHCNNCVLQFVDLSSTIIKIDPFGFLPGILSLGGSISLIGSQRILYSAKPIEDNVLEVNDISTLDP
  211  FNGFNVSIVSDSFPNVRTSRFTYNKNKLEMQDLTIPTTDLNIKLFFNDSGSLKFGGISTRTKSSIYITGD
  281  TKLHIENYLLDSIGKTSNKEYDDTKLTFSSDNPNNVTDIIIGKNQRFRNSLYIEFSNSVTIKNSVIIGNT
  351  GSSRSSLVFFQSNVNISGNLIVGTAGSSMIAQYGTEYIESSNNYFSLVLPNEFPTSPAFMDYGHEGNGIY
  421  SLSPNIFSTNDIFNGQKILYNFNFISRENVTGFDKDCFAPCDINSIVVPSLSQEPIDFSKNLVNPISINP
  491  VDYFLIVNSDEELKNTFFTIKDSTISSKIIVNLKGSGLVLKNIIGVNNFKLTGQVKRLDIYDSFFGSSTT
  561  PSLGDNGIKSSAVIIKNSSFYIDDFQLQNNQIYGSSIVPYLYELLNVDEFILVDSIFPTSQYQIPVGGKL
  631  DLIVKLVIISPVISRITCELESDNETITSSAISESVNKECKFPLTFKNDGSVNLKVTVKLKNVGDSEIMY
  701  IINFPKITIFSNYSFYSGWSMENSTENGIISVDGNSFKKGCSISSNDNSNNCTISNNIKYITNLPKLNIS
  771  NDLNELFLNGITSINSNELVTITTRIDSETKYYQIQLFFIHQPIDDQPTPLSIYIENQPVFLLEPLESNS
  841  APIFKNLTFNFDNINSLENINISFATRGNTYLTSMAIYSSLVIEYPIPVEKINLLPIIVPICVTVLVLLS
  911  ILIVFFGARYYKHKKRRRLEIAVFGVELYSKKPSNNKSPVPSTPLIINSNQEQSSSNHISITVNRNSDIQ
  981  TQSENNNLEPTTVETTTTTTTTAAGAILQTNVTLEMENLEIKPIINNNEPFFEDYSDEYDDSSDGAELNE
 1051  DDVEDYINIIRGGNIKIEAEGELFVRQFERNTFDQEFFEYKKSKIPSPHLVPSLAHGDFIENPLNIIDIL
 1121  LDPVTNEIPLEHYLKTYKNRNGQSGKYRTNTQTHVLIADTIFQHCNDPDLPVSVSPYICDFGMNGRKSDL
 1191  GEVCHDTITFKNKLSEPLYALFLFPEGDDSSITSSPFQSIELKPNVETSISLSVTLFSTTKYNEKVIVRF
 1261  ETLDVKQSWSTFVFLRLESELTN**IIDFNEIIIKNYISEGTFGIVYRGIWRSSPVAVKLLKNEYVDHDEIE**
 1331  **KEISLLERLRHPNILLYVGRFIFSGIHGIVTDYCSKGCLSKYIHENKYKITIIQKIRILIDISKACAYLH**
 1401  **RNGITHRDLKPENILITSLNPKSLVCAKVSDFGTSKETNERYNLQTRRGTVAYMSNEILSQGSYTKSTDI**
 1471  **YSFGMLCYELFSERIPYYSEPHWRIRELVLKNERPSLDQEIFKDTDISDIVTRCWSKNPSQRPTF**DYLSK
 1541  YLKHLLKKYDINNIIYDKDKKNKKKNKNNNNNNNNNNNNNNNNNNNNNNNNNNNNNDHDDNNNNINDSDCDNNKN
 1611  NNNNNNNNNNNNNNNNNNNNNNNNNNNNN
```

Figure 1
Identification of the *gdt*2 gene. (A) Schematic map of the *gdt*2 gene. The coding sequence is shown as open boxes, the site of REMI insertion is indicated with an arrow. The site of the Blasticidin insertion in the knockout construct is shown with triangle. Sequences used as probes for Southern and Northern blots are schematically shown below. (B) Southern blot analysis of one of the *gdt*2 knockout clones, g14, and wild type Ax2 cells. Genomic DNA was digested with BcII and hybridized with the probe shown in (A). (C) Predicted amino acid sequence of GDT2. The GDT2 protein is composed of 1637 amino acids. The signal sequence (AA 1-21) is boxed and the putative transmembrane domains (AA 129-145, 167-184 and 897-921) are underlined. The putative kinase domain (AA 1284-1535) is shown in bold.

and were found to be identical in phenotype to the original REMI mutant IR32.

Both *gdt*2⁻ and IR32 mutants made plaques with unusual morphology when grown on bacterial lawns. Likewise, each displayed accelerated aggregation when developed on non-nutrient agar (see below). This confirmed that the original REMI insertion caused the phenotype of IR32.

Sequence analysis of the gdt2 gene
Sequence analysis of the genomic contig revealed an open reading frame of 4.6 kb, interrupted by 5 small introns. The ORF within the *gdt*2 gene is predicted to produce a protein of 1637 amino acids, with a mass of 185 kDa. Analysis of the predicted protein by structure-predicting programmes (SMART, TMHMM and MEMSAT) revealed an N-terminal signal sequence for secretion/membrane inclusion, at least 1 and most likely 3 potential transmembrane domains, and a kinase homology domain at the C-terminal end of the protein (Fig 1C). As mentioned above, GDT2 showed substantial homology to GDT1 [12]. It is worth mentioning that the original *gdt*1 sequence deposited in the database was incomplete at its 5' end. Our analysis showed that GDT1 has an extra 101 amino acids at its N-terminus, the first 20 of which represent a putative signal sequence. The kinase domain of GDT1 is interrupted by a poly-Asn containing insert and presents many substitutions to well conserved amino acids, particularly in the catalytic loop. It is therefore presumed to be non-functional. The kinase domain of GDT2 is uninterrupted and includes all the typical amino acids for a classical serine/threonine protein kinases. It therefore seems likely that GDT2, unlike GDT1, is a fully functional protein kinase.

A family of gdt1 and gdt2 like genes
Analysis of the *Dictyostelium* genome revealed a family of genes which were closely related to *gdt*1 and *gdt*2. As shown in Fig. 2 and 3, we identified six additional full-length genes and one pseudogene (*gdt*1 DDB0215003, *gdt*2 DDB0201878, *gdt*3 DDB0186734, *gdt*4 DDB0201754, *gdt*5 DDB0186736, *gdt*6 DDB0201749, *gdt*7 DDB0184322, *gdt*8 DDB0201879 at The *Dictyostelium* Database [14]). Three of the full-length genes (*gdt*2, *gdt*3 and *gdt*4) encode complete, well conserved (and therefore presumably functional) kinase domains; another three (*gdt*1, *gdt*6 and *gdt*8) seem to encode proteins with degenerate protein kinase domains. GDT5 presents a C-terminal sequence unrelated to protein kinases, while GDT7 appears truncated and stops before the protein kinase domain. All the GDT proteins share extensive homology in first 1000 amino acids (700 for the truncated GDT7) despite some insertions or deletions. All the homologs start with a clear signal sequence for secretion/membrane inclusion. Additionally, all have at least one strongly predicted transmembrane domain located

between amino acids 600 to 1000 and two weaker ones in the region of amino acids 120 to 200. Since the proteins are well conserved, they should also share the same topology. Combining the transmembrane predictions with the multiple alignment leads to the proposed model of 3 transmembrane domains (Fig. 2). Neighbor joining phylogenetic analysis clustered these genes into two groups, with group 2 containing the three functional kinases and group 1 containing the others (Fig. 3A). In the genome the *gdt* genes form 3 clusters of two genes each plus two singletons (Fig. 3B). Each cluster includes a member of the groups 1 and 2, suggesting that the clusters arose by duplications of an initial tandem pair of genes.

Expression of the gdt2 gene during growth and development
To analyse expression of the *gdt*2 gene, we carried out Northern blot analyses using a *gdt*2 cDNA fragment as a probe. RNA samples were prepared from wild type cells in a vegetative stage and at different time points of development. As shown in Fig. 4, during exponential growth expression of *gdt*2 mRNA does not depend upon cell density. The *gdt*2 mRNA is expressed at about the same level in vegetative cells and throughout development, with a slight peak at the time when the cells are aggregating. This is in sharp contrast to *gdt*1, which has been shown to be expressed in vegetative cells only [12].

gdt2⁻ mutants develop prematurely
Colonies of *gdt*2⁻ cells on bacterial plates displayed an unusual morphology. Cells aggregated very close to the growing edge of the colony – or in some cases even within the bacterial lawn, while Ax2 cells made colonies with clear preaggregation zones (Fig. 5A).

A similar phenotype has been described in other mutants, in particular HBW3 [15], and *gdt*1 nulls [12]. Since both HBW3 and *gdt*1⁻ null mutants overexpress discoidin, we decided to measure discoidin expression in *gdt*2⁻ cells. Wild type cells and *gdt*2⁻ mutants were grown in bacterial suspension and harvested at a density of 1×10^6 cells/ml. Expression of discoidin I was monitored on Western blots using an anti-discoidin I antibody. Although levels of discoidin expression varied somewhat from experiment to experiment, a significant amount of discoidin was always detected in the mutant, even at very low cell densities (Fig. 5B). In wild type cells growing in bacterial suspension, discoidin can normally be detected at cell densities above 1×10^6 cells/ml, whereas in *gdt*2⁻ cells discoidin was detected even at a density of 1×10^5 cells/ml (data not shown).

gdt2⁻ cells sense cell density and concentration of food
In wild type cells, expression of discoidin is induced a few generations before starvation and then gradually increases
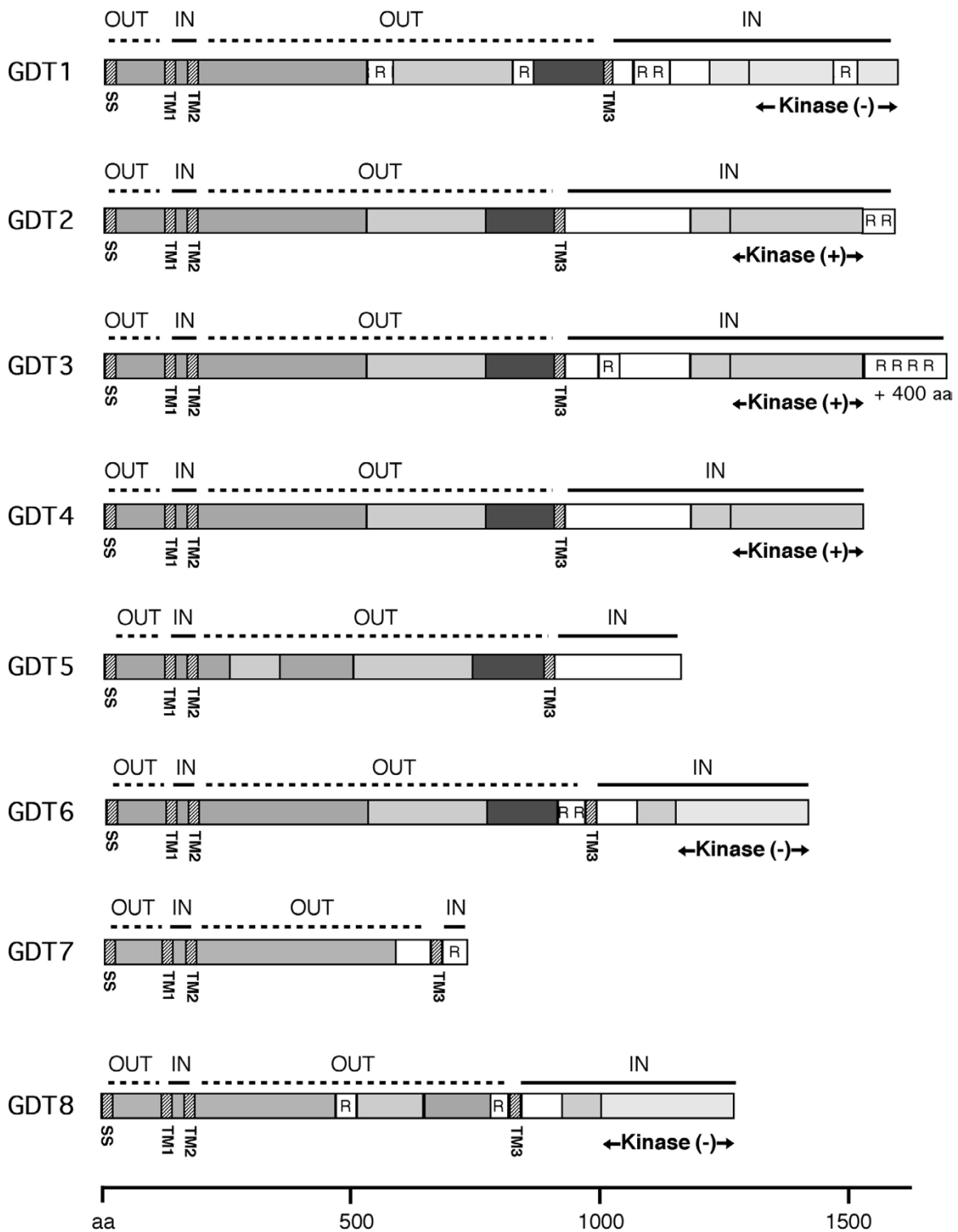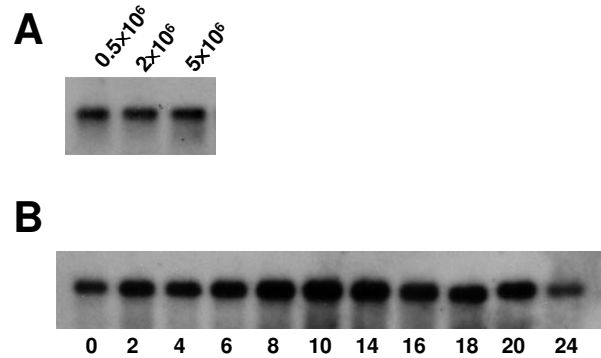
Figure 2
A family of proteins related to GDT1 and GDT2. 8 GDT proteins are shown schematically. SMART, TMHMM and MEM-SAT programs were used for sequence analysis. The level of grey indicates the extent of conservation. SS – signal sequence, TM – transmembrane domain, R – repetitive polyAsn-containing sequences, IN – predicted cytoplasmic domains, OUT – predicted lumenal/extracellular domains. The putative kinase domains are marked with "+" if they appear to be functional or with "-" if they are degenerate.

A

B
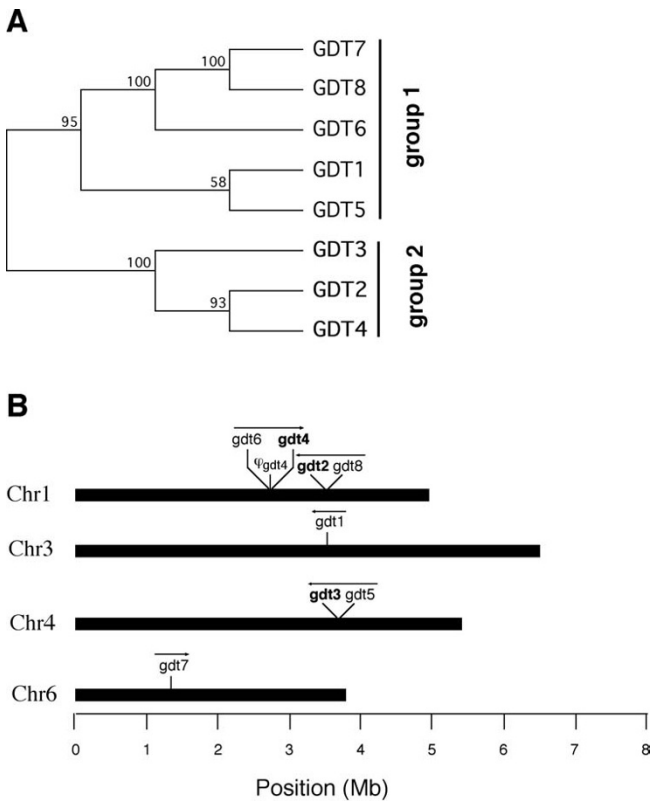
Figure 3
***Gdt* gene family in the *Dictyostelium* genome**. (A) Neighbor joining tree of the *gdt* gene family in the *Dictyostelium* genome. Only the topology of the tree is shown. Bootstrap support of the internal nodes is shown over the branches. (B) Localisation of the *gdt* genes on *Dictyostelium* chromosomes. Direction of the transcription is shown with arrows. Genes with predicted functional kinase domains are shown in bold.

A

B

Figure 4
Expression of the *gdt*2 gene. (A) Expression of *gdt*2 mRNA in vegetative cells. mRNA was prepared from axenically grown wild type Ax2 cells at the cell densities indicated. (B) Expression of *gdt*2 mRNA during development. Ax2 cells were harvested from axenic medium during exponential growth, washed and plated on non-nutrient agar for development. Cells were collected and mRNA prepared at the time points indicated. The blot was hybridised with the probe shown in Fig. 1A.

with further cell proliferation. To determine whether discoidin expression in *gdt*2⁻ mutant changes with increasing cell density, discoidin I expression was analysed at different stages of exponential growth. Wild type cells and *gdt*2 mutants were grown in bacterial suspension and harvested at densities of 1×10^6, 2×10^6 and 6×10^6 cells/ml. Despite the fact that discoidin expression was significantly increased in growing *gdt*2⁻ mutants compared to Ax2 wild type cells at all cell densities, the amount of discoidin protein still increased with cell density (Fig. 6A). Thus, *gdt*2⁻ cells are still able to estimate the number of neighbouring cells and accordingly produce and detect autocrine factors such as PSF.

Wild type cells start to express discoidin at a relatively low cell density when the nutrient supply is limited, but high nutrient supply restricts expression to higher cell densi-

ties. To understand whether the onset of development in *gdt*2⁻ mutants is regulated by amount of food, expression of discoidin I was measured in cells that had reached the same cell density growing in bacterial suspensions of different saturation. Ax2 and *gdt*2⁻ cells were grown in *Klebsiella* suspensions of different concentrations and harvested at a density of 1.2×10^6 cells/ml. In wild type cells, discoidin was detected at normal or reduced concentrations of bacteria and was not detected at all at 3× bacterial concentration. In the *gdt*2⁻ mutant, some protein was still detectable even in cells grown in the most concentrated bacterial suspension (Fig 6B). The expression of discoidin in the mutant still decreased with increasing concentration of food, although the starting level was higher. Therefore, *gdt*2⁻ cells are still able to sense the concentration of food.
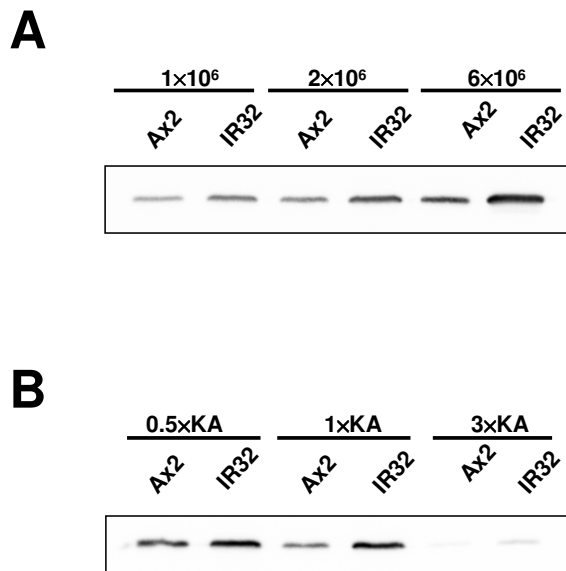
Taken together, these data demonstrate that although the *gdt*2⁻ mutants made the decision to start development even at unusually low cell densities and high concentration of the food source, the onset of development was still to some extent regulated by cell density and concentration of food.

gdt*2⁻ *mutants show accelerated aggregation
*gdt*2⁻ cells grow at the same rate as parental Ax2 cells in axenic culture and in bacterial suspension (data not

A

B
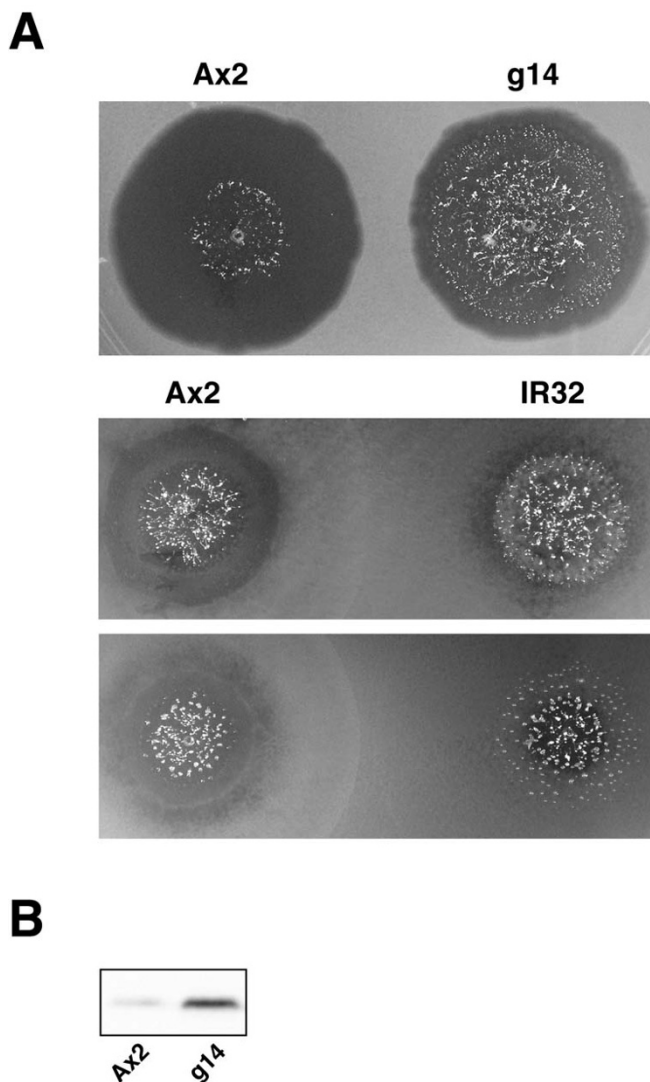
Figure 5
Phenotype of the *gdt*2⁻ mutant. (A) Colony phenotype.
500 cells of wild type and either g14 (the knockout clone) or
IR32 (the original REMI mutant) were spotted on the same
SM agar plate and left to grow for 5 days. Both REMI and
knockout clones show very narrow or absent clearing zones.
(B) Overexpression of discoidin I by *gdt*2⁻ mutant at low cell
densities. Ax2 and g14 cells were grown in bacterial suspen-
sion and harvested at a density of 1 × 10⁶ cells/ml. Discoidin I
was detected by Western blot analysis with an anti-discoidin
I antibody.

A

B

Figure 6
**Sensitivity of *gdt*2⁻ mutants to cell density and food
supply.** (A) Ax2 and IR32 cells were grown in 1 × KA sus-
pension and harvested at the cells densities indicated. (B)
Ax2 and IR32 cells were grown in 0.5×, 1× and 3 × KA sus-
pensions and harvested at a density of 1.2 × 10⁶ cells/ml. Dis-
coidin I was detected by Western blot analysis with an anti-
discoidin I antibody.

shown). To investigate whether development is impaired
in *gdt*2⁻ mutants, mutant and wild type cells were allowed
to develop on non-nutrient agar plates. Development was
monitored microscopically. *gdt*2⁻ cells consistently started

to aggregate 1–1.5 hours earlier than wild type Ax2 cells
(Fig. 7A), but the rest of their development proceeded
normally – they successfully finished aggregation and
formed fruiting bodies just before or in some experiments
at about the same time as wild type (data not shown).

We also observed aggregation of starved cells in sub-
merged culture. As expected, *gdt*2⁻ cells started to aggregate
earlier than wild type, with about the same time difference
as we had seen on non-nutrient agar (Fig. 7B).

This demonstrated that the disruption of *gdt*2 specifically
accelerates the growth/differentiation transition, but does
not significantly affect later development.

*gdt*2⁻ mutants are able to sense folate
Folate is a known chemoattractant for growing *Dictyostel-
ium* cells. It can also function as an extracellular signal that
inhibits development. Folate has been shown to inhibit
discoidin expression when added to axenically growing
wild type cells [16]. To determine whether the *gdt*2⁻ phe-
notype is due to a loss of sensitivity to folate, we
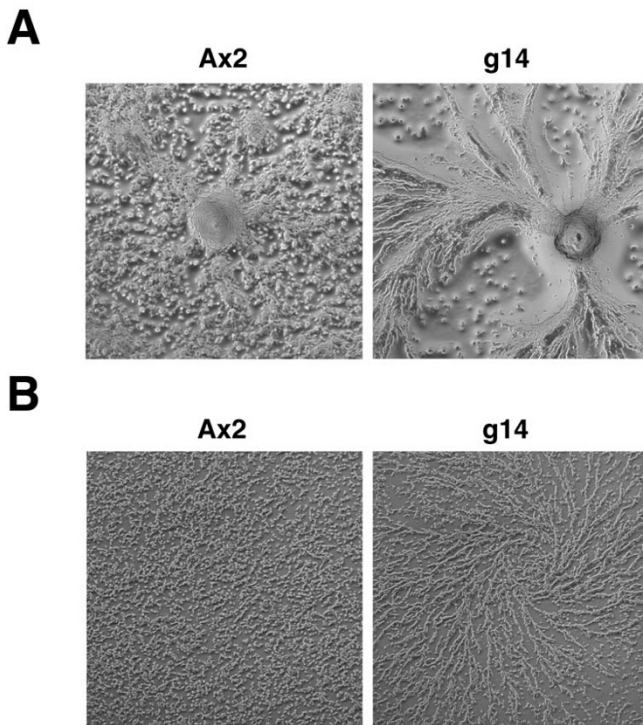
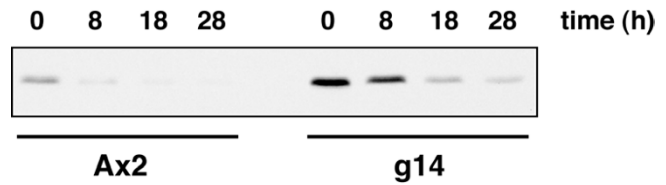**Figure 7**
Accelerated aggregation of *gdt*2⁻. (A) Aggregation on non-nutrient agar. (B) Aggregation in submerged culture. Cells were harvested at exponential growth phase, washed, plated at a density of 2×10^5 cells/cm^2 and incubated at 22°C. Photographs were taken after 7.5 hours (A) or 8 hours (B). Under both conditions *gdt*2⁻ mutant cells start to aggregate about 1–1.5 hours earlier than wild type cells.
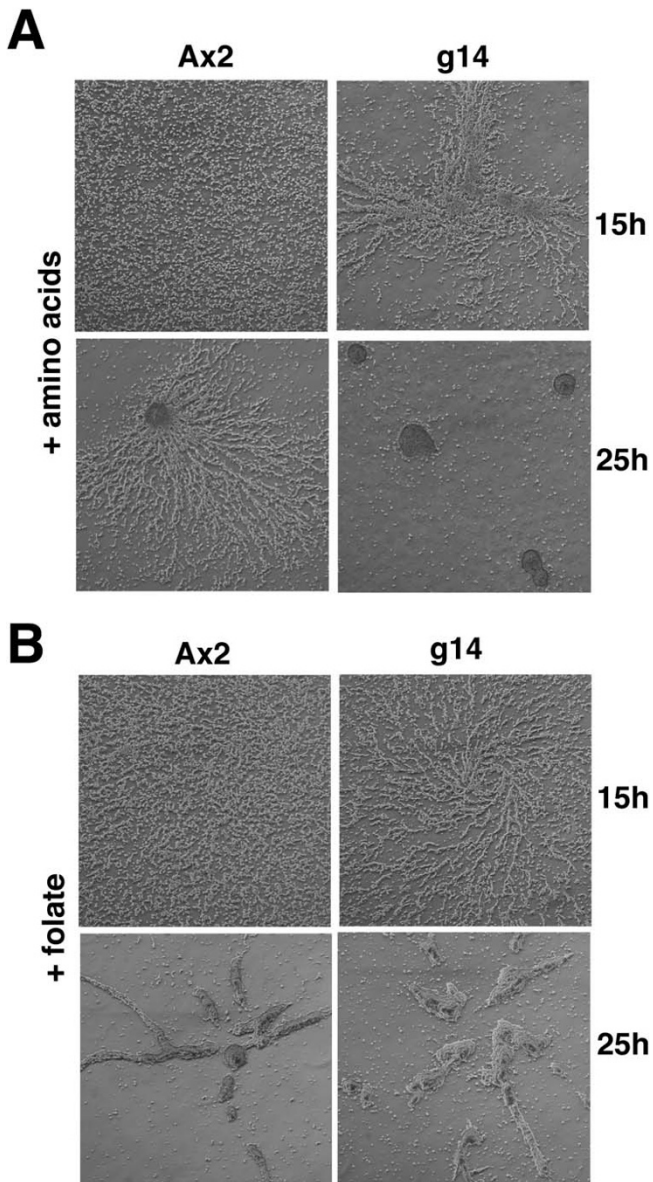

Figure 8
Sensitivity of *gdt*2⁻ mutants to folate. Ax2 and g14 cells were grown in axenic medium in presence of 1 mM folate while cell density was maintained at about 1×10^6 cells/ml. Samples were removed at the time points indicated. Discoidin I was detected by Western blot analysis with an anti-discoidin I antibody.

possible that they are impaired in sensing of some specific food components. Earlier studies had shown that amino acids specifically inhibit initiation of development [17]. In our experiments, *gdt*2⁻ cells starved in submerged culture started to form aggregation streams after 8 hours of incubation, whereas Ax2 cells started to aggregate about 1 hour later (see Fig. 7). When the buffer was supplemented with essential amino acids and glucose (see Methods), both wild type and mutant cells exhibited delayed aggregation compared to unsupplemented buffer, but the delay was more substantial for wild type cells. *gdt*2⁻ cells started to make streams after 13–14 hours of incubation, whereas wild type cells started to stream only after 18–19 hours. As shown in figure 9A, after 25 hours of incubation *gdt*2⁻ mutant cells had completed aggregation and made mounds, whereas Ax2 cells were still streaming. Thus, while the mutant was still able to sense amino acids, it was considerably less sensitive than the wild type.

To determine whether this effect was specific to amino acids, we used folate to inhibit aggregation. Folate at a concentration of 10 µM caused 1.5–2 hours delay in starting aggregation in submerged culture. Under this folate concentration the time difference in starting aggregation between the mutant and wild type was about 1 hour (data not shown). Higher concentrations of folate caused stronger inhibition of aggregation. Cells incubated in the presence of 1 mM folate started to aggregate after 15 hours (even later than cells incubated with amino acids), but the time difference in starting aggregation was again the same between the mutant and wild type – about 1 hour. After 25 hours both wild type and the mutant were breaking streams and making mounds (Fig. 9B).

The above experiments show that the *gdt*2 mutant is clearly different from wild type in its response to amino acids, but it is not significantly different in folate response.

investigated the ability of *gdt*2⁻ mutant to respond to different levels of folate in the medium. Our preliminary data showed that *gdt*2⁻ mutants responded normally to folate in a chemotaxis assay (data not shown). To test whether discoidin expression is regulated by folate in the *gdt*2⁻ mutant, cells were grown in axenic medium and treated with 1 mM folate for various times, while cell density was maintained at around 10⁶ cells/ml. In wild type cells discoidin expression was considerably reduced after 8 hours of folate treatment and was nearly undetectable thereafter (Fig. 8). *gdt*2⁻ cells, on the other hand, produced a higher amount of discoidin than wild type cells, but expression of discoidin clearly decreased under folate treatment. Thus, the phenotype of the mutant was not caused by an inability to sense folate levels.

Regulation of starvation by different nutrients
Since *gdt*2⁻ mutants exhibit accelerated aggregation, but can in general estimate the quantity of food, it seemed

Figure 9
Aggregation of *gdt2⁻* cells in the presence of amino acids and folate. Cells were harvested from axenic culture at exponential growth phase, washed and plated at a density of 2×10^5 cells/cm^2 in buffer containing either amino acids (A) or 1 mM folate (B). In the presence of amino acids *gdt2⁻* mutant cells aggregate 4–5 hours earlier than wild type, while in the presence of folate they aggregate only 1 hour earlier than wild type, as they do in unsupplemented buffer.

Protein kinase A and the gdt2 phenotype

A large body of work has shown that PKA is a major regulator of early *Dictyostelium* development. Overactivation of PKA leads to premature aggregation [18], while loss of the catalytic subunit results in cells that never initiate development [19]. Since *gdt2⁻* mutants showed premature aggregation, we investigated PKA expression and activity in the mutant and in the wild type.

Our preliminary data using H-89, cell permeable inhibitor of PKA [20-22], showed that discoidin expression was inhibited upon PKA inhibition in the mutant to about the same extent as in the wild type (data not shown). It suggests that the disruption of *gdt2* caused an increase in the level of discoidin expression but did not greatly affect its PKA-dependent regulation. Northern blots showed that mRNA of the catalytic subunit of PKA (PKA-C) was expressed at about the same level in the mutant as in wild type in vegetative cells (Fig. 10A). After 3–4 hours of starvation, expression of PKA-C mRNA increased in both wild type and the mutant, though it increased somewhat more in the wild type. Interestingly, the expression of PKA-C protein was slightly higher in the mutant than in wild type at corresponding time points of development (Fig. 10B). However, although there were some differences between PKA mRNA and protein levels later in development, both were very similar in growing cells at the stage when *gdt2⁻* mutants prematurely initiate development.

PKA activity is regulated by signalling through the regulatory subunit as well as by protein abundance. We directly assayed PKA activity in vegetative wild type and *gdt2⁻* cells grown on bacteria. The difference in PKA activity was indistinguishable (Fig. 10C). Taken together, this data indicate that GDT2 does not exert its effects by modulating PKA activity. It remains possible that it acts downstream of PKA and mediates a subset of cellular responses.

Discussion

The soil amoeba *Dictyostelium discoideum* has a relatively simple developmental programme compared to higher eukaryotic multicellular organisms. Nonetheless, the change from growth to development is regulated by a complex series of signals designed to ensure that aggregation only occurs under optimal conditions. In the prestarvation response, during which cells anticipate future starvation and prepare to develop, expression of a number of genes is induced, and when starvation occurs and cells are triggered to aggregate, changes in gene expression occur in a wholesale fashion. Recent studies of gene transcription profiles show that aggregation of unicellular amoebae to multicellular structures is accompanied by a change in the expression of more than 25% of the genes in the genome [23].

In this work we have described a new gene, *gdt2*, which is important for the control of this process. We isolated a REMI mutant which was able to develop in the presence of propranolol. The mutated gene was found to be a novel gene. The mutant showed premature expression of

A

B

C

Ax2   14±5 pmol/min/mg protein

gdt2⁻   15±4 pmol/min/mg protein
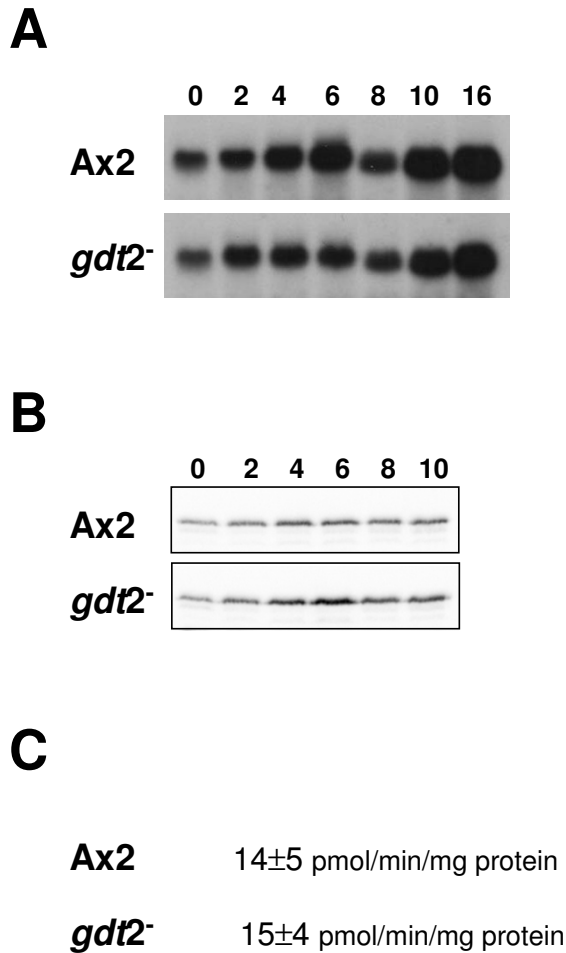
Figure 10
Protein kinase A expression and activity. mRNA (A) and protein (B) extracts were prepared from vegetative cells and cells at different time points of development. Northern blots (A) were probed to visualise PKA-C mRNA, and Western blots (B) were probed with anti PKA-C antibody. (C) PKA enzyme activity, measured in vegetative cells at a density of 2 × 10⁵ cells/ml growing on bacteria. Data shown represent mean ± SE from 3 independent experiments.

discoidin and premature aggregation. Morphological development was not affected in *gdt2*⁻ mutants, thus allowing us to assume that *gdt2* was predominantly involved in the growth-differentiation transition, but was not essential for later development.

It is clear that the prestarvation response in *Dictyostelium* depends on two major factors: cell density and the abun-

dance of food [2,24,25]. It is possible that the *gdt2*⁻ mutant was impaired in calculating the density of neighbouring cells and/or in sensing food. We have shown here that *gdt2*⁻ cells are able to estimate their own density (see Fig. 6), but we cannot exclude that the mutant has either enhanced PSF production or a higher sensitivity to PSF. However, it is more likely that the mechanisms of sensing some of food components are seriously impaired, although not abolished in the mutant. Our measurements of amino acid sensitivity support this hypothesis. In addition, the *gdt2* mRNA is expressed during growth and throughout development (Fig. 4), which may reflect a role for the *gdt2* gene product in dedifferentiation as well as the primary decision to initiate development.

Mutants lacking *gdt1* show similar phenotypes to *gdt2*⁻ mutants. The *gdt1*⁻ mutants also overexpress discoidin and show premature entry into the developmental cycle [12]. However, there are major differences. The *gdt1*⁻ mutants display slow growth compared to wild type cells, whereas *gdt2*⁻ cells grow at the same rate as wild type. mRNA expression patterns of *gdt1* and *gdt2* are also different. The expression of *gdt1* mRNA increases with cell density in vegetative cells and rapidly decreases after the onset of development [12,26]. We expected to see a similar pattern of *gdt2* mRNA expression, or at least a decrease in mRNA upon starvation. Surprisingly, we found the *gdt2* mRNA to be expressed at about the same level in vegetative cells and throughout development. Taken together, the similarities and dissimilarities between *gdt1* and *gdt2* mutants' phenotypes and gene expression patterns allow us to propose that these two genes possibly regulate some or all of the same cellular processes, but are regulated by parallel signal transduction pathways. These genes are possibly most important at different points in the developmental cycle. It would be interesting to see a phenotype of a double *gdt1*⁻/*gdt2*⁻ mutant, if a double knockout of these genes was possible.

Here we have also described a family including *gdt1*, *gdt2* and 6 more genes in the *Dictyostelium* genome. The similarities between these genes suggest that they all may be involved in regulation of the process of development, probably responding to different extracellular signals. However, the particular roles of the members of the family remain to be explored.

Conclusion
We have isolated a *Dictyostelium* REMI mutant, which initiates multicellular development earlier than the wild type. The genome rescue showed that the disruption occurred in a new gene, which was named *gdt2*. G*dt2* codes for a transmembrane protein with a kinase domain. Using developmental assays we have shown that *gdt2* is involved in control of the growth-differentiation

transition. We have also described a family of *gdt*1 [12] and *gdt*2 related genes in the *Dictyostelium* genome. The family is comprised of eight genes coding for transmembrane proteins with and without kinase domains. All *gdt* genes may be involved in regulation of the process of development in *Dictyostelium*.

Methods

Cell culture

Dictyostelium discoideum Ax2 and derived transformants were grown as appropriate in axenic medium (HL-5), in suspension with autoclaved *Klebsiella aerogenes* (KA), or on lawns of *Klebsiella aerogenes* growing on SM agar plates. Cells were grown and developed at 22°C.

Klebsiella were harvested from SM plates, suspended in KK2 buffer (20 mM potassium phosphate, pH 6.2) at an OD_{600} = 6.5 and then autoclaved. The resulting medium was used as 1 × KA suspension. 3× and 0.5× suspensions were made by concentrating or diluting 1 × KA, respectively. Prior to inoculation of cells, $MgCl_2$ was added to KA suspensions to a concentration of 2 mM. *Dictyostelium* cells were inoculated at 5×10^4 cells/ml, shaken at 150 rpm at 22°C and harvested at the cell densities indicated in the text and in figure legends.

For the folate sensitivity experiment, cells were grown in axenic medium until they reached a density of 10^6 cells/ml, after which folate was added to a concentration of 1 mM. Cell density was calculated every 8–10 hours and the culture was diluted with axenic medium containing 1 mM folate to keep the cell density around 10^6 cells/ml. Cells were harvested at time points indicated.

Developmental conditions

Vegetative cells were harvested from bacterial or axenic suspension cultures at densities of $1–2 \times 10^6$ cells/ml and washed free of bacteria/medium in KK2 buffer. For development on non-nutrient agar, cells were resuspended in KK2 supplemented with 2 mM $MgCl_2$, plated on agar plates ($2–8 \times 10^5$ cells/cm²) and allowed to develop. For aggregation experiments in submerged cultures, cells were resuspended in either KK2 or in aggregation buffer (5 mM Na_2HPO_4, 5 mM KH_2PO_4, 7.5 mM NH_4Cl, 0.41 mM $MgSO_4$ and 0.34 mM $CaCl_2$, pH 6.8). Two millilitre portions containing 6×10^6 amoebae were plated in Ø60 mm tissue culture dishes. For aggregation in presence of folate, folate was added to concentrations of 10 μM to 1 mM after cells were plated. For aggregation in presence of amino acids, cells were resuspended in aggregation buffer or in aggregation buffer supplemented with 10 mg/ml glucose and 0.5 mg/ml of each of the following amino acids: methionine, glycine, isoleucine, valine, arginine histidine, tryptophan, phenylalanine, threonine, lysine.

Cells were observed using Zeiss Axiovert 100 microscope with a 5× objective.

REMI library screening and genomic rescue of IR32 mutant

A REMI-mutagenised Ax2 library [27] was provided by A. Harwood (UCL, London). To obtain propranolol resistant mutants, the library was selected for growth and development on SM agar/KA plates in presence of 1 mM propranolol. Eight separate clones were isolated and one clone, IR32, was rescued.

A 0.5 kb fragment was recovered from the IR32 REMI mutant by plasmid rescue as described by Kuspa and Loomis [28]. Genomic DNA from IR32 was digested with BclI, circularized in a diluted solution and used to transform *E. coli*. A plasmid containing 0.5 kb of genomic sequence upstream of the insertion site was recovered.

Reconstruction of the mutant phenotype

The *gdt*2 knockout vector was constructed by inserting the Blasticidin S resistance (BSR) cassette from pRHI148 into single MscI site of a 2 kb *gdt*2 fragment, which was generated by PCR from genomic DNA and cloned into pBluescript. The resulting vector was cut with ApaI and NotI to generate the 3.3 kb *gdt*2/BSR fragment and used to transform *Dictyostelium* Ax2 cells as described [29]. The resulting transformants were screened by colony phenotype and confirmed by Southern blot.

Southern and Northern blot analyses

Genomic DNA was prepared using DNAzol reagent (Invitrogen) according to manufacturer's instructions, digested and blotted onto Hybond N+ membranes (Amersham Pharmacia Biotech).

Total RNA was extracted using TRI reagent (Sigma). RNA (20 μg per lane) was subjected to electrophoresis in 1.2% agarose/formaldehyde gels and transferred to Hybond N+ membranes. Equal amounts of RNA per lane were loaded as determined by measuring OD at 260 nm and confirmed by ethidium bromide staining.

Two fragments of the *gdt*2 gene were obtained by PCR and used as Southern and Northern blot probes (Fig. 1A). A fragment corresponding to C-terminal part of the catalytic subunit of PKA was also obtained by PCR and used for Northern hybridisation. The fragments for both Southern and Northern blots were radiolabelled using a random primer kit (Amersham Pharmacia Biotech) and [alpha-^{32}P]dATP (ICN). Blots were exposed to X-ray film for 16–48 hours using intensifying screens.

Western blot analysis and PKA assays

Total cell extracts were prepared by lysing *Dictyostelium* cells in SDS-PAGE loading buffer. Equal amounts of

protein (5 to 10 μg) were separated on 12% gel and blotted by tank transfer. Equal loading was confirmed by staining of the membranes with Ponceau S. Discoidin Iγ was detected using a monoclonal antibody 80-52-13 [15], a generous gift from W. Nellen (Department of Genetics, Kassel University, Germany) and peroxidase coupled anti-mouse antibody. The catalytic subunit of PKA was detected using a polyclonal antibody [30], a generous gift from F. Traincard (Pasteur Institute, Paris) and peroxidase coupled anti-rabbit antibody. Protein bands were visualized using an ECL Super Signal detection kit (Pierce). Total PKA activity was assayed as previously described [31], using extract from bacterially grown *Dictyostelium* cells. The PKA activities are expressed as the amount of phosphorylation of the Kemptide substrate that can be inhibited by the PKA inhibitor PKI.

Authors' contributions

MVC carried out most of the experimental work and wrote the manuscript. CA participated in genome analysis and performed PKA assays. RHI coordinated the study and participated in manuscript correction.

Acknowledgements

References

1. Clarke M, Yang J, Kayman S: **Analysis of the prestarvation response in growing cells of *Dictyostelium discoideum*.** *Dev Genet* 1988, **9**:315-326.
2. Clarke M, Dominguez N, Yuen IS, Gomer RH: **Growing and starving *Dictyostelium* cells produce distinct density-sensing factors.** *Dev Biol* 1992, **152**:403-406.
3. Gomer RH, Yuen IS, Firtel RA: **A secreted 80 × 10³ Mr protein mediates sensing of cell density and the onset of development in *Dictyostelium*.** *Development* 1991, **112**:269-278.
4. Yuen IS, Jain R, Bishop JD, Lindsey DF, Deery WJ, van Haastert PJM, Gomer RH: **A density-sensing factor regulates signal transduction in *Dictyostelium*.** *J Cell Biol* 1995, **129**:1251-1262.
5. Primpke G, Iassonidou V, Nellen W, Wetterauer B: **Role of cAMP-dependent protein kinase during growth and early development of *Dictyostelium discoideum*.** *Dev Biol* 2000, **222**:101-111.
6. Souza GM, Lu S, Kuspa A: **YakA, a protein kinase required for the transition from growth to development in *Dictyostelium*.** *Development* 1998, **125**:2291-2302.
7. Chae S-C, Inazu Y, Amagai A, Maeda Y: **Underexpression of a novel gene, Dia2, impairs the transition of *Dictyostelium* cells from growth to differentiation.** *Biochem Biophys Res Commun* 1998, **252**:278-283.
8. Nagasaki A, Sutoh K, Adachi H, Sutoh K: **A novel *Dictyostelium discoideum* gene required for cAMP-dependent cell aggregation.** *Biochem Biophys Res Commun* 1998, **244**:505-513.
9. Kon T, Adachi H, Sutoh K: **AmiB, a novel gene required for the growth/differentiation transition in *Dictyostelium*.** *Genes to Cells* 2000, **5**:43-55.
10. Souza GM, da Silva AM, Kuspa A: **Starvation promotes *Dictyostelium* development by relieving PufA inhibition of PKA translation through the YakA kinase pathway.** *Development* 1999, **126**:3263-3274.
11. Hirose S, Inazu Y, Chae S-C, Maeda Y: **Suppression of the growth/differentiation transition in *Dictyostelium* development by transient expression of a novel gene, dia1.** *Development* 2000, **127**:3263-3270.
12. Zeng C, Anjard C, Riemann K, Konzok A, Nellen W: **Gdt1, a new signal transduction component for negative regulation of the growth-differentiation transition in *Dictyostelium discoideum*.** *Mol Biol Cell* 2000, **11**:1631-1643.
13. Zeng C, Anjard C, Primpke G, Wetterauer B, Wille S, Nellen W: **Interaction of gdt1 and protein kinase A (PKA) in the growth-differentiation-transition in *Dictyostelium*.** *Differentiation* 2000, **67**:25-32.
14. **The *Dictyosteluim* Database** [http://dictybase.org]
15. Wetterauer B, Jacobsen G, Morandini P, MacWilliams H: **Mutants of *Dictyostelium discoideum* with defects in the regulation of discoidin-I expression.** *Dev Biol* 1993, **159**:184-195.
16. Blusch J, Nellen W: **Folate responsiveness during growth and development of *Dictyostelium* – separate but related pathways control chemotaxis and gene regulation.** *Mol Microbiol* 1994, **11**:331-335.
17. Marin FT: **Regulation of development in *Dictyostelium discoideum* : I. Initiation of the growth to developmental transition by amino acid starvation.** *Dev Biol* 1976, **48**:110-117.
18. Simon MN, Pelegrini O, Veron M, Kay RR: **Mutation of protein kinase-A causes heterochronic development of *Dictyostelium*.** *Nature* 1992, **356**:171-172.
19. Mann SKO, Firtel RA: **A developmentally regulated, putative serine/threonine protein kinase is essential for development in *Dictyostelium*.** *Mech Devel* 1991, **35**:89-101.
20. Chijiwa T, Mishima A, Hagiwara M, Sano M, Hayashi K, Inoue T, Naito K, Toshioka T, Hidaka H: **Inhibition of forskolin-induced neurite outgrowth and protein phosphorylation by a newly synthesized selective inhibitor of cyclic AMP-dependent protein kinase, N-[2-(p-bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide (H-89), of PC12D pheochromocytoma cells.** *J Biol Chem* 1990, **265**:5267-5272.
21. Wong AO, Le Drean Y, Liu D, Hu SJ, Du , Hew CL: **Induction of Chinook salmon growth hormone promoter activity by the adenosine 3'-5'-monophosphate (cAMP)-dependent pathway involves wo cAMP-response elements with the CGTCA motif and pituitary-specific transcription factor Pit-1.** *Endocrinology* 1996, **137**:1775-1784.
22. Anjard C, van Bemellen M, Reymond CD, Véron M: **A new spore differentiation factor (SDF) secreted by Dictyostelium cells is phosphorylated by the cAMP dependent protein kinase.** *Differentiation* 1997, **62**:43-49.
23. Van Driessche N, Shaw C, Katoh M, Morio T, Sucgang R, Ibarra M, Kuwayama H, Saito T, Urushihara H, Maeda M, Takeuchi I, Ochiai H, Eaton W, Tollett J, Halter J, Kuspa A, Tanaka Y, Shaulsky G: **A transcriptional profile of multicellular development in *Dictyostelium discoideum*.** *Development* 2002, **129**:1543-1552.
24. Clarke M, Kayman SC, Riley K: **Density-dependent induction of discoidin-I synthesis in exponentially growing cells of *Dictyostelium discoideum*.** *Differentiation* 1987, **34**:79-87.
25. Clarke M, Gomer RH: **PSF and CMF, autocrine factors that regulate gene expression during growth and early development of *Dictyostelium*.** *Experientia* 1995, **51**:1124-1134.
26. Primpke G, Salger K, Wetterauer B: **Absence of complementation in non-allelic mutants of *Dictyostelium discoideum* with defects in the transition from growth to development.** *Mol Genet Genomics* 2001, **266**:396-405.
27. Williams RS, Eames M, Ryves WJ, Viggars J, Harwood AJ: **Loss of a prolyl oligopeptidase confers resistance to lithium by elevation of inositol (1,4,5) trisphosphate.** *EMBO J* 1999, **18**:2734-2745.
28. Kuspa A, Loomis WF: **Tagging developmental genes in *Dictyostelium* by restriction enzyme-mediated integration of plasmid DNA.** *Proc Natl Acad Sci USA* 1992, **89**:8802-8807.
29. Wilkins A, Khosla M, Fraser DJ, Spiegelman GB, Fisher PR, Weeks G, Insall RH: ***Dictyostelium* RasD is required for normal phototaxis, but not differentiation.** *Genes Dev* 2000, **14**:1407-1413.
30. Chevalier M, de Gunzburg J, Veron M: **Comparison of the regulatory and catalytic subunits of cAMP dependent protein kinase from Dictyostelium discoideum and bovine heart**

using polyclonal antibodies. *Biochem Biophys Res Commun* 1986, **136:**651-656.

31. Anjard C, Soderbom F, Loomis WF: **Requirements for the adenylyl cyclases in the development of** *Dictyostelium*. *Development* 2001, **128:**3649-3654.