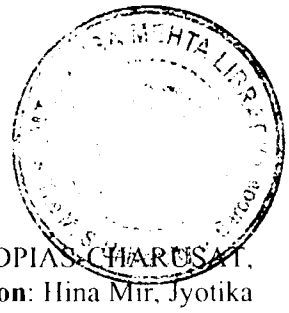

Presentations

International conference on **Molecular Medicine-2011** held at PDPIAS-CHARUSAT, Anand, 9th-11th January, 2011. **Best poster selected for oral presentation**: Hina Mir, Jyotika Rajawat and Begum R, Staurosporine induced cell death in *D. discoideum* is independent of PARP.

XXXIII **All India Cell Biology Conference** 10-13th December, 2009 at Central University of Hyderabad. **Poster presentation: Hina Mir**, Jyotika Rajawat, Iqbal Vohra, Payal Sharma, Milan Vachhani and Begum R, Involvement of Poly ADP-ribose polymerase (PARP) and Apoptosis Inducing Factor (AIF) in starvation induced cell death in *D. discoideum*.

National symposium for **Young Explorers in Indian Biology (YEIB)** TIFR, Mumbai, 14-16th September 2009. **Poster presentation: Hina Mir**, Iqbal Vohra, Jyotika Rajawat, Kaushal Patel and Begum R, Response of *Dictyostelium discoideum* to UV-C and involvement of Poly (ADP-ribose) polymerase.

International symposium of **developmental biology** held on 18-19th October, 2007 at Holiday Inn, Agra. **Poster presentation: Hina Mir**, Jyotika Rajawat, Jayesh Davda, Iqbal Vohra, Mansi Arora and Begum R, Role of Poly (ADP) ribose polymerase in UVC induced cell death in *D. discoideum*.

National Symposium on ***Apoptosis and Cancer***, organized at Department of Biochemistry, MSU, Baroda, December 28-29, **2007.**

National symposium on **development dynamics** organized by international symposium on developmental biology on 22-24 November, 2005, University of Kalyani, Kalyani, West Bengal. Poster presentation: Iqbal Vohra, Dipti Khanna, **Hina Mir**, Begum R "Effect of oxidative stress on *Dictyostelium discoideum* growth, development and cell death"

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Review Article

Signaling molecules involved in the transition of growth to development of *Dictyostelium discoideum*

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The social amoeba *Dictyostelium discoideum*, a powerful paradigm provides clear insights into the regulation of growth and development. In addition to possessing complex individual cellular functions like a unicellular eukaryote, *D. discoideum* cells face the challenge of multicellular development. *D. discoideum* undergoes a relatively simple differentiation process mainly by cAMP mediated pathway. Despite this relative simplicity, the regulatory signaling pathways are as complex as those seen in metazoan development. However, the introduction of restriction-enzyme-mediated integration (REMI) technique to produce developmental gene knockouts has provided novel insights into the discovery of signaling molecules and their role in *D. discoideum* development. Cell cycle phase is an important aspect for differentiation of *D. discoideum*, as cells must reach a specific stage to enter into developmental phase and specific cell cycle regulators are involved in arresting growth phase genes and inducing the developmental genes. In this review, we present an overview of the signaling molecules involved in the regulation of growth to differentiation transition (GDT), molecular mechanism of early developmental events leading to generation of cAMP signal and components of cAMP relay system that operate in this paradigm.

Keywords: *Dictyostelium discoideum*, GDT, Signal transduction, cAMP

troduction

discoideum often referred as 'slime mould' or ɔcial amoeba', is one of the simplest studied karyotes that possesses true multicellularity[1]. *D. scoideum* amoebae grow and divide asexually while eding on bacteria or a defined medium. The most ımmon stress that *D. discoideum* encounters is ıtrient depletion and responds to it by shutting down owth and cell division and initiating a velopmental program. Its development shows much the complexity seen in a metazoan. One ndamental difference is that metazoans develop ɔm a single cell, the zygote by a combination of cell vision, growth and differentiation but *D. discoideum* velopment, requires no growth and multicellularity achieved by aggregation of many unicellular ıoebae. Developmental fate of the cells is termined by the cell cycle phase amongst other rious factors[2-5].

Initiating events of *D. discoideum* development clude sensing starvation and cell density, which in rn results in the isolated cells acquiring the ability to gregate. The mechanism of density sensing by ırved cells ensures that aggregation occurs only ıen there are sufficient number of starved cells to form aggregates and subsequent structures of appropriate size for optimized spore dispersal[6-8]. There are reviews discussing the molecular aspects of late stages of *D. discoideum* development, but not on the early events of development. Recent investigations have revealed the involvement of several components in regulating the initiation of development[9-12], however little information exists on how the cells exactly sense starvation and in particular amino acid deprivation. This review discusses about the signaling molecules involved in the early development of *D. discoideum* with an attempt to address the molecular events associated in sensing amino acid starvation.

Pre-starvation facto (PSF)

Throughout the vegetative growth, *D. discoideum* amoebae secrete an autocrine factor known as pre-starvation factor (PSF). It is a 68 kDa glycoprotein that is secreted while cells are in growth phase and accumulates as an indicator of the ratio of the cell density to the food supply[7,13]. The PSF response is inhibited by the bacteria used as a food supply, however when the bacterial population drops, the PSF inhibition is relieved, and PSF induces genes such as discoidin-I and cyclic nucleotide phosphodiesterase (PDE) that trigger the developmental process[14] (Fig. 1). Discoidin-I is a soluble lectin synthesized by

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aggregating cells which helps in adhesion of the cells to substratum[15] and its expression acts as a marker for growth to differentiation transition. Nevertheless, once the nutrients are depleted, PSF production declines and a second cell density sensing pathway mediated by conditioned medium factor (Fig. 2) gets activated[7,8,12].

Conditioned medium factor (CMF)

Cells coordinate their development so that aggregation occurs only when the density of starved cells is sufficiently high (about ~10^5 cells/cm^2). The starved cells differentiate when present at high density, whereas cells at low density generally do not differentiate. CMF, a 80 kDa glycoprotein is sequestered in vegetative cells but it is secreted upon starvation regardless of the cell density[7]. Accumulation of CMF into the medium is also affected by *p*H, light, cAMP pulses or cell cycle phase at the time of starvation, but CMF itself is the major factor that potentiates its own accumulation. Observed cell density necessary for differentiation matches with the diffusion calculation predicted cell density. The behavior of cells at different cell densities and the accumulation rate, diffusion coefficient, and activation threshold of CMF suggest that it serves as a part of cell density sensing system allowing *D. discoideum* cells to coordinate the onset of cAMP pulse mediated aggregation[16]. CMF initiates signal transduction pathway (Fig. 2) that amplifies at the cAMP level[8].

CMF increases the frequency of pseudopod formation and hence is important for chemotaxis. In the presence of high levels of CMF, the cAMP pulse causes the cell to chemotax towards cAMP, relay cAMP signal and express specific genes. CMF is necessary for the cAMP induced Ca^{2+} influx, activation of adenylyl cyclase (ACA) and guanylyl cyclase (GCA) (Fig. 3). Binding of cAMP to cAR1 causes a transient influx of Ca^{2+} and activates an associated heterotrimeric G protein. CMF among

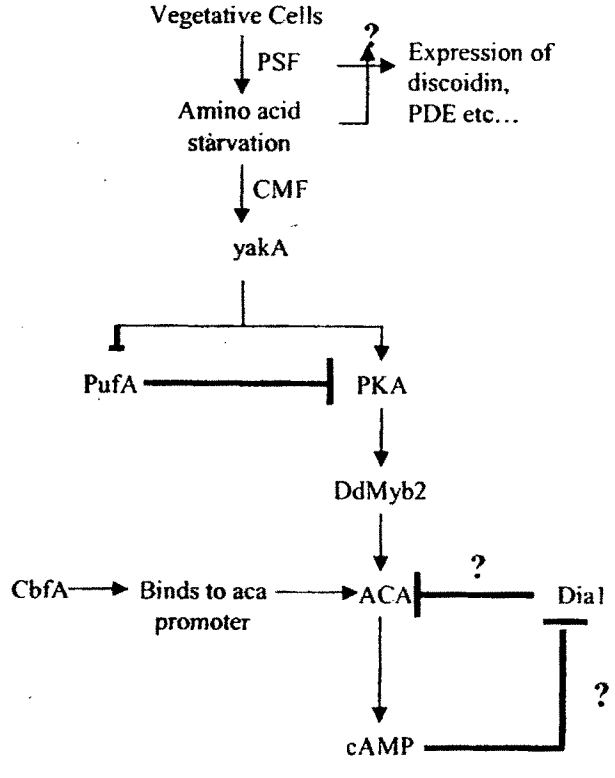


Fig. 1—A schematic description of the pathway controlling onset of *D. discoideum* development. PSF after sensing amino acid starvation leads to Yak A activation, releasing negative control of Puf A on *pka-C* mRNA translation which consequently results in increased production of cAMP by adenylyl cyclase. Question marks indicate the uncharacterized aspects of *D. discoideum* development.

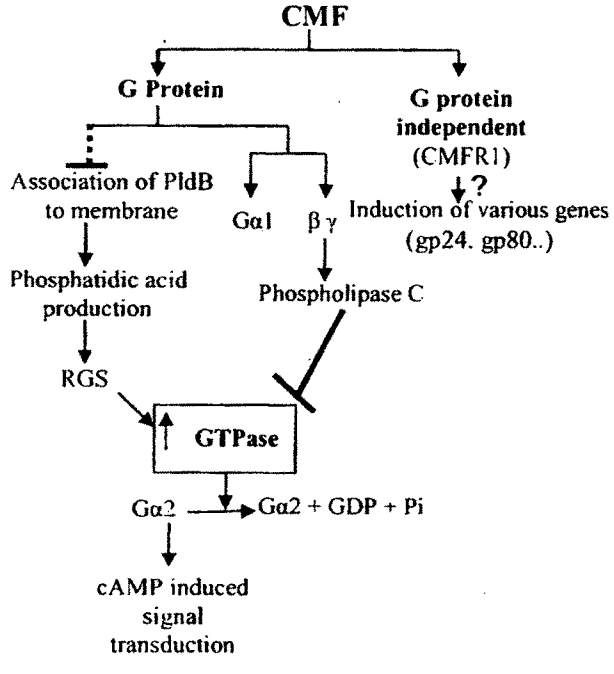
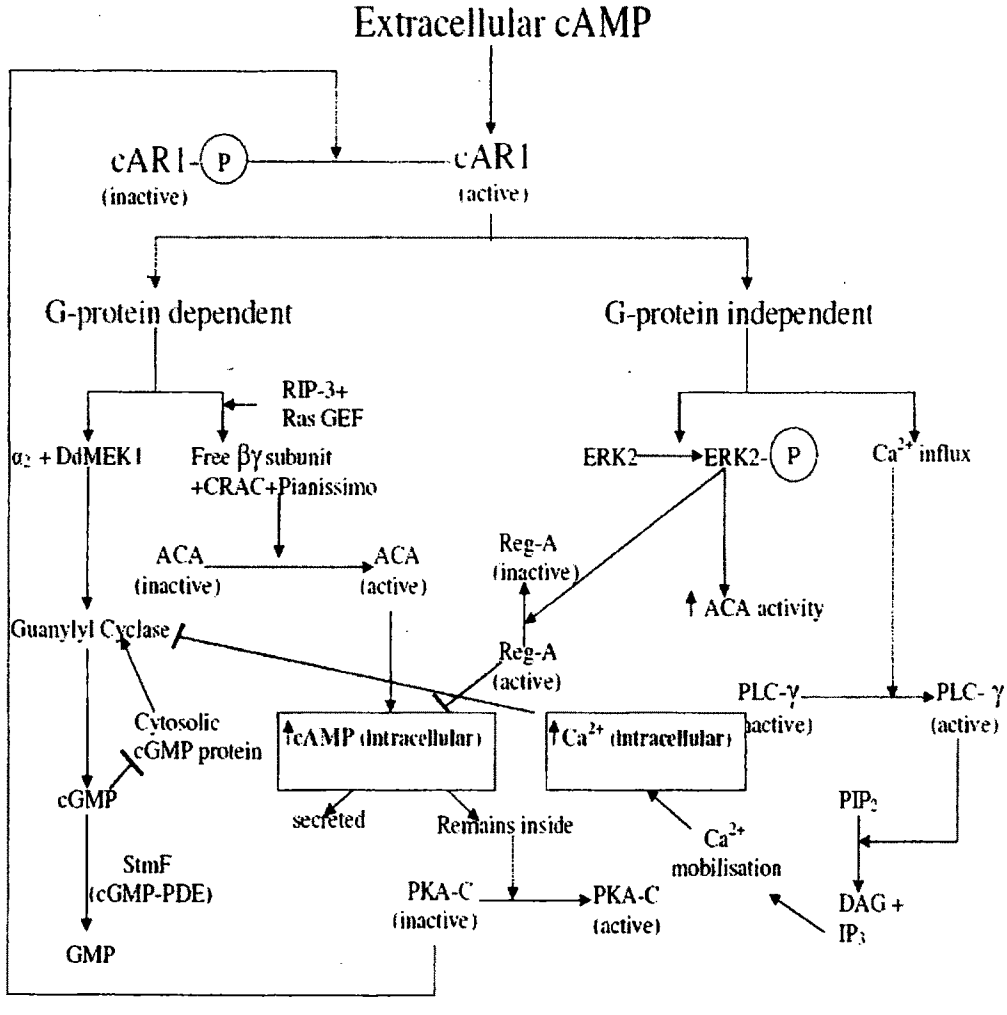


Fig. 2—Two hypothesized signal transduction pathways induced by CMF to mediate the cAMP induced chemotaxis in *Dictyostelium discoideum* cells. CMF has both G protein dependent and independent actions by which it affects cAMP signal transduction and gene expression, respectively. CMF either affects the GTPase by regulating the RGS activity through phospholipase D or by modulating phospholipase C activity through G beta-gama. The question mark indicates the uncharacterized aspect/s and the dotted line indicates an unknown mechanism.

ers regulates CRAC (cytosol regulator of adenylyl lase), which in turn assists the Gβγ subunit to ısiently activate ACA either by modulating its vity or by changing its subcellular localization, ile the Gα2 subunit activates GCA. Thus CMF liates activation of guanylyl cyclase and adenylyl lase via the cAMP receptor 1 (Fig. 3). Regulation of nylyl and adenylyl cyclases is independent, as tants lacking adenylyl cyclase have normal cAMP nulated guanylyl cyclase activation, and *vice versa*[8]. CMF signal transduction also involves G protein ıpled receptor as GTPγS partially inhibits the ding of CMF to membranes (Fig. 2). Gα1 null cells to show this GTPγS induced inhibition or CMF ulation of cAMP signal transduction, which indicates that a putative CMF receptor interacts with Gα1[17].

CMF affects cAMP signal transduction by regulating the Gα2-GTP conformation. The CMF leads to Gα1/βγ dissociation and thereby activates phospholipase C (PLC) (Fig. 2). Activated PLC inhibits the Gα2-GTPase and hence stabilizes the cAMP activated Gα2-GTP, ultimately promoting the cAMP signal transduction process[17,18]. Another hypothesis for the regulation of the GTPase activity by CMF involves RGS (Regulator of G protein signaling) protein and PldB (a phospholipase D). RGS proteins act as GTPase activating proteins for heterotrimeric G proteins and are regulated by phosphatidic acid (PA) produced by PldB (Fig. 2).



3—An overview of signal transduction pathway induced by cAMP during *D. discoideum* development. Detailed outline of G-protein ɔndent and independent pathways triggered by cAMP pulses are shown. G-protein dependent pathway mainly leads to an increase in ntracellular cAMP, whereas G-protein independent pathway results in increased intracellular Ca^{2+} levels. However, the two cascades ross talk at some points and the effects may not be mutually exclusive.

PldB is found to be indispensable for the proper aggregation as cells lacking *pldB* aggregate at even low cell density bypassing the need for CMF, while *pldB* overexpressing cells do not aggregate at high cell density, and neither do such cells respond to exogenous addition of CMF. PldB has a PH domain (phospholipid binding domain), which is responsible for translocating proteins bearing the PH domain to the plasma membrane during chemotaxis. PldB (through localization provided by its PH domain) could be involved in the localized regulation of Gα2, the G protein mediated cAMP chemotaxis. This localized control of G protein activity could influence the polarization of chemotaxing cell. It is also hypothesized that CMF could decrease the PldB association with the membrane, leading to decreased PA production. This would consequently decrease RGS activity and thereby allowing increased signaling through Gα2 (Fig.2). Also *pldB* null cells show higher levels of *cAR1* expression earlier than the wild type cells, which indicates that PldB plays an important role in the timing of development[19].

CMF also has a role in the induction of pre-stalk and prespore gene expression along with cAMP in a G protein-independent manner. A 50 kDa membrane protein, CMFR1, binds to CMF during affinity purification and may be responsible for these effects of CMF at the gene level (Fig. 2). Heterologous expression of *cmfr1* leads to increased CMF binding while disruption of *cmfr1* in *D. discoideum* cells leads to about 50% reduction in CMF binding and all of its associated G-independent signal transduction[20]. This limited effect of *cmfr1* disruption exclusively on G-protein independent functions suggests that CMF has more than one receptor[21]. CMFR1 upregulates cAMP binding and is involved in the regulation of gene expression in growth to differentiation transition. Among others gp24 and gp80 are under CMFR1 control (Fig. 2); gp24 is important for the initial formation of filopodia mediated intercellular contacts and aggregation, whereas gp80 is involved in regulating cellular streaming. Thus, CMF is required for proper aggregation of *D. discoideum* cells under starvation (Fig. 2).

Myb2

DdMyb2, a transcription factor contains three Myb repeats, a DNA-binding helix-turn-helix motif, a potential nuclear localization signal, two potential PKA-C phosphorylation sites, and glutamine-, proline- and acidic amino acid-rich regions of Myb-related transcription factors. *DdMyb2* null cells show undetectable levels of adenylyl cyclase *(ACA)* transcript and no cAMP production. Ectopic expression of *aca* rescues differentiation and morphogenesis of *DdMyb2* null mutants suggesting that development in *D. discoideum* starts by starvation-mediated DdMyb2 activation. Protein kinase A (PKA-C) might be involved in starvation-mediated DdMyb2 activation and thus regulating its translocation to the nucleus, which then binds to the upstream region of *aca* gene and induces adenylyl cyclase expression (Fig. 1). The adenylyl cyclase A thus formed produces the very first few molecules of extracellular cAMP that induces chemotaxis and aggregation in neighboring cells. Intercellular signaling by secreted cAMP then induces expression of other genes required for further stages of development (Fig. 3). Thus DdMyb2 that mediates the initial induction of adenylyl cyclase seems to play a central role in the growth to development transition in *D. discoideum*[22].

Several genes have been found to regulate the growth to differentiation transition (GDT) in *Dictyostelium discoideum*. Positive regulators of GDT are *yakA, pka, dia2, amiA* and *amiB* while negative regulators are *pufA, dia1 and gdt2*.

YakA

To begin differentiation cells must reach a specific point in G2 phase of the cell cycle and specific cell cycle regulators would facilitate the exit from cell cycle and mediate transition from growth to development. One such factor is YakA. YakA is a cytosolic protein kinase which phosphorylates itself as well as myelin basic protein. *yakA* is expressed at low levels in all cells during growth phase, and peaked at the onset of starvation and then decreases but is present throughout the development. *yakA* null cells have faster and smaller cell cycle compared to wild type, suggesting a role for YakA in cell cycle regulation and coordination between growth and cell cycle progression[11]. Pronounced defect in actin polymerization and cGMP accumulation in *yakA* null cells and the phenotypic similarity between *yakA*$^-$ and *gβ*$^-$ cells suggests that YakA has a role in G-protein mediated signaling pathways. *yakA* overexpression caused a growth arrest in *gβ*$^-$ cells and cAMP receptors are still coupled to G-proteins in the *yakA* mutant suggesting that YakA operates downstream to Gβ[23]. Studies with temperature sensitive *yakA* mutants suggest that YakA is not only required during onset but throughout the development.

The conditioned medium containing PSF induces : accumulation of yakA mRNA in wild type cells g. 1), suggesting that *yakA* expression may be ıtrolled by PSF. PSF signaling is independent of *kA* as discoidin-I was expressed normally in *yakA* ll cells. The regulation of YakA by PSF might ›vide a way for the cells to coordinate nutrient ıilability with cell division (Fig. 1).

YakA has a dual role in starvation sensing- growth est and induction of PKA-C. YakA controls cell ision during growth by ensuring that cells are of ›per size before they divide and also regulates the erval between two cell divisions. Overexpression of *kA* arrests the growth of *Dictyostelium* cells in G2 ase[11]. Induction of *yakA* upon starvation leads to a crease in vegetative phase gene expression such as *rD* and induces the expression of *pka, aca* and *R1*. Induction of *yakA* consequently relieves PufA diated translational block on pkaC mRNA (Fig.1). ıring the first 4-6 h of development there is about 5 d increase in *pkaC* mRNA, PKA-C protein and its kA mediated activation[9].

PKA-C controls the timing of early developmental ents by regulating expression of the key cAMP naling proteins such as cAR1 and ACA through yb2[22,24,25] (Fig. 1). It is possible that PKA-C or MP signaling is required for YakA mediated ponse during development but neither of them is quired for YakA induced growth arrest[11]. PufA is : key effector of YakA starvation response pathway ding to multicellular development.

fA

PufA is a translational regulator belonging to Puf ›tein family. PufA binds to the PufA regulatory ments (PREs) present in the 3′ end of *pkaC* mRNA l regulates its translation directly. There is an erse relationship between PKA-C protein and pufA pka-c PRE complex (Fig. 1). *pufA* null cells rescue velopmental defects in yakA null cells and have no vious cell cycle defect. *pufA* null mutation does not er the fast growth phenotype of *yakA* mutants nor ınge the growth rate of wild type cells but *yakA* ll and *pufA* null double mutants showed accelerated velopment during the initial hours of starvation npared to wild type cells. Thus inactivation of fA restores developmental gene induction but not growth phase gene repression in *yakA* null cells. ctivation of *pufA* rescues early developmental ges of *yakA* null cells but exhibit arrestation at culmination phase[9]. This reflects additional function for PufA during later stages of development, which is yet to be studied. Thus, the possible mechanism is that YakA shuts off *pufA* expression at the onset of development, therefore YakA is the master regulator switch between vegetative and developmental gene expression. Inhibition of PufA by YakA relieves the negative control on PKA-C thereby causing an increase in cAMP production via adenylyl cyclase and thus leads to development.

Gdt2

Gdt2 is a serine/threonine protein kinase and it is expressed in vegetative cells and also throughout development at the same level but with a slight peak at the time of aggregation. *gdt2* null mutants develop prematurely and in such cells discoidin can be detected at a density of 1×10^5 cells. As these cells can sense folate, it is more likely that *gdt2* null mutants have an impaired mechanism in sensing amino acid/s. *gdt2* null mutants have no effect on PKA activation, suggesting that Gdt2 is downstream to PKA[26]. Gdt2 is involved in the control of growth to differentiation transition via an unknown pathway.

Dia1

Cells in any phase of the cell cycle can start differentiating from PS (Putative Shift) point. Several genes are expressed in response to initial differentiation from PS point. One such gene is *dia1*, which is expressed at 2h after starvation, reaching a peak at 4h followed by a rapid decrease in its levels. *dia1* is adjacent to *impA* on chromosome 4, with their start codons separated by intergenic region of 654 bp. It is an example of bidirectional regulation where intergenic region regulates the expression of these two genes during growth and development. These two genes are inversely expressed before and after GDT. There are three regions within this 654 bp sequence, which play significant role in the regulation of *dia1* and *imp* gene. A pair of 7-bp direct repeats in the 92 bp region proximal to *impA* is essential for expression of both the genes. Middle region is essential for repression of *dia1* expression in growing cells. This region contains a sequence, which is recognized by a repressor and blocks transcription of *dia1* but not of *impA* during growth. This repressor disappears following a shift from nutrient to starving medium[27]. Expression of *dia1* is transient and is seen only during initial stage of development. *dia1* overexpression

suppresses the progression of differentiation and such cells showed delay in aggregation (and some of them could not even aggregate), but the fruiting body formed by such cells was found to be normal. Antisense mediated gene inactivation of *dia1* has shown to enhance the progression of differentiation. Such cells become aggregation competent and formed aggregates within 5.5 h compared to 8 h in control cells. *dia1* expression affects genes involved in cAMP signaling and its overexpression reduces the expression of cAR1 upto 2-4 h, while *aca* mRNA was expressed weakly after 4-6h of starvation. Underexpression of *dia1* exhibited the precocious expression of cAR1 and *aca*. Thus *dia1* plays an inhibitory role during early development by reducing the expression of cAR1 and *aca* genes of cAMP relay system (Fig. 1). DIA1 protein seems to be negatively coupled with cAR1 and ACA associated events but exact mechanism is yet to be elucidated. Developmental defect of *dia1* overexpressing cells were nullified by mixing these cells with the wild type cells. cAMP pulses also restored the delayed aggregation of *dia1* overexpressing cells, suggesting that cAMP secreted from wild type differentiating cells would remove the inhibitory effect of DIA and allow *dia1* overexpressing cells to develop normally. Thus, cAMP most likely acts as a suppressor of the DIA1 function[28] (Fig. 1). DIA2 and DIA3 are also required for proper expression of early genes such as cAR1 and *aca*[29].

AmiA and AmiB

AmiA and AmiB positively regulate the GDT possibly via regulation of adenylyl cyclase expression[28]. AmiB is also necessary for aggregation as *amiB* null cells failed to repress the vegetative gene *cprD* (a cysteine proteinase) during growth, suggesting that they cannot sense starvation[10]. *amiB* null cells exhibited changes in the distribution of actin, Apr and myosin II resulting in defective locomotion possibly due to altered cytoskeletal regulation[30]. Genetic studies suggested that amiA is involved in bridging communication between cAMP receptor and adenylyl cyclase[31]. Interestingly, *amiA* null cells also showed partial disruption in cell division[32]. The exact mechanism of action of AmiA and AmiB in the control of growth to differentiation transition is yet to be studied.

CbfA

CbfA (C-module DNA binding factor) interacts *in vitro* with a regulatory element in retrotransposon TRE5-A. It is a transcription factor which binds to AT-rich target sites in *Dictyostelium* genome and regulates the expression of its target genes. CbfA is not required for pre-starvation response as yakA pathway functions normally in *cbfA*^{am} amber mutant cells. It seems to act downstream of the yakA pathway (Fig. 1) and controls transcription of *acaA* and other genes directly or indirectly. CbfA depleted cells were unable to aggregate and *cbfA*^{am} cells failed to activate cAMP induced genes in early development. *cbfA*^{am} cells when supplied with cAMP pulse, causes induction of *acaA* and showed further development. Sensing of cell density and starvation are independent of CbfA[33], however, CbfA binds to the *acaA* promoter to provide a basal transcription activity that is required for induction of *acaA* expression after the onset of *D. discoideum* development[34].

Protein kinase (PKA)

PKA, a cAMP dependent protein kinase plays multiple roles during *D. discoideum* development and it is the central component in signal transduction pathway. It phosphorylates a variety of proteins and thereby affects their activity. Inactive form of PKA consists of catalytic subunit (PKA-C) associated with regulatory subunit (PKA-R). Exponentially growing *D. discoideum* cells consist of both the subunits, as PKA is not required for growth. There is a five-fold increase in catalytic subunit levels in the first 6 h of development and is maintained till culmination[9]. The signal transduction pathway that initiates from cell surface binding of cAMP to accumulation of mRNA appears to act through PKA (Fig. 1) since *acaA* is not expressed in *pkaC*-null cells. PKA plays a central role in timing of the burst of adenylyl cyclase activity. PKA regulates this adenylyl cyclase activity by phosphorylating adenylyl cyclase or any of its coupling components. PKA is regulated by *regA* which encodes a phosphodiesterase that can reduce the cAMP available to PKA-R and hence free form of PKA-R will associate with PKA-C resulting in an inactive form[35] (Fig. 3).

cAMP

In the social amoeba *D. discoideum*, cAMP via PKA controls almost all the major life cycle transitions including growth to development transition. Aggregation of *D. discoideum* amoebae into multicellular structures is organized by cyclic AMP (cAMP), which acts as a chemo-attractant, as a

:ond messenger, and as a morphogen. Once the MP accumulates in sufficient amount it triggers the scade of events (Fig. 3).

mponents of the cAMP relay system

During starvation, *D. discoideum* amoebae become ponsive to cAMP, which is released in a pulsatile hion and governs the process of aggregation. The lls achieve competence to relay cAMP signals thin a period of 6 h of starvation. Initially at lower ncentration of cAMP, the receptors undergo citation leading to a cascade of processes, but as the MP concentration rises extracellularly, the eptors become desensitized due to the odification/sequestration/internalization/degradation receptors/uncoupling of receptors and target oteins, etc. Desensitization can be reversed if the MP signal remains absent for a fixed period of time d this reversal is facilitated by the removal of MP by extracellular phosphodiesterase (ePDE)[36] ig. 3). Rapidly developing (rde) mutants of *ctyostelium discoideum*, in which cells precociously ferentiated into stalk and spore cells without rmal morphogenesis, were investigated genetically d biochemically. Genetic complementation tests monstrated that the rde mutants could be classified o at least two groups (groups A and C). easurements of cell-associated and extracellular osphodiesterase activities and intracellular and total MP levels revealed that cAMP metabolism in both oups are significantly altered during development. oup A mutants showed precocious and excessive oduction of phosphodiesterase and cAMP during entire course of development; intracellular cAMP vels in group C mutants were extremely low, and ore and stalk cell differentiation occurred without apparent increase in these levels[37].

MP receptors

Serpentine G-protein-coupled cAMP receptors are key components in detection and relay of the tracellular cAMP waves that control chemotactic l movement during *D. discoideum* development. ring development the cells sequentially express ir closely related cAMP receptors of decreasing inity (cAR1-cAR4). Of these cAR1 and cAR3 are gh affinity receptors expressed before and during gregation, respectively[38,39], whereas cAR2 and R4 are low affinity receptors expressed after gregation in pre-stalk cells[40,41].

cAR1 is the first one to be expressed during early aggregation and its expression continues in the later stages of development in all cells. It is necessary for aggregation because cells lacking cAR1 fail to aggregate. cAR1 is involved in activation of ACA, GCA and ERK2 with nanomolar concentrations of cAMP[42-44] during aggregation. Expression of aggregative genes by cAMP pulses is mediated by cAR1[45]. Desensitization of cAR1 occurs due to prolonged stimulation of the receptors by micromolar concentration of cAMP, by internalization and degradation of the receptor[46]. The half time of this process is 15-30 min and once the cAMP stimulus is removed, it takes several hours for the receptor to reaccumulate. cAR3 shows the highest affinity for cAMP and it is expressed during aggregation. However, deletion of *cAR3* has no obvious phenotype[47]. In the slug, the expression of *cAR3* becomes confined to the pre-spore cells. Expression of *cAR2* starts at the mound stage in the cells forming prestalk zone. cAR4 is expressed in a pre-stalk specific manner at the slug stage. Deletion of cAR4 leads to defects during culmination[41]. This activation of ACA, GCA and ERK2 may also be brought about by other cARs (other than cAR1) during other developmental stages. cAMP induces an increase in the intracellular Ca^{2+} levels directly by increasing the Ca^{2+} influx[48,49] and indirectly by stimulating phospholipase C (PLC). The influx of Ca^{2+} can be induced by all four receptors[50]. cAMP signals can activate PLC in null mutants of cAR1/cAR3 in aggregative cells which do not show cAR2 and cAR4 expression. This suggests the possibility of Gα2 associated fifth cAR.

Adenylyl cyclases (AC)

Genes encoding three distinct adenylyl cyclases have been characterized and are shown to be expressed at different stages of *D. discoideum* development[51,52]. Adenylyl cyclase A (ACA), a G-protein-coupled adenylyl cyclase is one of the first genes to be expressed upon starvation. It produces extracellular cAMP, which is the signaling molecule required for the chemotaxis and aggregation of neighbouring cells. The osmosensory adenylyl cyclase, *acgA* is expressed only during germination of spores[51,53,54], while adenylyl cyclase R (ACR) produces internal cAMP necessary for terminal differentiation of spores[55]. ACG is required for the maintenance of spore dormancy and is known to have intrinsic osmosensing property.

ACA is activated when the G-protein coupled surface receptor CAR1 binds extracellular cAMP (Fig. 3). However, during culmination, ACR activity is tenfold higher than ACA activity. Studies showed that ACR activity is essential for morphogenesis as well as the maturation of spores[53,56,57]. Thus, *acrA* cannot fulfil the role of *acaA* in production of extracellular cAMP necessary for chemotaxis while *acaA* cannot fulfil the role of *acrA* in production of internal cAMP necessary for terminal differentiation of spores. The discovery of ACR helped to resolve many controversial observations on the roles of extra- and intra-cellular cAMP in the regulation of gene expression. ACR seems to be independent of G-proteins[52], and capable of activating PKA-C in the absence of ACA.

Developmental studies with *acaA* null cells showed that adenylyl cyclase is essential for the chemotactic response. The behavioral defects of *acaA* null cells were interestingly similar to those of null mutants of *regA*, which encodes the intracellular phosphodiesterase that hydrolyzes cAMP and, hence, functions opposite to adenylyl cyclase A (ACA). Thus ACA and RegA are components of a receptor-regulated intracellular circuit that controls protein kinase A activity (Fig. 3) and the suppression of lateral pseudopods in the front of a natural wave depends on a complete circuit. Hence, deletion of any component of the circuit (i.e., RegA or ACA) would result in the same chemotactic defect[58].

Guanylyl cyclases (GCAs)

The cAR1 activated Gα2 subunit leads to guanylyl cyclase activation[50,59]. Activated guanylyl cyclase causes an increase in cGMP concentration, which in turn leads to pseudopod extension via myosin phosphorylation[60,61] (Fig. 3). Null mutants of *cAR1* and *Gα2* do not show cAMP induced guanylyl cyclase activation. Activation of guanylyl cyclase by cAR1 requires Gα2 subunit and MAP Kinase-DdMEK1[62]. In addition to cAMP signal, activation of guanylyl cyclase requires binding to a cytosolic cGMP binding protein. When the concentration of cGMP is low this protein is free to bind to the enzyme and activate it, while at high concentration of cGMP it binds to cGMP thereby preventing the guanylyl cyclase activation. Increase in the concentration of cGMP due to activation of guanylyl cyclase is transient because cGMP is rapidly degraded by cGMP-phosphodiesterase (PDE), which is activated by cGMP itself. Also the activity of guanylyl cyclase is inhibited by Ca^{2+}at nanomolar range, which is easily achieved by Ca^{2+} influx and Ca^{2+} mobilization (Fig. 3). Adaptation at the receptor level also keeps cGMP concentration in check. There are also evidences indicating the presence of two different MAP kinase cascades involved in aggregation, one consists of ERK2, mediating the cAMP effects, and the other contains DdMEK1, essential for the cAMP mediated activation of guanylyl cyclase.

Phospholipase C (PLC)

The activated Gα2 subunit also activates PLC γ that catalyses the conversion of phosphatidylinositol-(4,5)-bisphosphate (PIP2) to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3), the latter product in turn leads to Ca^{2+} mobilisation causing an increase in intracellular Ca^{2+} concentration. However, a novel metabolic pathway independent of PLC has also been demonstrated[63]. Ca^{2+} is required for PLC activity, which could be provided either directly by inducing Ca^{2+} influx or indirectly by Ca^{2+} mobilization brought about by IP3 (Positive Feedback Effect) (Fig. 3). Contribution of Ca^{2+} requirement to the cAMP mediated regulation of phospholipase C activity is not clearly understood. IP3 can also be generated by breakdown of IP5[64,65] suggesting that IP3 signaling is important for aggregation, but evidence to the contrary exists, that IP3 dependent calcium signaling may not be required during aggregation[66]. The interaction between cAMP and CMF signaling involves IP3 and PLC activity (Fig. 3). CMF binding to its receptor activates PLC, while PLC inhibits the GTPase activity of Gα2, prolonging the lifetime of the response[17] (Fig. 2).

Gα2 null mutants do not show PLC activation, but *cAR1/cAR3* double null mutants show PLC activation in aggregation stage indicating the presence of fifth cAR and thus cAR5-Gα2 combination is important for the activation of PLC. 3'-deoxy-3'-aminoadenosine 3':5'-monophosphate (3'NH-cAMP) is a partial antagonist of cAMP which can only inhibit PLC. This inhibitory effect of 3'NH-cAMP is lost in cAR1 and Gα1 null mutants[64] suggesting that cAR1- Gα1 combination is involved in adaptation of PLC. Recently it has been identified that PLC also plays an essential function in germination of spores. Under adverse environmental conditions PLC activity is inhibited and so reduced IP3 levels prevent germination of spores. Inhibition of spore germination by high osmolarity is probably a dual control of ACG and PLC[67].

AP kinase ERK

Two MAP-kinase genes *erk A* and *erk B*, encoding ?K1 and ERK2, respectively, have been identified. ?K1 is required for vegetative growth and during ılticellular development[68], while ERK2 is essential r cell aggregation. ERK2 regulates the receptor ediated adenylyl cyclase activation however, it is ıt yet clear whether this activation is direct or direct. ERK2 mediates the coupling of extracellular muli such as cAMP and folic acid to adenylyl clases through two pathways (Fig. 3). In gregating cells exogenous cAMP leads to rapid and nsient activation of ERK2[50,51], in the presence of ıR1, this activation is also observed in Gα 4 null utants indicating the presence of novel receptor ediated pathway for ERK2 activation[69]. Activation ERK2 is under negative regulation of Ras signaling ıthway. PKA-C and CRAC are involved in laptation of ERK2[49,70]. *erkB* null mutants show gregation defect which is suppressed by *pka-c* erexpression, implying that PKA lies downstream ERK2 mediated responses[68] (Fig. 3). Apart from ese classical signaling molecules reactive oxygen ecies are also involved in the *D. discoideum* velopment. Interestingly, oxidative stress and UV-C adiation were found to affect the development of *discoideum*[71-73]. These aspects of *D.discoideum* are so very intriguing however, the details are beyond e scope of this review.

ounting Factor

In sporulating organism like *Dictyostelium scoideum* a balance is maintained for differentiating many spores as possible and supporting the spores stalks that are sufficiently tall and strong to elevate e spore mass above the substratum and to assist the spersion of spores to an environment that is richer in od. In response to cAMP pulse *D. discoideum* cells rm fairly uniform aggregates at a sufficient density, ggesting that a mechanism exists to restrict gregate size within an optimal range. Individual lls would be able to sense the number of cells in a oup by secreting and sensing a diffusible factor own as counting factor[13,74,75], which is required for oper function of a cell counting mechanism that gulates organism size. Counting factor is a large mplex of >450 kDa of at least five polypeptides, th molecular masses of about 60, 50, 45, 40, and 30 Da, and its oversecretion leads to the formation of ıaller fruiting bodies.

Computer simulations indicate that a stream stays intact if the cell-cell adhesion is high and the random cell motility forces are relatively low[76]. If the adhesion forces are less than the random motility forces, the cells will instead begin to disperse, disrupting the integrity of the stream. Thus the size of the groups depends inversely on the extent and length of time the adhesion forces are less than the motility forces[77,78]. cAMP stimulated cGMP pulse is repressed by CF (Fig. 4). Cells oversecreting CF have attenuated cGMP pulse whereas cells with mutated countin have increased cGMP compared to wild type cells. These results were further supported by the addition of anticountin-Ab to wild type cells which led to increased cGMP, while addition of recombinant countin yielded decreased cGMP levels. CF is able to bring back the group size to normal in streamer F cells (mutants forming abnormal fruiting bodies due to lack of cGMP PDE) and *countin* null cells show high GCA activity. Hence this repression of cGMP levels by CF is mediated by modification at the GCA activity level rather than any changes in the cGMP PDE activity (Fig. 4).

CF potentiates the cAMP-stimulated cAMP pulse without affecting the kinetics of the cAMP receptor, cAMP-induced GTP binding to membranes, the subsequent GTP hydrolysis, the GTPγS inhibition of cAMP binding, or the binding of the cytosolic regulator of adenylyl cyclase (CRAC) to membranes[76]. The binding of CRAC to membranes is due to cAMP activating a phosphatidylinositol 3-kinase, which creates phosphatidylinositol 3,4,5-

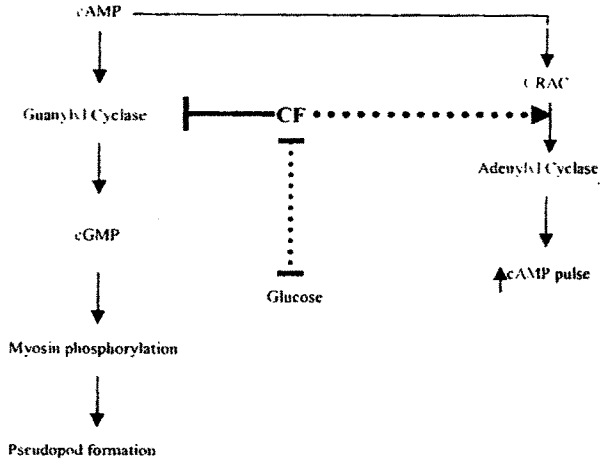


Fig. 4—A schematic representation of the counting factor (CF) induced regulation of chemotaxis and pseudopod formation during *D. discoideum* development. CF and glucose have opposing effects. Dotted lines indicate unknown mechanism of action of CF.

trisphosphate and phosphatidylinositol 3,4-bisphosphate on the inner surface of the plasma membrane; a pleckstrin homology domain on CRAC then binds to these lipids[79,80]. CF is not regulating group size by regulating the cAMP receptor or its activation of G proteins. It appears that CF regulates cAMP signal transduction at a step downstream of the cAMP receptor and G protein activation[81] (Fig. 4).

One of the component polypeptides of CF was purified and termed countin. Disrupting the expression of *countin* essentially abolishes CF activity, indicating that either countin is a key component of CF that directly affects cells or countin is simply a necessary part of the CF. Optimal concentration of recombinant countin did not cause an increase in group number or a significant decrease in adhesion as purified CF, suggesting that other components of CF are needed for a maximal change in group number. Purified CF potentiates the cAMP stimulated cAMP pulse within 60s while 60s exposure of cells to countin can decrease myosin polymerization and an increase in actin polymerization, myosin phosphorylation, and GTPγS stimulated activity of adenylyl cyclase. This suggests that countin, like CF, stimulates a rapid signal transduction pathway that has a direct effect on actin polymerization and a modulating effect on the cAMP receptor to adenylyl cyclase pathway (Fig. 4). *countin* null cells have a considerably higher cell-cell adhesion than parental cells. Also recombinant countin modulates the GTPγS stimulated activity of adenylyl cyclase without affecting the basal or Mn^{2+}-stimulated activities. CF possibly affects the cAMP-stimulated cAMP pulse at a step between the binding of CRAC to membranes and adenylyl cyclase[82] (Fig. 4).

Disrupting the expression of *cf50,* another component of CF, has essentially the same effect as disruption of *countin* with respect to group size, adhesion and motility, but unlike the effect of disrupting *countin*, disrupting *cf50* affects the initial cell-type choice. However, recombinant CF50 does not seem to increase group number to the extent that CF can. Thus neither CF50 nor countin is the sole effector molecule in the CF complex, but both the molecules can independently affect group size, as when the medium of countin null cells are immunodepleted with antibodies against CF50 or vice versa the cells form larger fruiting bodies compared to the single null cells.

Countin also shows its effect on cAMP pulse. One minute treatment of wild type cells with countin increases ACA activity by GTPγS and thereby increases cAMP pulse while *Erk2* is repressed. Effect of CF50 is found to be exactly opposite on these two factors. CF50 deletion reduces the percentage of CP2 positive (prestalk) cells and increases the percentage of SP70 positive (prespore) cells. Such changes in differentiation are not seen with countin deletion. Altogether these facts implicate that though countin and CF50 have negative effect on the group size, they have different and unique effects on the initial cellular differentiation and cGMP pulse and Erk2 activation. This implicates that countin and CF50 may activate two different signal transduction pathways, which have some different/unique effects, converging at some downstream point to give common effect on the group size[82,83].

Relation of CF and glucose has been unravelled to certain extent and this further elucidates the mechanism of group size regulation. Like CF, glucose affects stream breakup rather than altering territory size or mound breakup. Glucose partially negates the effects of countin and CF50 addition. Glucose affects two main downstream targets of CF, cell-cell adhesion and motility. Either increasing the glucose levels or decreasing CF would increase gp24 levels, adhesion, and myosin polymerization and decrease actin polymerization and motility. CF increases the cAMP induced cAMP pulse while addition of glucose decreases the cAMP pulse size. Also *countin* null cells have a large and prolonged cGMP pulse, whereas exposure of cells to high glucose results in prolonged pulse. All these facts together suggest that glucose per se or one of its metabolites may affect CF signal transduction pathway and the difference in cGMP pulse response in countin null cells and cells exposed to glucose points out that CF affects a pathway in addition to the one involving glucose[84]. Recently, CF has been shown to affect the activity of microsome associated glucose-6-phosphatase enzyme[85]. However, the importance of glucose-6-phosphatase and CF interaction in the regulation of the organism size is not yet clear.

CF45, a component of CF is expressed in vegetative and early developing cells and cells lacking CF45 form huge groups. Like *countin* null and *cf50* null cells, *cf45* null cells have high glucose levels, high cell-cell adhesion and low motility. Exogenous CF45 rescues the huge group size in *cf45* null cells to

me extent; however, the group size is not mparable to the wild type cells. High extracellular 'els of countin causes *cf45* null cells to form small)ups. *countin* null and *cf50* null cells oversecrete '45 than wild type yet, form larger groups. Thus '45 functions as a part of CF complex but not as the le factor to determine the group size and also that : three proteins, countin, CF45 and CF50, affect :h other's secretion or stability and they seem to ve overlapping as well as exclusive functions[86].

CF60, fourth component of the ~450 kDa CF mplex, has been recently identified[87]. After :retion of CF complex (counting factor) by the irving cells, CF60 dissociates from the complex in : absence of CF50. Its activity is dependent upon '50 while independent of countin. Decreased pression of CF60 also led to formation of large)ups while overexpression resulted in very small)ups[87].

Recently, an autocrine proliferation repressor,)rA has been identified in *Dictyostelium* system. iis 60 kDa protein has similarity to bacterial)teins of unknown function. It serves as a part of out 150 kDa complex. AprA has been reported to)w down the proliferation and thereby cell cycle and ordinate cytokinesis with mitosis. a*prA* null cells 'm larger fruiting bodies. The correlation between effect on growth and on the formation of larger iiting bodies is yet to be characterized. Similarity in enotypes between *yakA* null cells and *aprA* null lls poses an interesting question regarding the)ociation of these proteins in the AprA induced ;nal transduction pathway leading to regulation of :e during *Dictyostelium* development [88].

)nclusion

Relatively simpler life cycle of *D. discoideum* ikes it a good model organism for studying cellular)vement, chemotaxis, cell-cell interaction, cellular 'ferentiation and cell death. These processes are /olved during multicellular development. cAMP is : key molecule responsible for the signaling thways in *D. discoideum*. Binding of cAMP to its :eptor results in certain short term responses, which uld be G-protein dependent or G-protein lependent processes. G-protein independent)onses are due to the Ca^{2+} influx and osphorylation of cAR1[57,58] while the other cAMP)onses are mediated by hetero-trimeric G-protein. ng-term responses of cAMP include expression of certain genes which are consistent for cell specificity such as prestalk specific *rasD* gene. Most of the genes are induced by nanomolar pulses of cAMP while certain genes require mM levels. Changing levels of cAMP pulses are involved in regulating cAMP induced developmental gene expression[89]. Regulation of intracellular cAMP levels is also involved in pathways required for the pulses of ACA activation during aggregation. Activation and adaptation of ACA is normally mediated by cAR1. Evidence suggests that besides cAR1, three components such as DdMyb2, AmiB and PKA-C, function in the same or related pathways to regulate *aca* expression and many such molecules during transition from growth to differentiation are yet to be identified.

Though many aspects of the signal transduction pathways have been elucidated, yet there are some unanswered questions, which might be addressed well with the completed *Dictyostelium* genome sequence. The genome sequence reveals that *Dictyostelium* is complex, highly evolved and contains coding sequences for approximately 12,500 proteins[90] and as many as 20% of all predicted proteins in the *D. discoideum* genome are arranged in a number of large gene families that are involved in processes such as motility and signaling. *D. discoideum* cells are also accessible for imaging, and the use of tags such as green-fluorescent protein (GFP) fused to proteins of interest or certain fluorescent dyes (like DAPI)[91] allow to visualize their location during chemotaxis and to know the fate of cells during development under various stress conditions. Interestingly *D. discoideum* is known to exhibit caspase independent form of programmed cell death after differentiating into pre-stalk cells. Besides various developmental studies *D. discoideum* is also a good model organism to study evolutionary aspects of cell death under oxidative stress[92]. Such cell death mechanism would throw light on the evolutionary changes in programmed cell death.

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