A Frizzled like protein in *Dictyostelium discoideum*

INAUGURAL-DISSERTATION

zur
Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Universität zu Köln

vorgelegt von

Deenadayalan Bakthavatsalam
aus
Chennai, Indien

Köln, 2004

Referees/Berichterstatter: Prof. Dr. Angelika A. Noegel

Prof. Dr. Martin Huelskamp

Date of oral examination: 10.02.2005
Tag der mündlichen Prüfung

The present research work was carried out under the supervision of Prof. Dr. Angelika A. Noegel, in the Institute of Biochemistry I, Medical Faculty, University of Cologne, Cologne, Germany from Nov 2001 to Feb 2005.

Diese Arbeit wurde von Nov 2001 bis Feb 2005 am Biochemischen Institut I der Medizinischen Fakultät der Universität zu Köln unter der Leitung von Prof. Dr. Angelika A.Noegel durchgeführt.

Acknowledgements

In the preparation of this doctoral thesis I gratefully acknowledge the contribution made by one and all, supervisors, colleagues, well-wishers and friends.

First of all I would like to express my heartfelt gratitude to my supervisor Prof. Angelika Noegel for her diligent guidance and constant encouragement throughout the course of my study. She strove to seek the very best in me with her expertise and knowledge, which provided the backbone of this research. Thank you very much for giving me this privilege.

I am also greatly indebted to Dr. Ludwig Eichinger for his unfailing patience and valuable suggestions especially with all the microarray studies. Thanks a lot Ludwig for sparing your valuable time whenever needed.

I also owe so very much to Dr. Francisco for his continual support, enthusiasm and encouragement throughout the course of my work. Thank you Francisco for your invaluable support and timely help. Special thanks are due to Dr. Derrick and Dr. Schlatterer for helping with the characterization of the mutant.
I thank Dr. Gomer for useful discussions and my thanks also to Dr. Devreotes, Dr. Nellen and Dr. Gundersen for providing antibodies.

I would like to mention my special thanks to Patrick for his never-ending support in microarray preparations and analysis. My thanks are also due to Dr. Budi Tunggal for all the help with the computers. I thank the secretaries Bettina and Dörte for their help in administration issues.

I am also thankful to Maria, Berthold, Rosie, Kathrin, Rolf and Martina for helping in more than one way and contributing towards this work.

I also highly acknowledge the financial support received from the DFG.

I owe a special thanks to my friend Sunil for being beside me throughout my stay.

Last but not at all the least I would like to thank my parents for never losing faith in me and being by my side always. Thank you mom and dad for everything.

My thanks will not end without mentioning Sharada for her constant support and helping hand in preparing the thesis. Thank you Sharada for your patience and smiling face during tough times and always being an inspiration.

I also thank all my friends and colleagues in the department for making my stay memorable. I will never forget the Christmas and Carneval parties.

Table of Contents

Abbreviations

APS	ammonium persulphate
ATP	adenosine 5'-triphosphate
bp	base pair(s)
BCIP	5-bromo-4-chloro-3-indolylphosphate
BSA	bovine serum albumin
Bsr	blasticidin resistance cassette
cAMP	cyclic adenosine monophosphate
cDNA	complementary DNA
CIAP	calf intestinal alkaline phosphatase
dNTP	deoxyribonucleotide triphosphate
DABCO	diazobicyclooctane
DEPC	diethylpyrocarbonate
DMSO	dimethylsulphoxide
DNA	deoxyribonucleic acid
DNase	deoxyribonuclease
DTT	1,4-dithiothreitol
ECL	enzymatic chemiluminescence
EDTA	ethylenediaminetetraacetic acid
EGTA	ethyleneglycol-bis (2-amino-ethylene) N,N,N,N-tetraacetic acid
ELISA	enzyme linked immunosorbent assay
G418	geneticin
GFP	green fluorescent protein
GST	glutathione S-transferase
HRP	horse radish peroxidase
IgG	immunoglobulin G
IPTG	isopropyl-β-D-thiogalactopyranoside
kb	kilo base pairs
MES	morpholinoethansulphonic acid
β-ME	beta-mercaptoethanol
MOPS	Morpholinopropanesulphonic acid
Mw	molecular weight
NP-40	nonylphenylpolyethyleneglycol
pNPP	para-nitrophenyl phosphate
OD	optical density
ORF	open reading frame
PAGE	polyacrylamide gel electrophoresis
PCR	polymerase chain reaction
PMSF	phenylmethylsulphonylfluoride
RACE PCR	random amplification of cDNA ends polymerase chain reaction
RT-PCR	reverse transcript polymerase chain reaction
RNA	ribonucleic acid
RNase	ribonuclease
rpm	rotations per minute
SDS	sodium dodecyl sulphate
TEMED	N,N,N',N'-tetramethyl-ethylendiamine
U	unit
UV	ultra violet
vol.	volume
v/v	volume by volume

w/v	weight by volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Units of Measure and Prefixes

Unit	**Name**	**Symbol**	**Prefix (Factor)**
°C	degree Celsius	k	kilo (10^{3})
kDa	Dalton	c	centi (10^{-2})
g	gram	m	milli (10^{-3})
hrs	hour	µ	micro (10^{-6})
ml	millilitre	n	nano (10^{-9})
m	meter	p	pico (10^{-12})
min	minute		
sec	second		
V	volt		
mol	mole		

1. Introduction

1.1 G Protein Coupled Receptors

Multicellular organisms have highly evolved based on the capacity of their cells to communicate with each other and with their environment. Over years of discovery the membrane-bound receptors have been categorized into four or five families belonging to one large G protein-coupled receptor (GPCR) superfamily. GPCRs are among the oldest signal transduction machinery present in plants (Plakidou-Dymock et al., 1998), yeast (Dohlman et al., 1991) and slime mold (*Dictyostelium discoideum*) (Devreotes, 1994), which control the activity of enzymes, ion channels and transport of vesicles via the catalysis of the GDP–GTP exchange on heterotrimeric G proteins (Gα–βγ). A large number of genes code for GPCRs in vertebrates and *Caenorhabditis elegans* that are mainly involved in the recognition and transduction of messages as diverse as light, Ca^{2+}, odorants, small molecules including amino-acids, nucleotides and peptides, as well as proteins (Figure I). Although sequence comparison between the different GPCRs revealed the existence of different receptor families sharing low sequence similarity they have a central core domain constituted of seven transmembrane helices (TM I-VII).

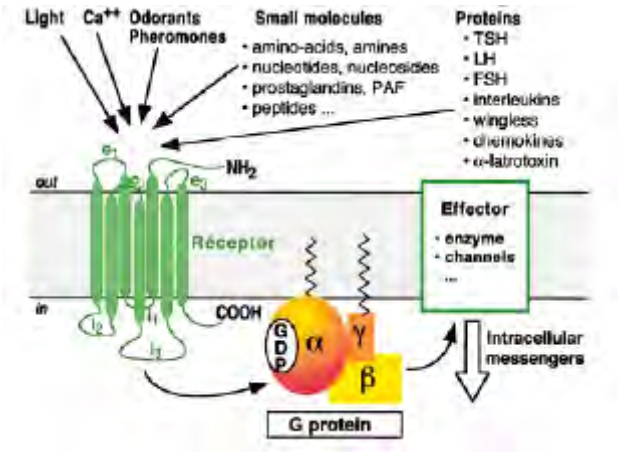


Figure I. Illustration of the GPCR mediated signal transductions (Bockaert and Pin, 1999).

Two cysteine residues in the extracellular loops (e1 and e2) are conserved in most GPCRs that form a disulfide link which is probably important for the packing and for the stabilization of a restricted number of conformations of these seven TMs (Figure I). GPCRs differ in the length and function of their N-terminal extracellular domain, their C-terminal intracellular domain and their intracellular loops. Each of these domains provides specific properties to the various receptor proteins and any conformational change in the core domain will affect the

intracellular pocket formed between the TM III-VI that plays a major role in binding and activating the G proteins (Bockaert and Pin, 1999).

1.2 Frizzled proteins

Frizzled proteins belong to the cell surface proteins functioning as the receptor for the Wnt ligand and range in length from about ~500 to 700 amino acids. The amino terminus of Frizzled protein is predicted to be extracellular and contains a cysteine-rich domain (CRD) that is required for binding of Wnt molecules (Bhanot et al., 1996), followed by a hydrophilic linker region of 40-100 amino acids. The proteins also contain seven hydrophobic domains that are predicted to form transmembrane α-helices. The intracellular carboxy-terminal domain has a variable length and is not well conserved among different family members (Wang et al., 1996). Moreover, the presence of seven hydrophobic domains has categorized the protein to be related to the GPCR superfamily. The sequence similarity to GPCRs is low, because similarity is restricted to the seven transmembrane regions that have a high frequency of hydrophobic residues.

1.2.1 Functions of Frizzled proteins

Frizzled proteins are exclusively found at the cell surface to bind Wnt and get internalized to regulate the extracellular level of Wnt protein (Chen et al., 2003). The binding of Wnt ligands to the Frizzled receptors induces a canonical and two non-canonical β-catenin dependent Wnt/Fz pathways, the planar cell polarity (PCP) and the Wnt/Ca^{2+} pathway.

I Canonical Wnt/β-catenin pathway

The canonical Wnt/β-catenin pathway is characterized by stabilization of β-catenin in response to ligand binding. Wnt proteins act on the target cells by binding to the Frizzled (Fz)/low-density lipoprotein (LDL) receptor-related protein (LRP) complex at the cell surface. These receptors transduce a signal to several intracellular proteins which mainly includes Dishevelled (Dsh) and the transcriptional regulator, β-catenin. Cytoplasmic β-catenin levels are normally kept low through continuous proteasome-mediated degradation but the degradation pathway is inhibited when cells receive Wnt signals and consequently β-catenin accumulates in the cytoplasm and nucleus. This accumulated β-catenin in the nucleus interacts with the transcription factors such as the lymphoid enhancer-binding factor 1/Tcell-specific transcription factor (LEF/TCF) to affect transcription (Figure III). A large number of Wnt targets have been identified that include members of the Wnt signal transduction

pathway itself, which provide feedback control during Wnt signaling (Logan and Nusse, 2004).

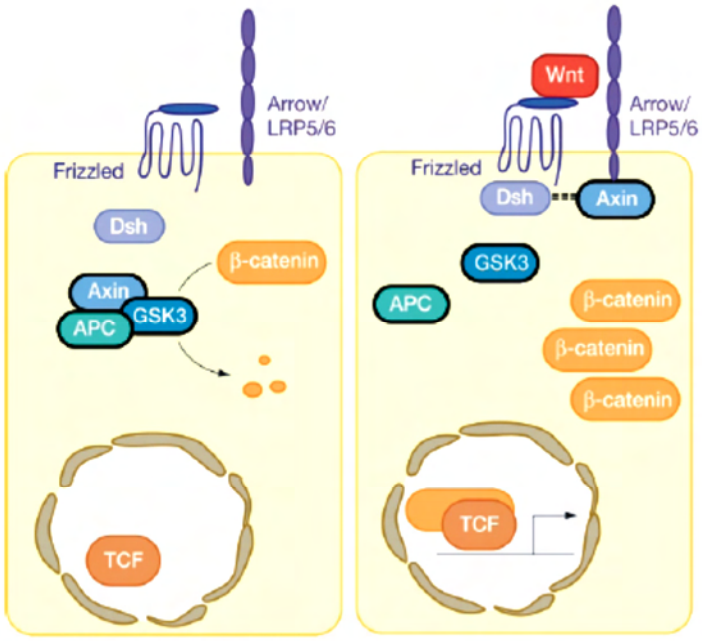


Figure III. Canonical Wnt signaling (Logan and Nusse, 2004)

II Non-canonical planar cell polarity

The PCP pathway regulated genes when mutated result in cell polarity defects in a planar tissue. A non-canonical Frizzled (*fz*) activity controls the coordinated cell polarity decisions in the *Drosophila* cuticle where each cell produces a single hair on its apical surface at the distal vertex of the cell, which then grows out distalwards (Figure IIA). In the absence of *fz*, hairs form in the centre of the apical surface of the cell and no longer invariably grow out distalwards (Figure IIB) (Wong and Adler, 1993). This constitutes the cell-autonomous activity of *fz* in the wing.

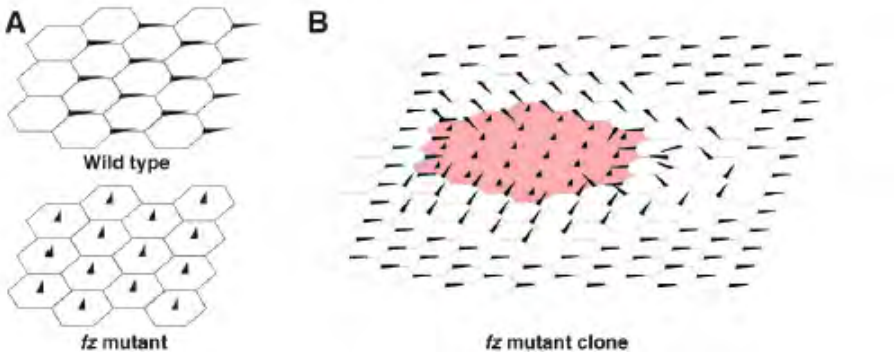


Figure II. Planar cell polarity in *Drosophila* wing (Strutt, 2003).

III Non-canonical Wnt/Ca^{2+} pathway

In the Wnt/calcium pathway Frizzled appears to act through heterotrimeric guanine nucleotide–binding proteins (G proteins) activating phospholipase C (PLC) (Slusarski et al., 1997a) and phosphodiesterase (PDE) (Ahumada et al., 2002), which leads to increased concentrations of free intracellular calcium $[Ca^{2+}]_i$ (Slusarski et al., 1997b) and to decreased intracellular concentrations of cyclic guanosine monophosphate (cGMP) (Ahumada et al., 2002), respectively (Figure IV). However the newly discovered role of cGMP in Wnt signaling is the least well understood. Therefore Frizzleds appear to function in multiple pathways, including the PCP, Wnt/calcium, and canonical Wnt/ß-catenin pathways and induce different signal transduction pathways that regulate some of the major cellular processes in a cell.

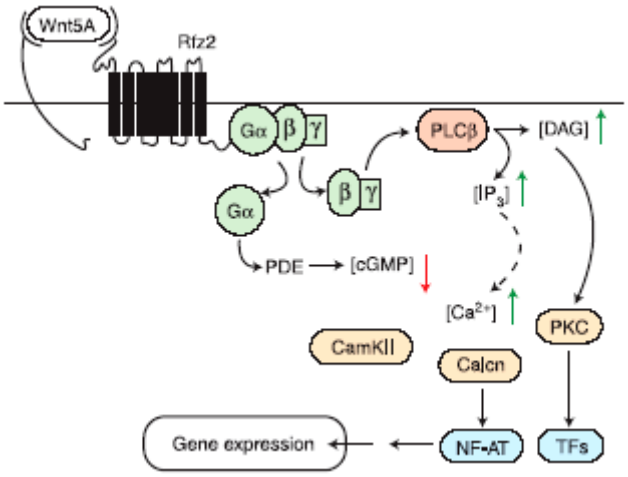


Figure IV. Frizzled mediated Wnt-Ca^{2+} signaling (Wang and Malbon, 2003).

1.2.2 Frizzled role in development and diseases

Frizzled receptors regulate specification of cell fate, cell adhesion, migration, polarity, and proliferation by binding to Wnt molecules. Their roles in development have been analysed by the phenotypes exhibited in different model systems as a result of gene knockout or mutations which is summarized in Table I (Huang and Klein, 2004). Noticable is a human hereditary disorder, called familial exudative vitreopathy (FEVR), which is caused by mutation in the Frizzled (Fz4) receptor that results in defective vasculogenesis in the peripheral retina (Robitaille et al., 2002).

1.3 Do Frizzled receptors exist in *Dictyostelium*?

Frizzled's role is remarkable with extended functions from cell polarity in *Drosophila* to the human diseases. The highly conserved signal transduction pathways identified in many

primitive organism like *Hydra vulgaris* to highly evolved species such as humans prompted us to search for Frizzleds in *Dictyostelium*. Although *Dictyostelium* has a β-catenin, a canonical β-catenin dependent pathway is not described.

Loss-of-function phenotypes of *frizzled* genes

Species	Genotypes*	Phenotypes	
Drosophila	$fz^{-/-}$	Disruption of planar cell polarity in sensory bristles, dorsal epidermis, and ommatidia	1
Drosophila	$Dfz2^{-/-}$	Viable	2
Drosophila	$Fz^{-/-}$; $Dfz2^{-/-}$	Wg signal transduction is abolished in embryos and the wing imaginal disk	3
Drosophila	$fz^{-/-}$; *Dfz2* deficiency	Mimics loss of *wg* in embryonic epidermal patterning, neuroblast specification, midgut morphogenesis, and heart formation	4
Drosophila	fz^{RNAi}; $Dfz2^{RNAi}$	Defects in embryonic patterning that mimic *wg* loss of function	5
Drosophila	$Dfz3^{-/-}$	Suppresses a hypomorphic *wg* mutation	6
C. elegans	$mom\text{-}5^{-/-}$	Embryos lack endoderm and overproduce pharyngeal tissue	7
C. elegans	$mig\text{-}1^{-/-}$	Abnormal migration of the Q neuroblast	8
C. elegans	$Lin\text{-}17^{-/-}$	Disruption of a variety of asymmetric cell divisions	9
Mouse	$mfz3^{-/-}$	Severe defects in major axon tracts within the forebrain	10
Mouse	$mfz4^{-/-}$	Defects in cell survival in the cerebellum; vascular defects in retina, cochlea, and cerebellum	11
Mouse	$mfz5^{-/-}$	Embryonic lethal (at day 10.75) because of defects in yolk-sac angiogenesis	12
Human	$hFZD4^{+/-}$	Familial exudative vitreoretinopathy	13
Xenopus	$Xfz3^{MO}$	Loss of neural crest induction	14
Xenopus	$Xfz7^{AS}$	Depletion of maternal *Xfz7* disrupts dorsal anterior development	15
Xenopus	$Xfz7^{MO}$	Severe gastrulation defect arising from inability of involuted anterior mesoderm to separate from the ectoderm	16

Table I. Frizzleds function in different model systems. All the phenotypes exhibited by the organisms due to the lack of Frizzled or mutation in Frizzled presented here is taken from (Huang and Klein, 2004). The number represents the respective references, **1**-(Adler, 2002), **2** and **3**-(Chen and Struhl, 1999), **4**-(Muller et al., 1999), **5**-(Kennerdell and Carthew, 1998), **6**-(Sato et al., 1999), **7**-(Rocheleau et al., 1997), **8**-(Harris et al., 1996), **9**-(Sawa et al., 1996), **10**-(Wang et al., 2002), **11**-(Wang et al., 2001), **12**-(Ishikawa et al., 2001), **13**-(Robitaille et al., 2002), **14**-(Deardorff et al., 2001), **15**-(Sumanas et al., 2000) , **16**-(Winklbauer et al., 2001).

However, cAR1 mediated signaling has been compared to the cell fate determination pathway in *Xenopus*, *Drosophila* and *C. elegans* (Kim et al., 2002). The PCP pathway may be present in *Dictyostelium* as the cells during development exhibit cell polarity (Williams and Harwood, 2003), but cell polarity is not directly associated to the components of PCP signaling. Calcium is an important part of *Dictyostelium* development (Bumann et al., 1984) and all the components of the Wnt/Ca^{2+} pathway are present except the Wnt protein and the Frizzled protein. The Wnt proteins are extracellular signaling molecules that play a central role in cell fate determination in *C. elegans* (Nusse and Varmus, 1992). Although Wnt proteins were not identified in *Dictyostelium*, CMF, an extracellular glycoprotein was identified that determines the cell density for aggregation and the cell type jointly with cAMP during development

(Mehdy and Firtel, 1985; Yuen et al., 1995). But when searched for Frizzled in the *Dictyostelium* we identified 25 Frizzled like receptors.

1.3.1 *Dictyostelium* as a model system

D. discoideum is a eukaryote, which can exist as a unicellular and multicellular organism. Various signal-transduction pathways regulates processes such as chemotaxis, cell adhesion and cell fate determination and are closely related to metazoan pathway (Firtel and Chung, 2000; Gerisch, 1968; Kim et al., 2002). Therefore *Dictyostelium* has been identified as a good model system for studying many biological processes. *Dictyostelium* cells feed on bacteria and grow as amoebae but with depletion of food they initiate development and achieve multicellularity by the aggregation of cells (Figure V) (Brown and Firtel, 1999).

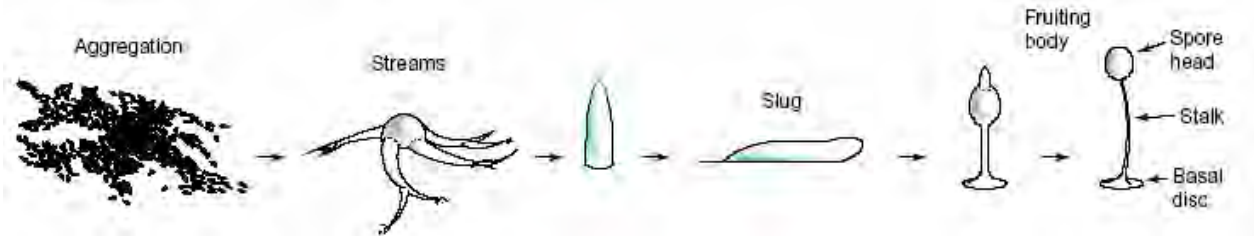


Figure V. Life cycle of *Dictyostelium* (Williams and Harwood, 2003)

1.3.1.1 Early development

The amoebae feed on bacteria and grow exponentially until the depletion of the food source. The status of the food supply is monitored by secretion of a prestarvation factor (PSF). The PSF expression is high till the depletion of the food source and the level goes down with the onset of starvation. The genes that are expressed at low level in response to PSF during the late exponential growth are expressed at higher level in starving cells (Rathi and Clarke, 1992). Discoidin-I is one of them which is induced in response to PSF (Rathi et al., 1991) and continues to be stimulated during early development by conditioned medium factor (CMF), a factor secreted by starving cells (Gomer et al., 1991). The expression level of discoidin-I is downregulated by cAMP during aggregation which is important for the formation of head-to-tail streams by aggregating cells (Crowley et al., 1985; Vauti et al., 1990). Although these factors PSF and CMF are produced at different times in the *Dictyostelium* life cycle, cells need both glycoproteins to measure their own density and to aggregate.

The developing *Dictyostelium* cells exhibit chemotactic response by extending pseudopods towards cAMP during aggregation (Soll et al., 2002). During this process the cells form radial branching streams, which results from the cAMP wave propagation depending on the

cell density (van Oss et al., 1996). These streams then move coordinately towards the aggregation centre to form a mass of 10^5 cells. When the cells move in streams they adhere to each other and express first the cell adhesion protein gp24. In later stage of aggregation a second adhesion molecule, csA, is expressed which is induced by cAMP pulses secreted by the starving cells. Cell-cell adhesion was found to be important for induction of developmentally regulated genes during the aggregation (Faix et al., 1992). The cAMP synthesized by aggregating cells is sensed by a cell surface receptor (cAR1) which through G protein signaling stimulates cAMP production through an adenylyl cyclase (Fontana et al., 1991; Klein et al., 1988). The signaling molecule cAMP acts as a chemoattractant, secreted at the aggregation centres towards which cells make directed migration (Alcantara and Monk, 1974). Similar chemotactic responses are critical for a wide variety of biological processes including immune system responses, neuronal path finding, angiogenesis and metastasis. Signalling molecules downstream of the receptors that contribute to chemotactic responses include guanylyl cyclase, phosphatidylinositol 3-kinases (PI 3-kinases), phospholipases and mitogen-activated protein kinases (Segall, 1999). Moreover, *Dictyostelium* and mammalian cells have a common mechanism for migration.

1.3.1.2 Calcium role in early development

The binding of cAMP to its receptor cAR1 also induces a transient entry of external Ca^{2+}, however G-proteins are not required for this process (Milne et al., 1995). Ca^{2+} influx increases the intracellular Ca^{2+} and activates phospholipase C (PLC) to elevate inositol 1, 4, 5-triphosphate (IP3), cAMP and cGMP. Although Ca^{2+} levels control the motile response it is not required for chemotaxis (Malchow et al., 1996a; Traynor et al., 2000). A slow periodic Ca^{2+} oscillation was observed during early development in suspension (Bumann et al., 1984) and a wave of Ca^{2+} is propagated from the tip of the slug towards the rear end in the multicellular slug stage that may help to regulate gene expression and coordinate cell movement in *Dictyostelium* (Pinter and Gross, 1995).

1.3.1.3 cAMP signaling

When cells are in the preaggregative stage a low level of cAMP is produced in a pulsatile fashion that augments the expression of aggregative genes coding for cAMP receptor (cAR1) and contact sites A (csA) (Gerisch and Malchow, 1976). cAR1 null cells show a defect in aggregation, however it is not clear whether cells produce a signal or whether the signal produced is not transduced to regulate gene expression. At the aggregation stage the cAMP

produced binds to cAR1 and induces the G protein Gα2 which binds to cAR1 and frees the Gβ causing PI-3 kinase activation that stimulates activation of the adenylyl cyclase (ACA) gene. ACA is expressed at high levels exclusively at the aggregation stage and is necessary for synthesis of the chemoattractant cAMP. The cAMP produced mediates chemotaxis and induces gene expression through cAR1 (van Haastert and Kien, 1983; Wu et al., 1995).

The hydrolysis of cAMP is very important for dynamic signaling. *Dictyostelium* employs 5 different classes of phosphodiesterase to breakdown cAMP, of which the cAMP phosphodiesterase gene (PDE) shows a pronounced expression during aggregation and is induced by cAMP pulses contributing to the negative feedback loop of oscillatory cAMP signaling (Meima et al., 2003). The level of active PDE is regulated at transcription level and by the phosphodiesterase inhibitor (PDI), which is induced at low levels of cAMP and inhibits the PDE by binding. PDI expression is also controlled by the presence of high levels of PDE (Franke and Kessin, 1992). The expression level of ACA decreases with the progression of aggregation to the mound stage. At this stage the postaggregative and cell-type specific genes stimulate a developmental change to form cell types that are the precusor cells found within the mature fruiting body.

1.4 Aim of work

We identified for the first time Frizzled in *Dictyostelium*, and found FrzA, one of the 25 Frizzled like receptors. It has a unique C-terminus with a Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain. The N-terminus preceeding the Frizzled transmembrane domain is rather short and lacks the extracellular CRD region. Our studies included RACE PCR, RT-PCR and characterisation of the genomic and cDNA database, to assemble the full-length FrzA cDNA. The sequence homology of FrzA showed its close relation to the smoothened receptor, which is a homologue of Frizzled having a structural and functional similarity to the GPCR. To characterize the cell biology of the FrzA protein further we generated a polyclonal antibody against the C- terminus of FrzA. To get further insight in to the function of this protein, a knockout strategy was initiated. Although the knock out cells survive as amoebae, they do not aggregate during the developmental cycle to develop into fruiting bodies. To understand the role of FrzA in *Dictyostelium* during early development, we analysed the $FrzA^-$ mutant in detail with regard to,

1. Aggregation
2. Cell-cell adhesion
3. Cell migration
4. cAMP and calcium signaling
5. Cell fate determination

1. Materials

1.1 Laboratory materials

Cellophane sheet, Dry ease	Novex
Centrifuge tubes, 15 ml, 50 ml	Greiner
Coverslips (glass), ∅ 12 mm, ∅ 18 mm	Assistant
Corex tube, 15 ml, 50 ml	Corex
Cryo tube, 1 ml	Nunc
Electroporation cuvette, 2 mm electrode gap	Bio-Rad
Gel drying frames	Novex
Hybridisation bag	Life technologies
Microcentrifuge tube, 1.5 ml, 2.2 ml	Sarstedt
Micropipette, 1-20 µl, 10-200 µl, 100-1,000 µl	Gilson
Micropipette tips	Greiner
Multi-channel pipette	Finnigan
Needles (sterile), 18G–27G	Terumo, Microlance
Nitrocellulose membrane, BA85	Schleicher and Schuell
Nitrocellulose-round filter, BA85, ∅ 82 mm	Schleicher and Schuell
Nylon membrane, Biodyne B	Pall
Parafilm	American National Can
Pasteur pipette, 145 mm, 230 mm	Brand, Volac
PCR softtubes, 0.2 ml	Biozym
Petri dish (35 mm, 60 mm, 100 mm)	Falcon
Petri dish (90 mm)	Greiner
Plastic cuvette, semi-micro	Greiner
Plastic pipettes (sterile), 1 ml, 2 ml, 5 ml, 10 ml, 25 ml	Greiner
Quartz cuvette, Infrasil	Hellma
Quartz cuvette, semi-micro	Perkin Elmer
Saran wrap	Dow
Scalpels (disposable), Nr. 10, 11, 15, 21	Feather
Slides, 76 x 26 mm	Menzel
Syringes (sterile), 1 ml, 5 ml, 10 ml, 20 ml	Amefa, Omnifix
Syringe filters (Acrodisc), 0.2 µm, 0.45 µm	Gelman Sciences
Tissue culture flasks, 25 cm^2, 75 cm^2, 175 cm^2	Nunc
Tissue culture dishes, 6 wells, 24 wells, 96 wells	Nunc

Whatman 3MM filter paper	Whatman
X-ray film, X-omat AR-5, 18 x 24 mm, 535 x 43 mm	Kodak

1.2 Instruments and equipments

Centrifuges (microcentrifuges):	
Centrifuge 5417 C	Eppendorf
Centrifuge Sigma	B. Braun Biotech Instruments
Cold centrifuge Biofuge fresco	Heraeus Instruments
Centrifuges (table-top, cooling, low speed):	
Centrifuge CS-6R	Beckman
Centrifuge RT7	Sorvall
Centrifuge Allegra 21R	Beckman
Centrifuges (cooling, high speed):	
Beckman Avanti J25	Beckman
Sorvall RC 5C plus	Sorvall
Centrifuge-rotors:	
JA-10	Beckman
JA-25.50	Beckman
SLA-1500	Sorvall
SLA-3000	Sorvall
SS-34	Sorvall
Dounce homogeniser, 10 ml and 60 ml	B. Braun
Electrophoresis power supply, Power-pac-200, -300	Bio-Rad
Electroporation unit, Gene-Pulser	Bio-Rad
Freezer (-80°C)	Nunc
Freezer (-20°C)	Siemens, Liebherr
Gel-documentation unit	MWG-Biotech
Heating block, DIGI-Block JR	neoLab
Heating block, Dry-Block DB x 20	Techne
Hybridising oven	Hybaid
Ice machine	Ziegra
Incubators:	
CO_2-incubator, BBD 6220, BB 6220	Heraeus
CO_2-incubator, WTC Binder	Biotran

Incubator, microbiological	Heraeus
Incubator with shaker, Lab-Therm	Kuehner
Laminar flow, Hera Safe (HS 12)	Heraeus
Magnetic stirrer, MR 3001 K	Heidolph
Microscopes:	
Light microscope, CH30	Olympus
Light microscope, DMIL	Leica
Light microscope, CK2	Olympus
Fluorescence microscope, DMR	Leica
Fluorescence microscope, 1X70	Olympus
Fluorescence microscope, CTR/MIC	Leica
Confocal laser scan microscope, DM/IRBE	Leica
Stereomicroscope, SZ4045TR	Olympus
Oven, conventional	Heraeus
PCR machine, PCR-DNA Engine PTC-2000	MJ Research
pH-Meter 766 Calimatic	Knick
Refrigerator	Liebherr
Semi-dry blot apparatus, Trans-Blot SD	Bio-Rad
Shakers	GFL, Kuehner
Sonicator (water bath), Sonorex RK 52	Bandelin
Speed-vac concentrator, DNA 110	Savant
Spectrophotometer: Ultraspec 2000, UV/visible	Pharmacia Biotech
BioPhotometer	Eppendorf
Scanarray Express Scanner	Perkin Elmer
UV- transilluminator, TFS-35 M	Faust
Vortex, REAX top	Heidolph
Waterbath	GFL
X-ray-film developing machine, FPM-100A	Fujifilm

1.3 Kits

Nucleobond AX 100 and 500	Macherey-Nagel
NucleoSpin Extract 2 in 1	Macherey-Nagel
Nucleotrap	Macherey-Nagel
1 kb DNA-marker	Gibco-BRL

pGEM-T Easy	Promega
High molecular weight protein marker	Amersham Biosciences
Low molecular weight protein marker	Amersham Biosciences
SMART RACE PCR Kit	Clontech
Qiagen RNeasy Mini or Midi Kit	Qiagen

1.4 Enzymes, antibodies, substrates, inhibitors and antibiotics

Enzymes used in the molecular biology experiments:

Calf Intestinal Alkaline Phosphatase (CIP)	Boehringer
Klenow fragment	Boehringer
Lysozyme	Sigma
Proteinase K	Sigma
Restriction endonucleases	Amersham, Life technologies, New England Biolabs
Reverse transcriptase, M-MLV	Promega
Ribonuclease H (RNase H)	Boehringer
T_4 DNA ligase	Boehringer
Taq-polymerase	Life technologies/Boehringer

Primary antibodies:

Goat anti-GST antibody	Pharmacia
Mouse anti-GFP monoclonal antibody, K3-184-2	(Noegel et al., 2004)
Polyclonal anti-cAR1	Gift from Dr. Peter Devreotes
Polyclonal anti- Gα2	Gift from Dr. Gundersen

Secondary antibodies:

Goat anti-mouse IgG, peroxidase conjugated	Sigma
Goat anti-rabbit IgG, peroxidase conjugated	Sigma
Mouse anti-goat IgG, peroxidase conjugated	Sigma
Sheep anti-mouse IgG, Cy3 conjugated	Sigma

Inhibitors:

Diethylpyrocarbonate (DEPC)	Sigma
Leupeptin	Sigma

Pepstatin	Sigma
Phenylmethylsulphonylfluoride (PMSF)	Sigma
Benzamidine hydrochloride	Sigma
Proteinase Inhibitor cocktail	Sigma

Antibiotics:

Ampicillin	Grünenthal
Blasticidin S	ICN Biomedicals
Chloramphenicol	Sigma
Dihydrostreptomycinsulphate	Sigma
Geneticin (G418)	Life technologies
Kanamycin	Sigma, Biochrom
Tetracyclin	Sigma

1.5 Chemicals and reagents

Most of the chemicals and reagents were obtained either from Sigma, Fluka, Difco, Merck, Roche, Roth or Serva. Those chemicals or reagents that were obtained from companies other than those mentioned here are listed below:

Acetic acid (98-100%)	Riedel-de-Haen
Acrylamide (Protogel: 30:0,8 AA/Bis-AA)	National Diagnostics
Agar-Agar (BRC-RG)	Biomatic
Agarose (Electrophoresis Grade)	Life technologies
Chloroform	Riedel-de-Haen
Dimethylformamide	Riedel-de-Haen
Ethanol	Riedel-de-Haen
Glycerine	Riedel-de-Haen
Glycine	Riedel-de-Haen
Isopropypl-β-D-thiogalactopyranoside (IPTG)	Loewe Biochemica
Methanol	Riedel-de-Haen
Morpholino propane sulphonic acid (MOPS)	Gerbu
Peptone	Oxoid
Sodium hydroxide	Riedel-de-Haen
Yeast extract	Oxoid

Roti-Phenol	Roth
TRITC Phallodin	Sigma

Radiolabelled nucleotide:

α-^{32}P-deoxyadenosine triphosphate, (10 mCi/ml)	Amersham

1.6 Media and buffers

All media and buffers were prepared with deionised water filtered through an ion-exchange unit (Membra Pure). The media and buffers were sterilized by autoclaving at 120ºC and antibiotics were added to the media after cooling to approx. 50ºC. For making agar plates, a semi-automatic plate-pouring machine (Technomat) was used.

1.6.1 Media and buffers for *Dictyostelium* culture

Ax2-medium, pH 6.7:

(Claviez et al., 1982)

7.15 g yeast extract
14.3 g peptone (proteose)
18.0 g maltose
0.486 g KH_2PO_4
0.616 g $Na_2HPO_4.2H_2O$
add H_2O to make 1 litre

Phosphate agar plates, pH 6.0:

9 g agar
add Soerensen phosphate buffer, pH 6.0 to make 1 litre

Salt solution:

(Bonner, 1947)

10 mM NaCl
10 mM KCl
2.7 mM $CaCl_2$

Starvation buffer, pH 6.5:

(Shaulsky et al., 1998)

10 mM MES, pH 6.5
10 mM NaCl
10 mM KCl
1 mM $CaCl_2$
1 mM $MgSO_4$

SM agar plates, pH 6.5:

(Sussman, 1951)

9 g agar
10 g peptone
10 g glucose
1 g yeast extract
1 g $MgSO_4.7H_2O$
2.2 g KH_2PO_4
1 g K_2HPO_4
add H_2O to make 1 litre

Soerensen phosphate buffer, pH 6.0:

(Malchow et al., 1972)

2 mM Na_2HPO_4
14.6 mM KH_2PO_4

1.6.2 Media for *E. coli* culture

LB medium, pH 7.4:

(Sambrook, 1989)

10 g bacto-tryptone
5 g yeast extract
10 g NaCl
adjust to pH 7.4 with 1 N NaOH
add H_2O to make 1 liter

For LB agar plates, 0.9% (w/v) agar was added to the LB medium and the medium was then autoclaved. For antibiotic selection of *E. coli* transformants, 50 mg/l ampicillin, kanamycin or chloramphenicol was added to the autoclaved medium after cooling it to approx. 50ºC. For blue/white selection of *E. coli* transformants, 10 µl 0.1 M IPTG and 30 µl X-gal solution (2% in dimethylformamide) was plated per 90 mm plate and the plate was incubated at 37ºC for at least 30 min before using.

SOC medium, pH 7.0:

(Sambrook, 1989)

20 g bacto-tryptone
5 g yeast extract
10 mM NaCl
2.5 mM KCl

dissolve in 900 ml deionised H_2O
adjust to pH 7.0 with 1 N NaOH
The medium was autoclaved, cooled to approx. 50ºC and then the following solutions, which were separately sterilized by filtration (glucose) or autoclaving, were added:

10 mM $MgCl_2.6H_2O$
10 mM $MgSO_4.7H_2O$
20 mM Glucose
add H_2O to make 1 litre

1.6.3 Buffers and other solutions

The buffers and solutions that were commonly used during the course of this study are mentioned below

10x MOPS, pH 7.0/ pH 8.0: 41.9 g MOPS
16.7 ml 3 M sodium acetate
20 ml 0.5 M EDTA
add H_2O to make 1 litre

10x NCP-buffer, pH 8.0: 100ml 1M Tris/HCl, pH 8.0
87.0 g NaCl
5.0 ml Tween 20
add H_2O to make 1 litre

PBG, pH 7.4: 0.5 % bovine serum albumin
0.1 % gelatin (cold-water fish skin)
in 1x PBS, pH 7.4

1x PBS, pH 7.4: 8.0 g NaCl
0.2 g KH_2PO_4
1.15 g Na_2HPO_4
0.2 g KCl
dissolve in 900 ml deionised H_2O
adjust to pH 7.4

	add H_2O to make 1 litre, autoclave
20x SSC, pH 7.0:	3 M NaCl
	0.3 M sodium citrate
TE buffer, pH 8.0:	10 mM Tris/HCl, pH 8.0
	1 mM EDTA
10x TAE buffer, pH 8.3:	27.22 g Tris
	13.6 g sodium acetate
	3.72 g EDTA
	add H_2O to make 1 litre

1.7 Biological materials

Bacterial strains:

E. coli BL21 (DE)	Studier and Moffat, 1986
E. coli DH5α	Hanahan, 1983
E. coli XL1 blue	Bullock *et al.*, 1987
Klebsiella aeorgenes	Williams and Newell, 1976

Dictyostelium discoideum strain:

Ax2-214	An axenically growing derivative of wild strain, NC-4 (Raper, 1935) commonly referred to as Ax2.

1.8 Plasmids

pDNeo II	Witke et al, 1987
pDGFP-MCS	Weber et al, 1999
p1aBsr8	Gräf et al, 2000b
pGEM-T Easy	Kit: Promega
pGEX-4T1, 2 and 3	Pharmacia Biotech

1.9 Oligonucleotide primers

Oligonucleotide primers were designed on the basis of sequence information available and ordered for synthesis from Sigma and Metabion companies. Following is a list of the primers

used for PCR or sequence analysis or both during the course of the present investigation. The position and orientation of the primers are indicated in the text.

FullFrzAfp	GGT ACC AAA ATG TCT TTT GCT GGA AGA ATT TCA TTA GAT GC
FullFrzArp	CTC GAG TTG TTG AGT AGC AGA TTT ATG
FrzTMrp	CTC GAG TGC AAT ACC ACG TAA TGA ACA
Myrpipkfp	GGT ACC ATG GGT TCA TCA AAA TCA AAA CCA AAA GAT CCA....
	...TCA CAA CGT CGT CGT TGT TCA TTA CGT GGT ATT GC
PIPKfp	GAATTCT T GTT CAT TAC GTG GTA TTG C
PIPKrp	TTA GTT GAG TAG CAG ATT TAT G
FrizcDNAfp	CTA TTG GAT GGA GTG GTA TGA
FrizcDNArp	ACT CGC TTG GTT GGG TGC ATC
14extn	GAAC CCATCTTTAA AATTTACTAC
15extn	GATTCAAGGGT TTATGAATGC
10extn	CTGAAGAGGG TTACCCTCTA AAG
11extn	CATCA GGGTTAAACCATATGGATG
8extn	CTAC CCCCAACAAT TCTCACATTT G
9extn	CCAAA TTATGCTCCC ATACCTTG
RtCMFfp	CCAAACCACTGTTGATGAAACAC
RtCMFrp	GGTGGAGAATGAACAATTGTACG
RtCMFRfp	TCGTTCAACCGGTGTTTGGAC
RtCMFRrp	GATGGTGGGTAGAAAACAACACC
frizkoFPa15p	CCA ACC CAA GTT TTT TTA AAC C
frizkoFPneo1	GAT TGT CGC ACC TGA TTG C
frizkoFPneo2	GTT TCC CGT TGA ATA TGG CTC AT
frizkoRPtest 1	CTT GAA GTG AAC GAA TTA GTG GTG
frizkoRPtest 2	GCA ATA CCA CGT AAT GAA CAT G

2. Cell biological methods

2.1 Growth of *Dictyostelium*

2.1.1 Growth in liquid nutrient medium (Claviez et al., 1982)

Dictyostelium discoideum Ax2 and the derived transformants were grown in liquid Ax2 medium containing dihydrostreptomycin (40 μg/ml) and other appropriate selective antibiotics (depending upon the mutant) at 21°C either in shaking-suspension in an Erlenmeyer flask with shaking at 160 rpm or the cells were grown in petri dishes.

2.1.2 Growth on SM agar plates (Bonner, 1967)

In general, *Dictyostelium* cells were plated onto SM agar plates overlaid with *Klebsiella aerogenes* and incubated at 21ºC for 3-4 days until *Dictyostelium* plaques appeared on the bacterial lawns. To obtain single clones of *Dictyostelium*, 50-200 cells were suspended in 100 μl Soerensen phosphate buffer and plated onto *Klebsiella* overlaid SM agar plates. Single plaques obtained after incubation at 21ºC for 3-4 days were picked up with sterile tooth-picks,

transferred either to new *Klebsiella* overlaid SM agar plates or to separate petri dishes in Ax2 medium supplemented with dihydrostreptomycin (40 µg/ml) and ampicillin (50 µg/ml) to get rid of the bacteria and any other appropriate selective antibiotic (depending upon the mutant).

2.2 Development of *Dictyostelium*

Development in *Dictyostelium* is induced by starvation. For analysis of development in suspension culture and on phosphate agar, cells grown to a density of 2-3 x 10^6 cells/ml were pelleted by centrifugation at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC and were washed two times in an equal volume of cold Soerensen phosphate buffer in order to remove all the nutrients present from the Ax2 media.

2.2.1 Development in suspension culture

After washing twice in Soerensen phosphate buffer, the cells were resuspended in Soerensen phosphate buffer at a density of 1 x 10^7 cells/ml and were shaken at 160 rpm and 21ºC for the desired time periods.

2.2.2 Development on phosphate-buffered agar plates

The washed cells were then resuspended as 1 x 10^8 per ml in Soerensen phosphate buffer and evenly distributed at 5 x 10^6 (high cell density) or 10^6 (low cell density) per cm^2 or other varying cell densities onto the phosphate-buffered agar plates. The plates were air-dried and any excess liquid was carefully aspirated without disturbing the cell layer. The plates were then incubated at 21°C, different stages of development were observed or used for preparation of protein or RNA samples and the microscopic images were captured at indicated time points.

2.3 Transformation of *Dictyostelium* (Mann et al., 1994)

Electroporation method

Dictyostelium discoideum Ax2 cells were grown axenically in suspension culture to a density of 2-3 x 10^6 cells/ml. The cell suspension was incubated on ice for 20 min and centrifuged at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC to collect the cells. The cells were then washed with an equal volume of ice-cold Soerensen phosphate buffer, followed by another wash with the electroporation buffer. After washings, the cells were resuspended in electroporation buffer at a density of 1 x 10^8 cells/ml and 500 µl of this was pipetted into a prechilled 2 mm cuvette with 20-30 µg of plasmid DNA. The cells were electroporated using

a Biorad Genpulser Xcell preset program for *Dictyostelium* or Biorad Genespulser II with a condition set at 3 µF, 0.9V. The cells were left on ice for 10 mintues after electroporation and later spread on a petri plate. The healing solution was then added dropwise to the plate and the cells were left on a shaker for 1 h at 50 rpm. Axenic medium was then added to this plate, after 16-20 hrs, the respective antibiotic was added to select the transformants.

Electroporation buffer (EB)

10 mM Na/K phosphate buffer, pH 6.1

50 mM sucrose

Healing Solution (10 ml)

15 µl 1M $CaCl_2$

15 µl 1M $MgCl_2$ in EB

2.4 Preservation and revival of preserved *Dictyostelium* cells

Dictyostelium cells were allowed to grow densely in Ax2 medium to a concentration of 4-5 x 10^6 cells/ml. 9 ml of the densely grown culture was collected in a 15 ml Falcon tube on ice and supplemented with 1 ml Horse serum and 1 ml DMSO. The contents were mixed by gentle pipetting, followed by preparing aliquots of 1 ml in cryotubes (1 ml, Nunc). The aliquots were incubated on ice for 60 min, followed by incubation at –20ºC for at least 2 hrs. Finally the aliquots were transferred to –80ºC for long term storage.

For reviving the frozen *Dictyostelium* cells, the aliquot was taken out from –80ºC and thawed. In order to remove DMSO, the cells were transferred to a Falcon tube containing 30 ml Ax2 medium and centrifuged at 2,000 rpm (Sorvall RT7 centrifuge) for 2 min at 4ºC. The cell pellet was resuspended in 10 ml of Ax2 medium and 200 µl of the cell suspension was plated onto SM agar plates overlaid with *Klebsiella*, while the remaining cell suspension was transferred into a 100-mm petri dish (Falcon) and appropriate antibiotics were added. Cells in the petri dish were allowed to recover overnight at 21ºC and the medium was changed the next day to remove the dead cells, whereas, the SM agar plates coated with cell suspension and bacteria were incubated at 21ºC until plaques of *Dictyostelium* cells started to appear.

3. Molecular biological methods

3.1 Purification of plasmid DNA

In general, for small cultures (1 ml) of *E. coli* transformants, the alkaline lysis method of Holmes and Quigley (1981) was used to extract plasmid DNA. This method is good for screening a large number of clones simultaneously for the desired recombinant plasmid. Alternatively, for pure plasmid preparations in small and large scales, kits provided either by Machery-Nagel (Nucleobond AX kit for small scale plasmid preparations) were used when the pure plasmid DNA was required for sequencing, PCR or transformation. These kits follow basically the same principle: first an overnight culture of bacteria containing the plasmid is pelleted and the cells are lysed by alkaline lysis. The freed plasmid DNA is then adsorbed on a silica matrix, washed with ethanol, and then eluted into TE, pH 8.0. This method avoids the requirement of caesium chloride or phenol-chloroform steps during purification.

3.2 Digestion with restriction enzymes

All restriction enzymes were obtained from NEB, Amersham or Life technologies and the digestions were performed in the buffer systems and temperature conditions as suggested by the manufacturers. The plasmid DNA was digested for 1-2 hrs and the chromosomal DNA for 12-16 hrs.

3.3 Dephosphorylation of DNA fragments

To avoid self-ligation of the vector having blunt ends or that has been digested with a single restriction enzyme, 5' ends of the linearised plasmids were dephosphorylated by calf-intestinal alkaline phosphatase (CIAP, Boehringer). Briefly, in a 50 µl reaction volume, 1-5 µg of the linearised vector-DNA was incubated with 1unit calf-intestinal alkaline phosphatase (CIAP) in CIAP-buffer (provided by the manufacturer) at 37ºC for 30 min. The reaction was stopped by inactivating the enzyme by heating the reaction-mixture at 65ºC for 10 min. The dephosphorylated DNA was extracted once with phenol-chloroform and precipitated with 2.5 volumes ethanol and 1/10 volume of 3 M sodium acetate, pH 5.2.

3.4 Setting up of ligation reaction

DNA fragment and the appropriate linearised plasmid was mixed in approximately equimolar amounts. T4 DNA ligase (Life technologies/Boehringer) and ATP was added as indicated below and the ligation reaction was left overnight at 10-12ºC.

Ligation reaction:
Linearised vector DNA (200-400 ng)
DNA-fragment
4 µl 5x Ligation buffer
1 µl 0.1 M ATP
1.5 U T4 ligase
add H_2O to make 20 µl

5x Ligation buffer:
supplied along with the T4 ligase enzyme by the manufacturer

3.5 Isolation of *Dictyostelium* genomic DNA

Genomic DNA from *Dictyostelium* was prepared according to the method described by Nellen *et al.* (1987), with slight modifications. *Dictyostelium* cells were allowed to grow on *Klebsiella*-covered SM plates or as suspension culture (Materials and Methods, 2.2) at 21ºC. When the plates were covered with densely grown *Dictyostelium*, cells were collected in 15 ml ice-cold water, pelleted and washed twice with ice-cold water to get rid of *Klebsiella*. Alternatively, the pellet of 1 x 10^8 *Dictyostelium* cells grown in shaking suspension was washed twice with ice-cold Soerensen phosphate buffer. The pellet of *Dictyostelium* cells was finally resuspended in 5 ml cold Nucleolysis buffer. The nuclei fraction was obtained by centrifugation at 3,000 rpm (Sorvall RT7 centrifuge) for 10 min. The nuclear pellet obtained was carefully resuspended in 1 ml TE, pH 8.0, with 0.5% SDS and 0.1 mg/ml proteinase K and incubated at 37ºC for 3-5 hrs. The genomic DNA was extracted twice with phenol/chloroform (1:1 v/v), precipitated by adding 2.5 vol. 96% ethanol and 1/10 vol. 3 M sodium acetate, pH 5.2. The DNA precipitate was carefully spooled with a Pasteur pipette, washed with 96% ethanol, air-dried and dissolved in the desired volume of TE, pH 8.0.

Nucleolysis buffer:
10 mM magnesium acetate
10 mM NaCl
30 mM HEPES, pH 7.5
10% sucrose
2% Nonidet P40

Estimation of DNA concentration:
1 OD at 260 nm = 50 µg DNA

3.6 DNA agarose gel electrophoresis

Agarose gel electrophoresis was performed according to the method described by Sambrook *et al.* (1989) to resolve and purify the DNA fragments. Electrophoresis was typically performed with 0.8% (w/v) agarose gels in 1x TAE buffer submerged in a horizontal electrophoresis tank containing 1x TAE buffer at 1-5 V/cm. Only for resolving fragments less than 1,000 bp, 1% (w/v) agarose gels in 1x TAE buffer were used. DNA-size marker (Life technologies) was always loaded along with the DNA samples in order to estimate the size of the resolved DNA fragments in the samples. The gel was run until the bromophenol blue dye present in the DNA-loading buffer had migrated the appropriate distance through the gel. The gel was examined under UV light at 302 nm and was photographed using a gel-documentation system (MWG-Biotech)

DNA-size marker:

1 kb DNA Ladder (Life technologies or NEB)

3.7 Recovery of DNA fragments from agarose gel

DNA fragments from restriction enzyme digests or from PCR reactions were separated by agarose gel electrophoresis and the gel piece containing the desired DNA fragment was carefully and quickly excised while observing the ethidium bromide stained gel under a UV transilluminator. The DNA fragment was then purified from the excised gel piece using the Macherey-Nagel gel elution kit (NucleoSpin Extract 2 in 1), following the method described by the manufacturers.

3.8 Southern blotting

Southern blotting (Southern, 1975) is a technique used to transfer DNA from its position in an agarose gel to a nitrocellulose/nylon membrane. After transfer, the membrane can be hybridised with a radiolabelled probe to identify specific fragments. The ethidium bromide stained agarose gel was photographed using a ruler under UV light to document migration of DNA fragments with respect to the DNA-size marker. DNA was depurinated by incubating the gel in 2 vol. of 0.25 M HCl for 20 min at room temperature with gentle shaking. The gel was rinsed in deionised H_2O to remove excess HCl and was then incubated in 2 vol. of denaturation solution (0.5 M NaOH, 1.5 M NaCl) for 30 min in order to denature the DNA. Now the transfer was performed by capillary transfer technique. Briefly, the gel was transferred directly from the denaturation solution to a buffer reservoir containing a

supporting wick (made up of Whatman 3MM paper) and 20x SSC. A dry nylon membrane (Biodyne B membrane, Pall) of the same size as the gel was then directly placed on the alkaline gel. Three pieces of Whatman 3MM paper followed by blotting pads, all cut to the same size as the gel were placed on top of the nylon membrane. A glass plate supporting approximately 500 g weight was finally kept on top of the stack and transfer of DNA to the membrane was allowed to proceed for overnight. Next day, the position of the wells and the orientation of the membrane were marked before removing the membrane from the gel surface. The transferred DNA was then immobilized onto the membrane by baking at 80ºC for 2 hrs. After baking, the membrane was hybridised with a desired radiolabelled probe.

3.9 Total RNA and cDNA preparation

3.9.1 Isolation of total RNA from *Dictyostelium* cells

Total RNA was extracted from either Ax2 or FrzA$^-$ cells of different developmental stages of the *Dictyostelium* life cycle using the Qiagen RNeasy Mini or Midi kit. The manufacturers protocol for the isolation of RNA from the cytoplasm of animal cells was used for preparation. The RNA samples were used for northern blot analysis (3.10 and 3.11) and after reverse transcription for Real-Time PCR (3.16) and Microarray analysis (4.0).

3.9.2 Generation of cDNA

cDNA was generated using the M-MLV reverse transcriptase, RNAse H minus (Promega) according to the manufacturers protocol. Usually 1 µg of total RNA was taken in a RNase-free microcentrifuge tube, add 1µg of Oligo dT per microgram of RNA and make up the volume to 15µl with water. The tube is incubated at 70ºC for 5 min and cooled on ice. To this add 10µl of the master mix as given below and incubate the tube for 60 min at 42ºC. The generated cDNA was used for down-stream applications.

<u>Master Mix</u>

M-MLV 5x reaction buffer	5 µl
10mM dNTP	10µl
M-MLV RT	200 units

3.10 RNA formaldehyde-agarose gel electrophoresis

The denaturing formaldehyde agarose electrophoresis is used for separation and resolution of RNA (Lehrach et al., 1977).

Sample preparation for electrophoresis:

In general, 20 µg of purified total RNA was mixed with an equal volume of RNA-sample buffer and denatured by heating at 65ºC for 10 min. After denaturation, the sample was immediately transferred to ice and 1/10 vol. of RNA-loading buffer was added. Thereafter, the RNA samples were loaded onto a denaturing formaldehyde-agarose gel.

Formaldehyde-agarose gel preparation:

For a total gel volume of 150 ml, 1.8 g agarose (final concentration 1.2%) was initially boiled with 111 ml DEPC-H_2O in an Erlenmeyer flask, cooled to 60ºC and then 15 ml of the RNA gel casting buffer, pH 8.0 and 24 ml of a 36% formaldehyde solution were added. The agarose solution was mixed by swirling and poured into a sealed gel-casting chamber of the desired size. After the gel was completely set, denatured RNA samples were loaded and the gel was run in 1x RNA gel running buffer, pH 7.0, at 100 V until the bromophenol blue dye had migrated the appropriate distance through the gel. A test gel was sometimes run with 5 µg of total RNA to check the quality of the RNA samples. In such a case, 10 µg/ml ethidium bromide was added to the RNA sample buffer during sample preparation and after electrophoresis the gel was examined under UV light at 302 nm and was photographed using the gel-documentation system.

10x RNA-gel-casting buffer, pH 8.0:
200 mM MOPS
50 mM sodium acetate
10 mM EDTA
adjust pH 8.0 with NaOH
autoclaved

RNA-sample buffer:
50% formamide
6% formaldehyde
in 1x RNA-gel-casting buffer, pH 8.0

10x RNA-gel-running buffer, pH 7.0:
200 mM MOPS
50 mM sodium acetate
10 mM EDTA
adjust pH 7.0 with NaOH
autoclaved

RNA-loading buffer:
50% sucrose, RNase free
0.25% bromophenol blue
in DEPC-H_2O

Internal RNA-size standard:

26S rRNA (4.1 kb)

17S rRNA (1.9 kb)

3.11 Northern blotting

After electrophoresis, the RNA formaldehyde agarose gel was rinsed in sufficient amount of deionised H_2O for 5 min and then equilibrated in 10x SSC for 25 min. The resolved RNA was then transferred from the gel to a nylon membrane (Biodyne B membrane, Pall) using the transfer setup as described for Southern blotting (Materials and Methods, 3.8). After overnight transfer with 20x SSC, the transferred RNA was immobilised by baking the membrane in an oven at 80ºC for 2 hrs.

3.12 Radiolabeling of DNA

Prime-it kit (Stratagene) was used for radiolabeling of DNA fragments following the method suggested by the manufacturers. Briefly, 0.1-0.3 µg DNA was suspended in 24 µl ddH_2O (final volume). Then 10 µl of random-oligonucleotide-primer (supplied along with the kit) was added and the DNA template was denatured at 95ºC for 5 min. After denaturation, 10 µl of 5x dATP-primer buffer (supplied along with the kit), 5 µl of α-^{32}P-ATP (Amersham) and 1 µl Klenow enzyme (5 U/µl, supplied along with the kit) was added and the reaction-mixture was incubated at 37ºC for 10 min. After 10 min the reaction was immediately stopped by adding 2 µl stop-mix (supplied along with the kit). Now the reaction-mixture was diluted with 100 µl TE, pH 8.0, to increase the reaction volume and the reaction-mixture was overlaid on a 0.9 ml Sephadex G-50 spin column (Materials and Methods, 3.13). The free nucleotides present in the reaction-mixture were separated by centrifugation at 3,000 rpm (Sorvall RT7 centrifuge) for 2 min through the Sephadex G-50 spin column and the radiolabelled DNA probe was collected in a 1.5 ml eppendorf tube. The purified radiolabelled DNA probe was denatured by heating at 100ºC for 10 min, cooled on ice and used for hybridisation of Southern or Northern blots.

3.13 Chromatography through Sephadex G-50 spin column

This technique (Sambrook *et al.*, 1989), which employs gel filtration to separate high-molecular weight DNA from smaller molecules, was used to segregate radiolabelled DNA from unincorporated α-^{32}P-ATP. 30 g of Sephadex G-50 (Pharmacia) was slowly added to 250 ml of TE, pH 8.0, in a 500-ml bottle and the beads were allowed to swell overnight at

room temperature. Next day, the supernatant was decanted and was replaced with an equal volume of TE, pH 8.0. The beads were autoclaved and stored in a screw-capped bottle at 4ºC. For preparation of Sephadex G-50 spin column, the swollen Sephadex G-50 beads were packed in a disposable 1-ml syringe plugged with sterile glass wool and the column was spun at 3,000 rpm (Sorvall RT7 centrifuge) for 2 min. Sephadex G-50 was added until the packed column volume was 0.9 ml. The column was then used for segregation of radiolabelled DNA probe.

3.14 Hybridisation of Southern or Northern blot with radiolabelled DNA probe

Southern or northern blots were rinsed briefly with 2x SSC and incubated in a hybridisation-bottle in 15-20 ml of pre-hybridisation buffer for 1 hrs at 37ºC on a rotating incubator. After pre-hybridization, the denatured radiolabelled DNA probe was added directly to the pre-hybridization-buffer in the hybridization bottle and the blot incubated overnight at 37ºC. After hybridization, the blot was washed twice with wash buffer for 30 min followed by one wash with 2x SSC/0.1% SDS for 10 min and one wash with 0.2x SSC/0.1% SDS for 10 min, in the end one wash with 2x SSC at 37ºC. The blot was then wrapped in a plastic wrap and autoradiograph was performed by exposing the blot to X-ray film at –70ºC for the desired time.

Pre-hybridisation/Hybridisation buffer:

50% formamide
1% sodium lauryl sarcosinate
0.2% SDS
2 mM EDTA, pH 7.2
0.12 M phosphate buffer, pH 6.8
2x SSC
4x Denhardt's reagent

Wash buffer:

same contents as Pre-hybridisation/ hybridisation buffer except without 4x Denhardt's reagent

100x Denhardt's reagent:

2% ficoll 400
2% polyvinylpyrolidone
2% bovine serum albumin

3.15 Transformation of *E. coli*

3.15.1 Transformation of *E. coli* cells by the $CaCl_2$ method

Transformation of $CaCl_2$-competent *E. coli* cells:

Plasmid DNA (~50-100 ng of a ligase reaction or ~10 ng of a supercoiled plasmid) was mixed with 100-200 µl of $CaCl_2$-competent *E. coli* cells and incubated on ice for 30 min. The cells

were then heat-shocked at 42ºC for 45 sec and immediately transferred to ice to cool for 2 minutes. The cells were then mixed with 1 ml of pre-warmed (at 37ºC) SOC medium and incubated at 37ºC with shaking at ~150 rpm for 45 min. Finally, 100-200 µl of the transformation mix, or an appropriate dilution, was plated onto selection plates and the transformants were allowed to grow overnight at 37ºC.

3.15.2 Glycerol stock of bacterial culture

Glycerol stocks of all the bacterial strains/transformants were prepared for long-term storage. The culture was grown overnight in LB medium with or without the selective antibiotic (depending upon the bacterial transformant). 850 µl of the overnight grown culture was added to 150 µl of sterilized glycerol in a 1.5 ml microcentrifuge tube, mixed well by vortexing and the tube was frozen on dry ice and stored at –80°C.

3.15.3 DNA sequencing

Sequencing of the PCR-amplified product or plasmid DNA was performed at the sequencing facility of the Centre for Molecular Medicine, University of Cologne, Cologne by modified dideoxy nucleotide termination method using a 'Perkin Elmer ABI prism 377' DNA sequencer.

3.16 Quantitative PCR

Total RNA was extracted and cDNA was prepared as described under 3.9. Primers were selected such that the expected product size was between 250-350bp. Prior to use in real time experiment the quality of the cDNA and the primers were tested by PCR. Real Time PCR was carried out with the Quantited™ SYBR® green PCR kit (Qiagen) according to the manufacturers protocol. For each sample gene specific primers (10 pmole) and 1 µl of cDNA was used. As a quantification standard defined concentrations (10ng, 1ng, 100pg, 10pg and 1pg) of pT7-7 contianing the annexin 7 gene were used. Actin specific primers were used as positive control and to ensure comparable concentrations of cDNA in samples of wild type and mutant cells.

4.0 Microarray analysis

4.1 Principle

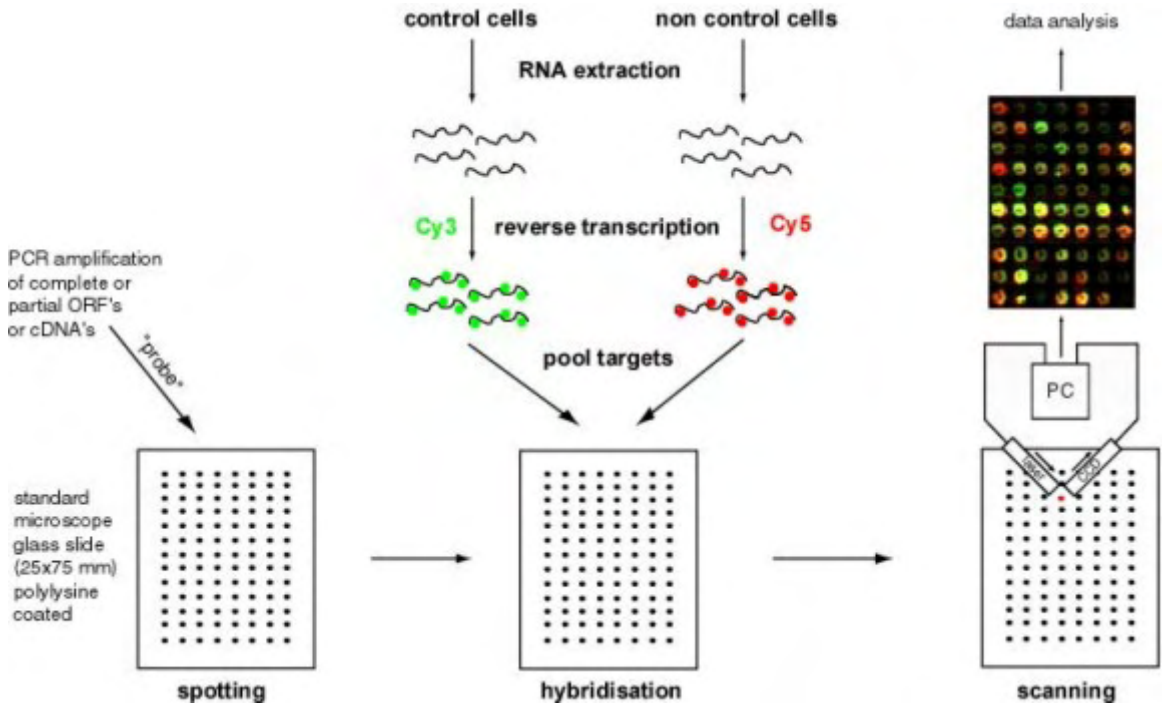


Figure A. Principle of DNA microarray analysis. cDNAs or partial ORFs are amplified by PCR and subsequently printed onto the microarray slides. The slides are hybridized with a pool of fluorescently labelled cDNAs derived from total RNA of control and experiment cells. The hybridized targets are then scanned to measure the intensity of each spots and the data further analysed.

4.2 The *Dictyostelium discoideum* DNA microarray

The *Dictyostelium* DNA microarray consists of an array of DNA probes that are derived from ESTs, published genes and controls. The array contains the sequences of 5423 non-redundant cDNA clones that were obtained from the *Dictyostelium* cDNA project (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html), 450 partial sequences of published genes and 33 controls (Table A). Positive controls are selected partial gene sequences from *D. discoideum*, genomic DNA (100 ng/µl) and the SpotReport-10 PCR products (Stratagene). Negative controls are Fish sperm DNA (100 ng/µl), phage λ Bst E III (100 ng/µl), human Cot-1 (10 ng/µl) and SpotReport Poly(dA) (1 ng/µl). Altogether, the array contains 5906 targets, each of which was printed at least in duplicate. Corning Ultra Gaps microrray slides were used for spotting and the spotted slides were baked for 2hrs at 80°C to covalently attach the DNA to the slides.

Description	Probes	Spots
Published genes	450	900
cDNAs	5423	10846
Controls	33	2874
Total	5906	14620

Table A. Properties of the *Dicytostelium* DNA microarray

4.2.1 Pre-hybridisation

The slides were pre-hybridised with blocking solution to reduce background signals through non-specific binding of labeled cDNAs.

Blocking solution (for 25 slides)		Final concentration
Water	92 ml	-
20x SSC	100 ml	5x SSC
Formamide	200 ml	50%
10% SDS	4 ml	0.1%
BSA (10mg/ml)	4 ml	0.1mg/ml

Procedure

- Pre-heat the blocking solution to 42°C
- Incubate the spotted slides in the pre-heated blocking solution for 45 min
- Rinse with water for 15 sec
- Briefly dip in Isoproponal
- Spin the slides at 235 x g for 2 min to dry them
- The slides are now ready for hybridisation

4.2.2 Sample preparation and cDNA generation

Total RNA from Ax2 cells and the FrzA$^-$ mutant was prepared, as described in (3.9.1). RNA was isolated at different time points of development in two independent experiments from cells starved at high cell density (HCD, 5 x 10^6 cells per cm^2) and low cell density (LCD; 1 x 10^6 cells per cm^2).

4.2.3 Spiking of internal mRNA controls

Quality control is an important issue of DNA microarray analysis. Therefore ten different mRNAs from A*rabidopsis thaliana* genes are used as internal controls. These are added (spiked) to the *D. discoideum* RNA prior to cDNA generation and labeling. mRNAs are provided in a spikemix with different known amounts of each mRNA (Table B). Two different mixes are used for the two labeling reactions (Cy3 and Cy5) of one microarray experiment.

Spikemix 4 single reaction		**Spike A** (pg)	**Spike B** (pg)	**Ratio**	**Total Amount** (pg)
1	**Cab**	300	150	2	450
2	**RCA**	250	250	1	500
3	**rbcL**	250	250	1	500
4	**LTP4**	5	5	1	10
5	**LTP6**	2	2	1	4
6	**XCP2**	1	1	1	2
7	**RCP1**	300	150	2	450
8	**NAC1**	400	40	10	440
9	**TIM**	375	75	5	450
10	**PRKase**	375	75	5	450

Table B. The composition of the mRNA spike mix.

Procedure:

- Add 1 volume of Spikemix to *D. discoideum* total RNA (e.g. 10 µl of Spikemix A to 10 µg of RNA from wild type and 10 µl of Spikemix B to 10 µg of RNA from the mutant).
- Precipitate the RNA mixes by adding 0.1 volumes 3 M sodium acetate, pH 4.8, and 2.5 volumes 100 % Ethanol.
- Store at –20°C for 2 hrs and centrifuge in a tabletop centrifuge at maximum speed for 30 min.
- Remove Ethanol by aspiration and wash with 70 % Ethanol.
- Centrifuge 15 min at maximum speed, aspirate and dry.
- Dissolve in 12 µl of Rnase free water.
- Proceed with cDNA generation and labeling.

4.3 cDNA generation and fluorescent labeling of cDNA

Total RNA (10 µg) in 12 µl of RNase free water was incubated with 1 µl of 500 ng/µl oligonucleotide (dT) for 10 min at 70°C and immediately cooled on ice. 5 µl of a mastermix (see below) and 0.7 µl of reverse transcriptasse (Stratascript RT 50 U/µl) was added and the sample incubated at 48°C for 25 min. Then another 1µl of 50 U/µl Stratascript RT was added and incubation continued at 48°C for 35 min. The reaction was stopped by adding 10 µl of 1M NaOH and then incubated at 70°C for 10 min. The samples were slowly cooled to room temperature. Tubes were spun briefly and 10 µl of 1M HCl was added for neutralisation.

Master mix	
Stratascript reaction buffer	2 µl
dNTP mix*	1 µl
0.1M DTT	1.5 µl
RNase block (40 U/µl)	0.5 µl

*The dNTP mix contains aminoallyl dUTP to which the fluorescent dyes (Cy™3 or Cy™5, Amersham) are covalently attached in the labeling reaction. The labeling was done with dyes according to the protocol of the Stratagene Fair Play kit with slight modifications.

4.4 Hybridization of microarray slides

The microarray slides have a labeling area with the slide number and a bar code that is used to identify each slide (Figure B). The array has 14620 spots per slide and represents almost 50% of the *Dictyostelium* genome. A corning hybridization chamber was used for hybridization.

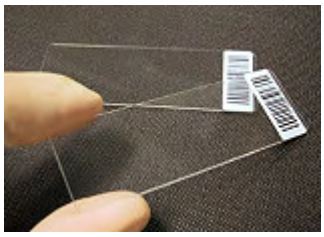


Figure B. Microarray slide with a bar code

4.4.1 Buffers and solutions

Hybridization solution (50 µl)		Hybridization buffer
Hybridization buffer	48 µl	Na-phosphate buffer, pH 6.8 0.12 M

Fish sperm DNA [10 mg/ml]	1 µl	EDTA	2 mM
Oligo dA (18 mer, 100 µM)	1 µl	Formamide	50 %
		Na-Laurylsarcosinate	1 %
		SDS	0.2 %
		Denhardt's reagent	4x

100 x Denhardt's reagent		20 x SSC	
Ficoll 400	2 %	NaCl	3 M
Polyvinylpyrrolidone	2 %	Na-citrate	0.3 M
Bovine serum albumin	2 %		

1.2 M Phosphate buffer, pH 6.8	
1.2 M Na_2HPO_4	2 vol.
1.2 M NaH_2PO_4	1 vol.

Procedure:

- Pipette 10 µl of 3xSSC into the two holes of the Corning hybridization chamber.
- Dissolve the precipitated targets in 65 µl of hybridisation solution.
- Incubate the target solution 10 min at 80°C.
- Centrifuge and pipette the 65 µl of targets on the microarray slide on the side opposite of the barcode.
- Take a cover-slip and slowly place it onto the microarray slide.
- Place the slide cover-slip up into the hybridisation chamber, close the chamber and submerge it in the water-bath at 37°C for overnight.

Washing

After hybridisation the microarray is washed to remove unbound target. During washing the transitions from the baths should be performed swiftly, so the microarray does not dry before processing is finished.

- Remove the microarray from the hybridisation chamber and plunge into 2xSSC with 0.1% SDS. Gently shake until the cover-slip comes loose and remove it with tweezers.
- Wash for 5 min in 1xSSC, 0.1% SDS.
- Wash for 5 sec in 0.1xSSC; repeat this step for five times.

- Wash for 5 sec with 0.01x SSC.
- Centrifuge slides in a rack at 235 x g (1000 rpm) for 5 min.

4.5 Scanning of microarray slides

The ScanArray 4000XL confocal laser scanner (Perkin Elmer Life Sciences) was used to detect the fluorescently labelled cDNA targets that bound to the immobilised DNA on the array. The microarray slide was scanned for Cy3 and Cy5 successively with a resolution down to 5 µm/pixel. The fluorescent dyes are excited by laser-light of pertinent wavelength and emission is detected by a photo-multiplier. To obtain images well suited for signal quantification image brightness has to be adjusted by adjusting the laser-power between 50% and 100% (photo-multiplier power should always be set at 70 to 80 %). The signals should be as bright as possible, but spots should not be saturated (indicated by white colouring). It might be necessary to scan at two different laser-power settings. One setting where most spots give bright signals, but a few like some of the positive controls are saturated, and another setting where no saturation is seen, but most spots give weak signals.

4.6 Data Analysis

The spot and background intensities of the scanned images were quantified using ScanArray Express (Version 2.2.22). The raw data thus obtained were subjected to a series analysis steps.

- The Scanarray express CSV files were converted into *.txt files and imported into the ArrayTools, which is an Add-in for Microsoft Excel designed for import and export of microarray data.
- Data were then exported to the program R for normalization and imported back to AarryTools.
- The normalized data were then imported to the Significance Analysis for Microarray (SAM) program to identify differentially expressed genes.
- The significant genes were then clustered based on their expression pattern using the clustering tool (K-means) in the program GeneSpring (Version 4.1).

5.0 Construction of vectors

5.1 Amplification and cloning of the partial and full-length cDNA

Different domains of FrzA gene were PCR amplified with primers listed in Materials and Methods (1.9) to facilitate cloning into GFP expression or GST expression vector.

10x PCR buffer:

100 mM Tris/HCl, pH 8.3

500 mM KCl

Reaction-mix:

1 µl Template

1 µl Forward primer (10 pmol/µl)

1 µl Reverse primer (10 pmol/µl)

5 µl 10x PCR buffer

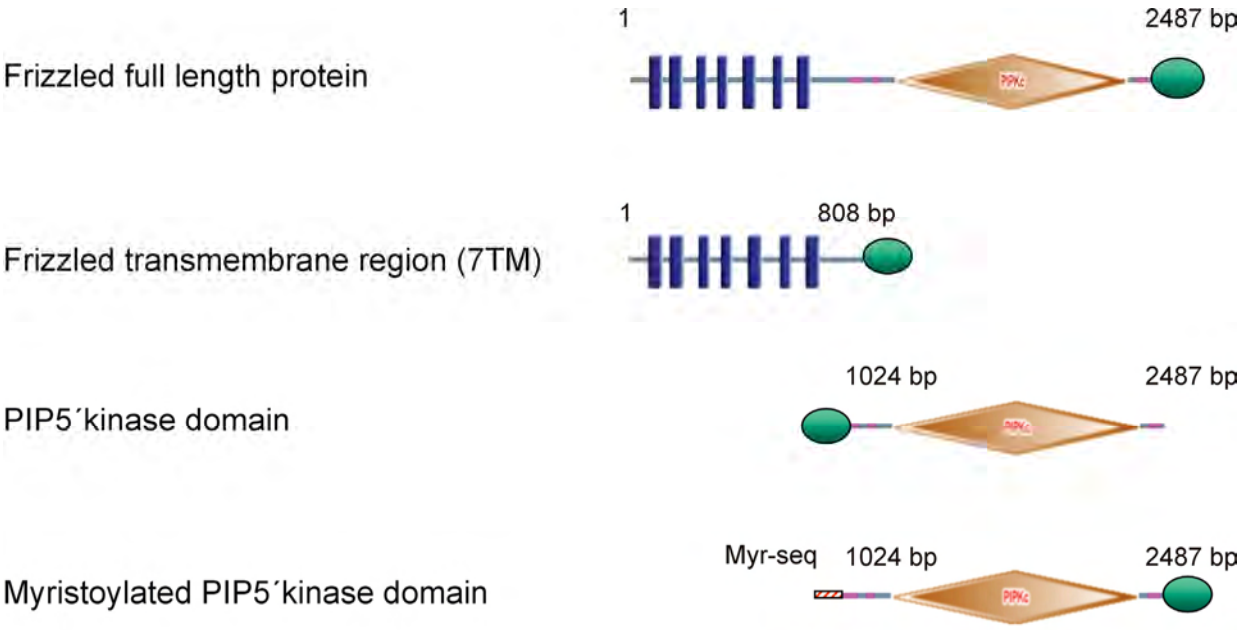
1 µl dNTP-mix (10 mM each)

1 µl Taq polymerase (1 U/µl)

add H_2O to make 50 µl

5.2 Vector for expression as a GFP-fusion protein

A vector for expression of different domains and full-length genes as a GFP- fusion protein in *Dictyostelium* under the control of actin-15 promoter and actin-8 terminator was constructed using the vector p1aBsr8 vector (Graf *et al.*, 2000) or pGFP-MCS (Weber et al, 1999). To facilitate subcloning into p1aBsr8 vector, various domains of FrzA gene (the positions relative to the start ATG are shown below) were PCR amplified with primers mentioned in Materials and Methods (1.9), and cloned into pGEM-T easy vector. The resulting plasmids were verified by sequencing and each of the domains were then taken as Kpn I + Sac I fragment and cloned in frame with the green fluorescent protein (GFP) as C-terminal fusion for p1aBsr8 vector, or cloned form of PIP5K in frame with the green fluorescent protein (GFP) as N-terminal fusion for pGFP-MCS vector. A sequence coding for the first 16 amino acids of chicken c-Src, composed of the myristoylation signal and the basic amino acid cluster sufficient for stable membrane association (Chung *et al.*, 1999) was added to the forward primer to amplify the PIP5K domain for cloning the myristolyated form of PIP5K domain in p1aBsr8 vector.

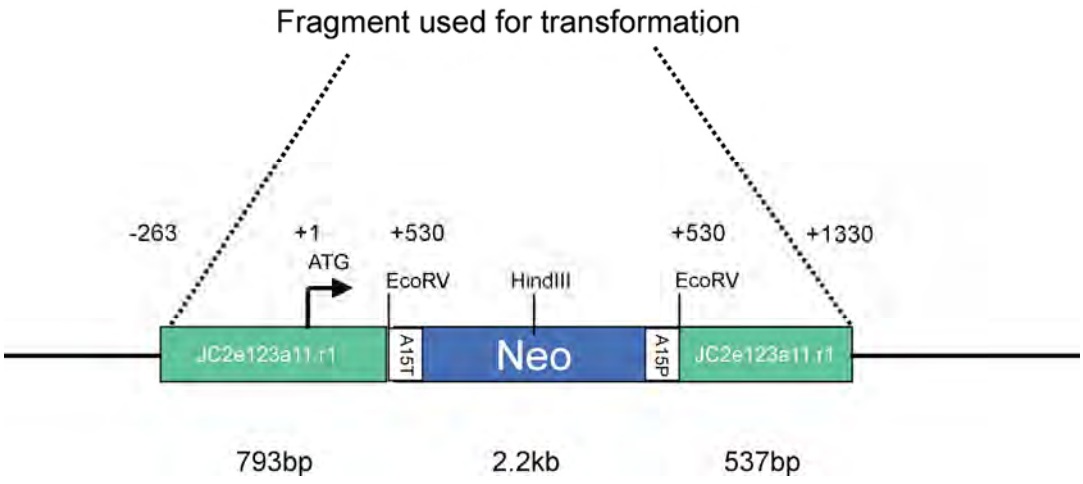


5.3 Vector for expression of PIP5K domain as a GST-fusion protein

The PIP5K domain encompassing amino acid residues 422-792 of the FrzA protein was amplified by PCR with primers PIP5kfp and PIP5krp and subcloned as Eco RI fragment encompassing amino acid residues 480-792 of the protein in pGEX-4T3 vector. The obtained pGEX-PIP5K expression vector was transformed into *E. coli* XL1 blue cells for the expression of GST-PIP5K fusion protein.

5.4 Gene replacement vectors

To create the gene replacement vector for the FrzA gene the Neomycin cassette from the pDNeo II plasmid was taken out as an Eco RV fragment and inserted into a FrzA read (JC2e123a11.r1) obtained from the IMB, Jena, Germany. The digested and eluted fragment shown below was used for transformation of Ax2 wild type cells.



5.4.1 Screening of FrzA$^-$ mutant

A PCR approach was used for screening for FrzA$^-$ mutant cells. Wild type Ax2 cells were electroporated with the gene replacement vector (Materials and Methods, 2.3) and the transformants were selected for resistance to Neomycin (3.5 µg/ml). Single cell transformants were then obtained by spreader dilution of the whole pool of transformants onto SM plates overlaid with *Klebsiella*. Thereafter, the single transformants were picked up and grown in separate wells in a 24 well tissue culture plate in the selection medium (as above) that has been supplemented with streptomycin (40 µg/ml) and ampicillin (50 µg/ml) to get rid of the bacteria. The positive clones were then further grown in separate flasks to prepare genomic DNA for the Southern blot analysis to confirm the recombination event.

Preparation of DNA for PCR reaction:

After the cells had grown confluent in the wells, cells were suspended in the medium present in the wells and transferred to a 1.5 ml microcentrifuge tube. The cells were then pelleted by centrifugation in a microcentrifuge at maximum speed for 15 s. The cells were washed twice with 1 ml of ice-cold H_2O and resuspended in 100 µl of lysis buffer. The cells were then incubated at 56ºC for 45 min followed by incubation at 95ºC for 10 min to liberate the genomic DNA.

PCR conditions:

15 µl of the processed cell suspension containing the liberated genomic DNA was used as a template for PCR. Reaction programme and composition of the reaction-mix are indicated:

Lysis buffer
0.5% Nonidet P-40
0.05 mg/ml proteinase K
in 1x PCR buffer

10x PCR buffer:
100 mM Tris/HCl, pH 8.3
500 mM KCl
15 mM $MgCl_2$

Reaction-mix (50 µl final volume)
15 µl template
2.5 µl a15p forward primer (2 pmol/µl)
2.5 µl knock out reverse primer (2 pmol/µl)
1.0 µl dNTP-mix (10 mM each)
5.0 µl 10x PCR buffer
1.0 µl Taq polymerase (1 U/µl)
23 µl H_2O

Reaction programme:
94ºC for 3 min
56ºC for 1 min
68ºC for 1 min
94ºC for 45 sec
56ºC for 45 sec (Step 5-3, 35 cylces)
68ºC for 10 min
4ºC till end

6. Biochemical methods

6.1 Cell Culture of *Dictyostelium*

Cells were grown in axenic medium (Materials and Method 2.1.1) to a density of 2 x 10^6 cells/ml. Growing *Dictoystelium* cells were then harvested by centrifugation to collect vegetative cells or washed twice in Soerensen phosphate buffer and starved by plating onto a phosphate agar plate (for protein and RNA preparation) or shaking in Soerensen phosphate buffer with required cell density for IP3 and cGMP assay or protein and RNA preparation or to collect developmental stages for protein sample preparation.

6.2 cAMP pulsing experiment

Cells were shaken in Soerensen buffer at a density of 1 x 10^7/ml either unpulsed or pulsed with 100 nM cAMP at 6 min intervals for 8 hrs at 160 rpm. Samples were then taken at 2 hrs intervals for protein and RNA preparation. Total protein of 1 x 10^7 cells was prepared by lysing the cells in 50 µl 1x SDS sample buffer and RNA was prepared after 8 hrs of shaking (with and without cAMP pulsing) as mentioned in Materials and Methods (3.9.1).

6.3 Subcellular fractionation

Dictyostelium cells were collected by centrifugation (1000 x g for 5 min) and resuspended in MES buffer supplemented with a protease inhibitor mixture (50 µg/ml leupeptin, 10 µg/ml pepstatin A, 2 mM benzamidine, 1 mM PMSF) and 0.1% Triton X-100. Triton X-100 soluble and insoluble fractions were separated by centrifugation at 100,000 x g for 10 min and extracted in 2x SDS sample buffer.

6.4 Preparation of a membrane-enriched cell fraction for cAR1 analysis (Brandon and Podgorski, 1997)

Cells at a density of 8-10 x 10^6 cells in a volume of 0.1 ml were rapidly diluted in 0.9 ml of ice-cold saturated ammonium sulfate, vortexed vigorously, and centrifuged at 6000 x g for 6 min at 4ºC. The supernatant was gently aspirated and the cell pellet was resuspended in 0.2 ml of ice-cold receptor assay buffer containing protease inhibitors (Theibert et al., 1984) to lyse the cells. A membrane-enriched cell fraction was collected by centrifugation at 16,000 x g for 10 min at 4ºC. The final pellet was resuspended in sample buffer (10% glycerol, 5% DTT, 3% SDS, 62.5 mM Tris, pH 6.8, 2% bromophenol blue) at 5 x 10^7 cell equivalents/ml. Approximately 2 x 10^7 cell equivalents were loaded per lane on a 12% SDS-PAGE gel.

6.5 PLC assay

Inositol 1,4,5-trisphosphate (IP-3) production was determined following the procedure described in Van Haastert, 1989, using a kit (Amersham Pharmacia Biotech) for the IP3 assay with a modification. The assay was performed in the presence or absence of a stimulus (1ng/ml rCMF) and 100 µl of supernatant from the neutralized cell extract was used.

6.6 cGMP assay

Competitive immunoassay for quantitative determination of cGMP levels was carried out using a cGMP assay kit (Amersham Pharmacia Biotech). Cells were prepared as in Materials and Methods (6.1) and the assay was done according to the manufacturers protocol.

6.7 SDS-polyacrylamide gel electrophoresis

SDS-polyacrylamide gel electrophoresis was performed using the discontinuous buffer system of Laemmli (1970). Discontinuous polyacrylamide gel (10-15% resolving gel, 5% stacking gel) was prepared using glass-plates of 10 cm x 7.5 cm dimensions and spacers of 1mm thickness. A 12-well comb was generally used for formation of the wells in the stacking gel. The composition of 12 resolving and 12 stacking gels is given in the table below:

Components	**Resolving gel**			**Stacking gel**
	10 %	**12 %**	**15 %**	**5%**
Acrylamide/Bisacrylamide (30:0.8) [ml]:	19.7	23.6	30	4.08
1.5 M Tris/HCl, pH 8.8 [ml]:	16	16	16	-
0.5 M Tris/HCl, pH 6.8 [ml]:	-	-	-	2.4
10 % SDS [µl]:	590	590	590	240
TEMED [µl]:	23	23	23	20
10 % APS [µl]:	240	240	240	360
Deionised H_2O [ml]:	23.5	19.6	13.2	17.16

Protein solutions were mixed with suitable volumes of 2x SDS sample buffer, whereas protein pellets were resuspended in a suitable volume of 1x SDS sample buffer. The samples were denatured by heating at 95ºC for 5 min and loaded into the wells in the stacking gel. A molecular weight marker, which was run simultaneously on the same gel in an adjacent well, was used as a standard to establish the apparent molecular weights of proteins resolved on

SDS-polyacrylamide gels. The molecular weight markers were prepared according to the manufacturer's specifications. After loading the samples onto the gel, electrophoresis was performed in 1x gel-running buffer at a constant voltage of 100-180 V until the bromophenol blue dye front had reached the bottom edge of the gel or had just run out of the gel. After the electrophoresis, the resolved proteins in the gel were either observed by Coomassie blue staining or transferred onto a nitrocellulose membrane for western blot analysis.

SDS-sample buffer:

1x	2x	
50	100	(mM) Tris/HCl, pH 6.8
2	4	(% v/v) SDS
10	20	(% v/v) glycerine
0.1	0.2	(% v/v) bromophenol blue
2	4	(% v/v) β-mercaptoethanol

Molecular weight markers:

Marker (Pharmacia)

LMW- 94, 67, 43, 30, 20.1, 14.4 kDa

HMW-116, 76, 70, 50 kDa

10x Gel-running buffer:

1.9 M glycine

0.25 M Tris/HCl, pH 8.8

1% SDS

6.8 Coomassie blue staining of SDS-polyacrylamide gels

After electrophoresis, the resolved proteins were visualised by staining the gel with Coomassie blue staining solution. The gel to be stained was placed in the Coomassie blue staining solution immediately after electrophoresis and the gel was allowed to stain at room temperature with gentle agitation for at least 30 min. After staining, the staining solution was poured off and destaining solution was added. The gel was then destained at room temperature with gentle agitation. For best results, the destaining solution was changed with fresh destaining solution several times until protein bands were clearly visible.

Coomassie blue staining solution:

0.1% Coomassie blue R250

50% ethanol

10% acetic acid

filter the solution before use

Destaining solution:

7 % acetic acid

20% ethanol

6.9 Drying of SDS-polyacrylamide gels

After destaining, the gel was immersed in gel-dry buffer for 10-15 min at room temperature. Two sheets of cellophane (Novex), slightly bigger than the size of the gel, were also immersed in gel-dry buffer. The gel was then carefully placed between two moistened sheets of cellophane avoiding trapping of air-bubbles, clamped between the gel-drying frames (Novex) and dried overnight at room temperature.

Gel-drying buffer:

25% ethanol

5% glycerine

6.10 Western blotting using the semi-dry method

The proteins resolved by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) were electrophoretically transferred from the gel to a nitrocellulose membrane by using the method described by Towbin *et al.* (1979) with little modifications. The transfer was performed using Towbin's buffer in a semi-dry blot apparatus (Bio-Rad) at a constant voltage of 10 V for 35-45 min. The instructions provided along with the semi-dry apparatus were followed in order to set up the transfer.

Towbin's buffer (transfer buffer):

39 mM glycine

48 mM Tris/HCl, pH 8.3

0.0375% SDS

20% methanol or ethanol

6.11 Ponceau S staining of western blots

To check for the transfer of proteins onto the nitrocellulose membrane, the membrane was stained in 10-15 ml of Ponceau S solution for 2-5 min at room temperature. After staining, the membrane was removed from the Ponceau S solution and rinsed with deionised water to destain until bands of proteins were visible and the background was clear. The position of the constituent proteins of the molecular weight marker and/or the protein of interest was marked and the membrane was again washed with several changes of deionised water to completely remove the stain. Now the membrane carrying the transferred proteins was used for immunodetection (Materials and Methods, 6.11) of specific proteins.

Ponceau S solution:	Ponceau S concentrate (Sigma):
1 ml Ponceau S concentrate (Sigma)	2% w/v Ponceau S in 30% w/v TCA
19 ml distilled H_2O	and 30% w/v sulfosalicylic acid

6.12 Immunodetection of nitrocellulose membrane bound proteins

The western blot was immersed in blocking buffer (1x NCP with 5% milk powder) and the blocking was performed with gentle agitation either overnight in the cold room or at room temperature for 1h. After blocking, the blot was incubated at room temperature with gentle agitation with either commercially available primary antibodies or hybridoma-supernatant at a proper dilution (in 1x NCP) for 1-2 h. After incubation with the primary antibody, the blot was washed 5-6 times with 1x NCP at room temperature for 5 min each with repeated agitation. Following washings, the blot was incubated for 1 h at room temperature with a proper dilution (in 1x NCP) of Horseradish peroxidase (HRP) conjugated secondary antibody directed against the primary antibody. After incubation with the secondary antibody, the blot was washed as described above. After washings, the substrate reaction was carried out depending upon the enzyme coupled to the secondary antibody. Enzymatic chemiluminescence (ECL) detection system (Materials and Methods, 6.12) was used for blots incubated with HRP-conjugated secondary antibody.

6.13 Enzymatic chemiluminescence (ECL) detection system

The blot was incubated in ECL-detection-solution for 1-2 min and then wrapped in a saran wrap after removing the excess ECL-detection-solution. Now an X-ray film was exposed to the wrapped membrane for 1-30 min and the film was developed to observe the immunolabelled protein.

ECL-detection-solution:

2 ml	1 M Tris/HCl, pH 8.0
200 µl	250 mM 3-aminonaphthylhydrazide in DMSO
90 µl	90 mM p-Coumaric acid in DMSO
18 ml	deionised H_2O
6.0 µl	30% H_2O_2 (added just before using)

6.14 Expression and purification of GST fusion protein

E. coli strain XL1 blue cells were transformed with expression vector pGEX-PIP5K (Materials and Methods, 3.15.1) for expression of GST-PIP5K as a glutathione S-transferase (GST)-fusion protein under the control of the IPTG-inducible *tac* promoter.

6.14.1 Small-scale protein expression

Small-scale expression of GST fusion proteins was performed to check the efficiency of expression of various recombinant clones as well as to standardise the conditions of expression before proceeding for the large-scale expression and purification of GST fusion proteins. Single colonies (5-10) of recombinant cells were picked and grown overnight in 10 ml of LB medium containing ampicillin (100 µg/ml) at 37ºC and 250 rpm. 5 ml of the overnight grown culture was inoculated into 45 ml of fresh LB medium containing ampicillin (50µg/ml). The culture was then allowed to grow at 37ºC till an OD_{600} of 0.5-0.6 was obtained. Now the induction of expression was initiated by adding IPTG. In order to standardise the conditions of maximum expression of the fusion protein, induction was performed with varying concentrations of IPTG (0.1 mM, 0.5 mM and 1.0 mM final concentration) at 37ºC. Samples of 1 ml were withdrawn at different hours of induction (3 and 5 hrs). The cells were then pelleted and resuspended in 100 µl of 1x SDS sample buffer. The samples were denatured by heating at 95ºC for 5 min and 10 µl of each sample was checked on a 10% SDS-polyacrylamide gel. Expression of the GST-PIP5K fusion protein was analysed by Coomassie blue staining of the SDS-polyacrylamide gel.

6.14.2 Large-scale protein expression

Large-scale expression of GST-PIP5K fusion protein was performed on the basis of results obtained with small-scale expression procedures as described above. An overnight culture was started with a recombinant clone, showing maximum level of expression, in 50 ml LB medium containing ampicillin (50µg/ml) at 37ºC and 250 rpm. The next day, 50 ml of the overnight grown culture was inoculated into 450 ml fresh LB medium containing ampicillin (50 µg/ml) and the culture was allowed to grow at 37ºC till an OD_{600} of 0.5-0.6 was obtained. Now the induction of expression was initiated by adding 1 M IPTG to a final concentration of 1.0 mM and the induction was performed for 5 hrs at 37ºC and 250 rpm.

6.14.3 Preparation of cell homogenates

After the induction, the culture was transferred to a 500 ml centrifuge bottle (Beckman) and the cells were collected by centrifugation at 4,000 rpm (Beckman Avanti J25, rotor JA-10) for 10 min at 4ºC. The pellet was resuspended in 10 ml of ice-cold lysis buffer containing lysozyme (1 mg/ml) and Triton X-100 (0.5%) and supplemented with fresh protease inhibitors, collected in a 50 ml tube and incubated on ice for 20 min. Incubation in lysis buffer was followed by a brief sonication (3 pulses of 10 s each with a 15 s rest between each pulse), keeping the tube immersed in ice. Sonication was followed by homogenisation using a Dounce homogeniser for 2-3 min in order to ensure complete and efficient cell lysis. The lysate was then subjected to gradual increases in the molarity of urea. At every extraction step the lysate is pelleted at 15,000 rpm (Beckman Avanti J25, rotor JA-25.50) for 15 min at 4ºC followed by next concentration of urea and continued till 8 M urea. The supernatant samples (10 µl) collected from each round of solubilization were dissolved in 1x SDS sample buffer and run on SDS-polyacrylamide gel to be analysed by Coomassie staining.

Lysis buffer:
50 mM Tris/HCl, pH 7.5
100 mM NaCl
5 mM $MgCl_2$
0.5 % Triton X-100
add fresh before use-
1 mM DTT
1 mg/ml lysozyme
protease inhibitors

Protease inhibitors:
1 mM PMSF
1.4 µg/ml pepstatin
5.0 µg/ml leupeptin

6.14.4 Analysis of GST fusion protein in fractionated cell lysate of *E. coli*

To analyse whether the fusion protein is in the cytosol or in inclusion bodies the cells were sonicated (3 pulses of 10 s each with a 15 s pause between each pulse) and centrifuged at high speed in a tabletop centrifuge for 1 min. Supernatant and pellet were dissolved in 1x SDS sample buffer individually and the proteins separated by SDS-polyacrylamide gel for analysis by both Coomassie blue staining and western blot analysis.

6.14.5 Affinity purification of polyclonal antibodies by the blot method

The recombinant protein, which was used to produce the polyclonal antibody, was transferred onto a nitrocellulose membrane and was stained with Ponceau S to confirm the transfer efficiency. The blot membrane corresponding to the recombinant protein was cut out and destained with 1x NCP followed by blocking in Solution I for 2 hrs. The serum was mixed in a ratio of 1:4 with 1x NCP and the stripes incubated in it at 4°C for 2 hrs. The unbound antibody was washed off by four to five times incubation with 1x NCP at 4°C. After washing, the antibodies bound to the recombinant protein on the membrane stripes are eluted with 1 ml Solution II, by incubation for 1min at 4°C. The eluted antibody was immediately neutralised with 100 µl of 1 M Tris/HCl, pH 8.0. The purified antibody was then stabilised with 0.5% BSA.

10x NCP (1L)

87 g NaCl

2 g KCl

100 ml 1 M Tris/HCl, pH 8.0

Solution I

5% milk powder

0.05% Tween 20 in PBS

Solution II, pH 2.6

0.1 M glycin

0.5 M NaCl

0.5% Tween 20

7. Cell biological methods

7.1 Measuring cell size

Ax2 wild type and FrzA$^-$ cells were washed once and allowed to settle on a coverslip for 15 min and random images of the cells were taken using a microscope. The images were then opened using Diskus software to measure the cell size for at least 600-700 cells for both Ax2 and FrzA$^-$. The values were processed using Microsoft Excel program.

7.2 Growth rate measurement

Cells from wild type and mutant were inoculated in equal volume of medium at a density of 1 x 10^6 cells/ml and grown at 21ºC with shaking at 160 rpm. Cells were counted at different time points using the Neubauer chamber.

7.3 Fluid uptake analysis

Cells were resuspended in fresh axenic medium at a density of 5 x 10^6 cells/ml in the presence of 2 mg/ml TRITC-dextran. Samples were harvested at indicated time points and the cells were pelleted after incubating for 3 min with 100 µl of trypan blue (2 mg/ml) to remove nonspecifically bound marker. The pellet was resuspended in phosphate buffer and the fluorescence was measured using a Fluorimeter (544 nm excitation/ 574 nm emission).

7.4 Conditioned medium preparation and starvation under submerged condition

Exponentially growing cells were harvested by centrifugation at 700 x g for 5 min, washed twice in ice-cold Soerensen buffer and resuspended in the same buffer at a density of 1 x 10^7 cells/ml for initiating starvation. For preparation of conditioned medium, cells were suspended at 1x10^7 cells/ml and starved for 16-20 hrs, at 21ºC with shaking at 160 rpm. Cell-free supernatant was prepared by a 700g centrifugation of the starved cell suspension for 5 min. The supernatant was immediately frozen at -80ºC and stored until use as starvation media for freshly harvested vegetative cells. Aggregation tests were performed by starving cells in a 6 well plate (NUNC, USA), in 1 ml of Soerensen buffer or in 1 ml of a cell-free conditioned medium or at a cell density of 10^4 or 10^5 cells per cm^2 in the presence of recombinant CMF (1ng/ml).

7.5 Light scattering measurements and $[Ca^{2+}]_i$ determination

The changes in light scattering of suspensions of amoebae were recorded with a Zeiss spectrophotometer (PM6) according to the method of (Gerisch and Hess, 1974), modified as in (Bumann et al., 1986), at a cell density of 2 x 10^7/ml in Soerensen phosphate buffer starting at 4 h after induction of development. $[Ca^{2+}]_i$ measurements were performed as described (Malchow et al., 1996b).

7.6 Prespore differentiation assay

The cells were grown and harvested as mentioned in Materials and Methods (6.1). Approximately 6000 cells were starved in the well of a Lab-Tek 4 chamber glass slide (roughly 1.1 cm^2 surface area; Miles, Naperville, IL) in 200 µl of buffer with or without 1ng/ml of CMF protein as described by Renu et al., (1994). The cells were subsequently fixed and stained for the prespore antigen D19 using the Mud-1 monoclonal antibodies as primary antibody and and the number of prespore positive cells was counted.

7.7 Video imaging and chemotaxis assay

Cells were prepared as in Materials and Methods (6.1) and starved for 6 to 8 hours. 25-30 µl of cell suspension were diluted in 3 ml of Soerensen buffer and mixed well by pipetting (25-30 times, with occasional vortexing). This is important to dissociate cells from aggregates. 1.5 ml of the diluted cells were then transferred onto a 5 cm glass cover-slip with a plastic ring placed on an Leica inverse microscope equipped with a 10x UplanFl 0.3 objective. Cells were stimulated with a glass capillary micropipette (Eppendorf Femtotip) filled with 0.1 mM cAMP (Gerisch and Keller, 1981), which was attached to a microcontroller. Time-lapse image series were captured and stored on a computer hard drive at 30 seconds intervals with a JAI CV-M10 CCD camera and an Imagenation PX610 frame grabber (Imagenation Corp., Beaverton, OR) controlled through Optimas software (Optimas Corp., Bothell, Washington). The DIAS software (Soltech, Oakdale, IA) was used to trace individual cells along image series and calculate the cell motility parameters (Soll et al., 2001). For processing images, Corel Draw version 11, Corel Photopaint and Adobe Photoshop were used.

7.8 Indirect immunofluorescence of *Dictyostelium* cells

7.8.1 Preparation of *Dictyostelium* cells

Dictyostelium cells were grown in shaking culture to a density of 2-4 x 10^6 cells/ml. Desired amounts of cells were collected in a centrifuge tube, washed twice with Soerensen phosphate buffer and finally resuspended in Soerensen phosphate buffer at 1 x 10^6 cells/ml. 400 µl of the cell suspension were then pipetted onto an 18 mm acid-washed glass cover-slip lying on a parafilm covered glass plate resting in a humid-box. Cells were allowed to attach to the glass cover-slip for 15 min. Thereafter cells attached onto the cover-slip were fixed immediately by one of the fixation techniques described below.

7.8.2 Methanol or PFA fixation

After the cells have attached to the coverslip, the supernatant was aspirated and the coverslip was either fixed by incubating the cells in 3% PFA for 20 min at room temperature or dipped instantaneously into the pre-chilled (-20ºC) methanol in a petri dish and incubated at –20ºC for 10 min. The coverslip was then washed with 500 µl PBS/glycine for 5 min to block free reactive groups followed by two washings with 500 µl of PBG for 15 min each. After washings, the cells were immunolabelled as described below.

Paraformaldehyde solution

0.3 g paraformaldehyde was dissolved in 5 ml ddH_2O by stirring at 40°C and adding 3-4 drops of 2M NaOH. After dissolving, the volume was adjusted to 10 ml with ddH_2O.

PBG , pH 7.4:	PBS Glycine
0.5 % bovine serum albumin	500ml 1x PBS
0.1 % gelatin (cold-water fish skin)	3.75g glycine
in 1x PBS, pH 7.4	filter sterilize and store at –20ºC

7.8.3 Immunolabeling

The cover-slip containing the fixed cells was incubated with 400 µl of the desired dilution (in PBG) of primary antibody for 1-2 h in the humid-box at room temperature. After incubation, the excess antibody was removed by washing the cover-slip five times with PBG for 5 min each. Now the coverslip was incubated for 1 h with 400 µl of a proper dilution (in PBG) of the appropriate secondary antibody followed by two washings with PBG for 5 min and then three washings with PBS for 5 min. After washings, the cover-slip was mounted onto a glass slide. Some time cells expressing GFP fusion protein were directly mounted on to a glass slide after fixation (Materials and Methods, 7.8.2) and observed under a fluorescence microscope or confocal laser scan microscope.

7.8.4 DAPI and phalloidin staining of fixed cells

DAPI staining of *Dictyostelium* nuclei and phalloidin staining of *Dictyostelium* F-actin was performed simultaneously. Staining of F-actin with TRITC- phalloidin demarcated the cell-boundary, which facilitated in determining the number of DAPI stained nuclei within a particular cell. Cells were harvested and the coverslip coated with cells were prepared as explained in Materials and Methods (7.8.1). Cells were then fixed onto the coverslip by methanol or paraformaldehyde fixation in case of TRITC-phalloidin as discussed in Materials and Methods (7.8.2). After fixation and usual washings, coverslips were incubated for 30 min with 400 µl of PBG containing DAPI (1:1000 dil.) and/or TRITC-phalloidin (1:1000 dil.). Thereafter, the coverslip was washed twice with 400 µl of PBG for 5 min each followed by three washings with 400 µl of PBS for 5 min each. After washings, the coverslips were mounted onto the glass slides (Materials and Methods, 7.6.5) for observation under a fluorescence microscope or confocal laser scan microscope.

7.8.5 Mounting

After immunolabeling of the fixed cells, the coverslip was swirled once in deionised water and the extra water was soaked off on a soft tissue paper. Now a drop of gelvatol was placed to the middle of a clean glass slide and the coverslip was mounted (with the cell-surface facing downwards) onto the drop of gelvatol taking care not to trap any air-bubble between the coverslip and the glass slide. Mounted slides were then stored in the dark at 4ºC for overnight. Thereafter, the mounted slides were observed under a fluorescence microscope or confocal laser scan microscope.

Gelvatol

2.4 g of polyvinyl alcohol (Mw 30,000-70,000; Sigma) was added to 6 g of glycerol in a 50 ml centrifuge tube and mixed by stirring. To the mixture, 6 ml of distilled H_2O were added and the mixture was incubated at room temperature. After several hours of incubation at room temperature, 12 ml of 0.2 M Tris/HCl, pH 8.5, was added and the mixture was heated to 50°C for 10 min with occasional mixing to completely dissolve polyvinyl alcohol. The solution was centrifuged at 5,100 rpm for 15 min. After centrifugation, 2.5% of diazobicyclo octane (DABCO), an anti-oxidant agent, was added to reduce the bleaching of the fluorescence. The solution was aliquoted in small volumes in 1.5 ml microcentrifuge tubes and stored at –20°C.

8. Computer analyses

Analyses of the sequences and homology searches were performed using the 'University of Wisconsin' GCG software package (Devereux *et al.*, 1984) and different gene bank databases and *Dictyostelium discoideum* gene databases. Structural predictions and multiple alignments of the protein sequences were made using Expasy Tools and ClustalW software respectively, accessible on the world-wide-web. For processing images, Corel Draw version 11, Adobe Illustrator, Adobe Photoshop and Microsoft Powerpoint softwares were used. Graphs were prepared using the Microsoft Excel software.

1. Results

1.1 Search for polarity genes in *Dictyostelium*

The *Dictyostelium* genome sequence is fully known and can be searched for gene sequences and/or domains of proteins that are involved in cell polarity. When we searched the *Dictyostelium* cDNA database at Tsukuba University, Japan, for domains that have been characterised to be present in the components of cell polarity pathways, we identified a gene with a Frizzled domain, a transmembrane region found in the Wnt receptors which are known to be involved in various cellular functions including the planar cell polarity signalling (Wallingford et al., 2000). We identified clone VFC361, harbouring a seven transmembrane Frizzled domain of the DdFrizzled gene (FrzA) as a singlet clone only in the cDNA database, Japan. Further experiments in our lab in combination with the information gained from the *Dictyostelium* genome project resulted in the complete sequence of the FrzA.

1.2 Sequence analysis and generation of the complete sequence for FrzA

The cDNA clone VFC361 was obtained from the *Dictyostelium* cDNA project, Japan, and sequenced in our laboratory. The sequence information thus obtained was then used to search for reads in the *Dictyostelium* genome project, IMB, Jena, Germany. Three genomic reads JC1a109d02.s1, JC2e123a11.r1 and JC2e123a12.r1, were identified and sequenced. RACE PCR was carried out to identify the real start of the gene giving a small fragment of 450 base pair that contained a start codon at 50 bp upstream of the cDNA sequence available. Meanwhile, the *Dictyostelium* genome project was also completed and the contigs were created from all reads at the Sanger Centre, UK, which had a contig, 4246, containing the complete information of the FrzA sequence. Independent sequencing and assembly of reads and data from the RACE PCR matched the contig from the Sanger Centre. Two contigs one upstream and the other downstream of contig 4246 were assembled to identify restriction enzyme sites for confirming the sequence information and screening the mutants (Figure 1B). Southern blot analysis for the gene FrzA was done based on the information. We observed that there was only one copy of the gene and the signal obtained was of the expected size (Figure 1A).

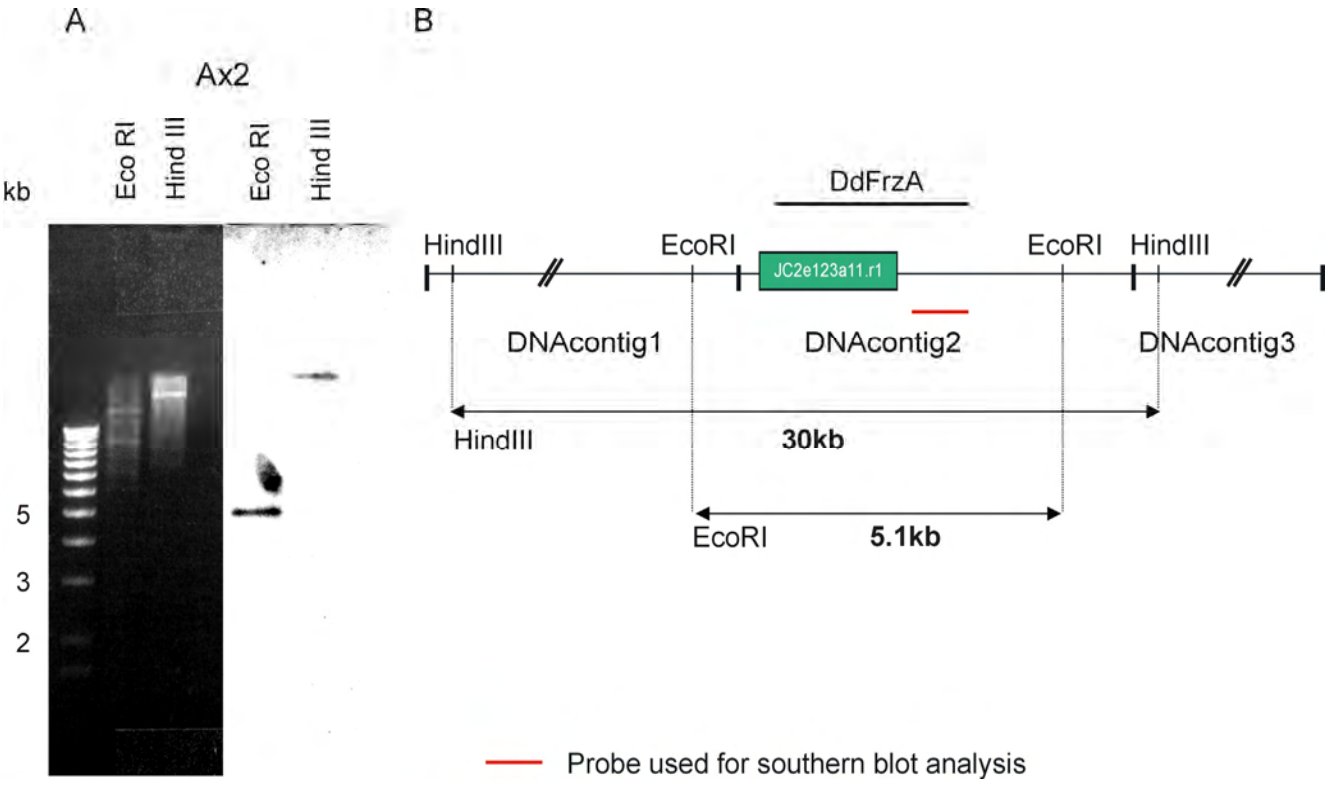


Figure 1. Southern blot analysis of Ax2 genomic DNA to confirm the information of the FrzA gene. A) Wild-type *D. discoideum* Ax2 DNA was digested with Eco RI and Hind III. The fragments were then separated in a 0.8 % agarose gel in TAE buffer and transferred to a nylon membrane as described in Materials and Methods (3.8) and probed with the 1.3 kb DNA probe derived from the 3' end of the gene giving a signal at 30 kb and 5.1 kb for Hind III and Eco RI, respectively. B) Image shows the assembly of the contig sequences obtained from the Sanger Centre, UK. Read JC2e123a11.r1 is also shown that comprises a part of the FrzA sequence.

1.2.1 *Dictyostelium* Frizzled like proteins

We identified 25 Frizzled like receptors when searching the *Dictyostelium* genome database using the transmembrane region of Frizzled. The seven transmembrane regions of *Dictyostelium* Frizzled like receptors were aligned using the ClustalX program and a phylogenetic tree was constructed using Treeview. *Dictyostelium* Frizzled like receptors clustered into five groups (Figure 2). Group I is more closely related to the smoothened Frizzled of *Drosophila* and human, diverging from DdcAR1. DdcAR1 (cAMP receptor) belong to a separate class of cAMP receptors unique to *Dictyostelium* (Bockaert and Pin, 1999) that diverged as a separate family before Group I. Figure 2 also shows that FrzA and DdFrzlike9, though belonging to Group I, are divergent from each other. Group I also diverges from other GPCR like GABA or Serotonin receptors at similar stage where GABA and Serotonin receptors diverge. FrzA has a unique domain architecture and emerges as a new class of proteins in *Dictyostelium* (Figure 3).

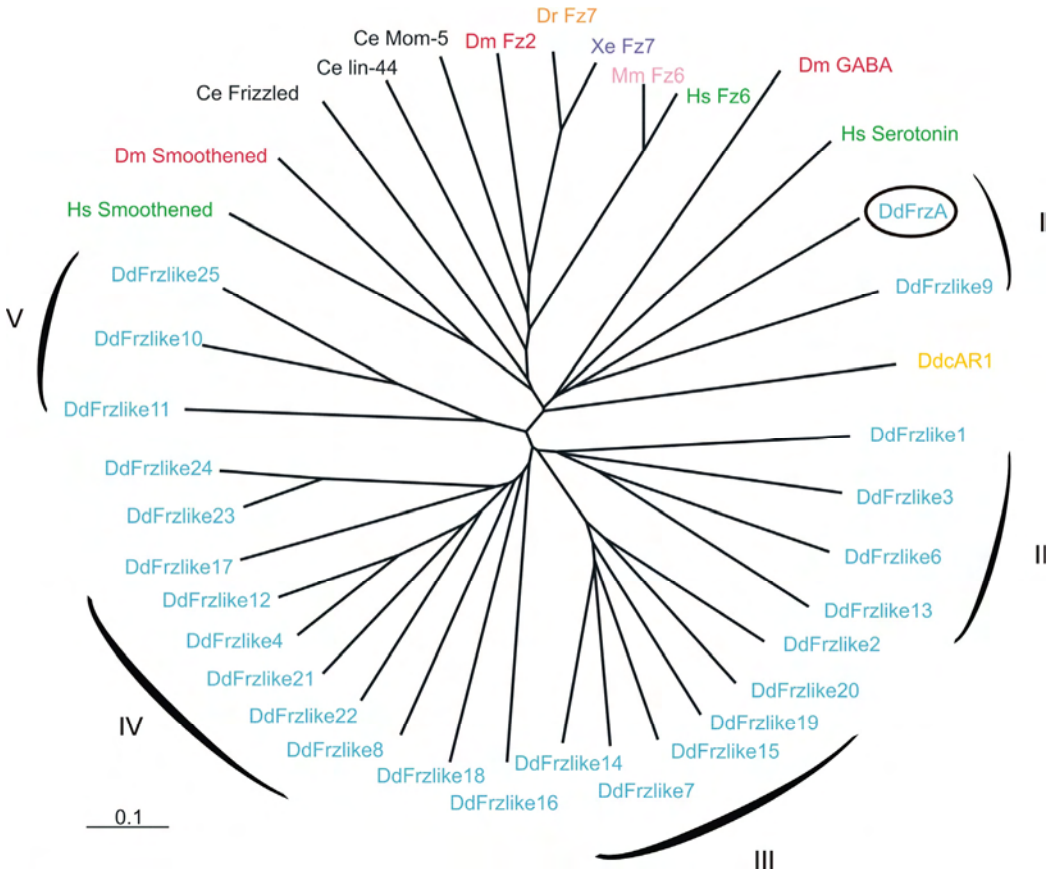


Figure 2. Phylogenetic tree of *Dictyostelium* Frizzled like receptors. Sequences of Dd *Dictyostelium discoideum*, Ce *Caenorhabditis elegans*, Xe *Xenopus laevis*, Mm *Mus musculus*, Hs *Homo sapiens*, Dr *Danio rerio* and Dm *Drosophila melanogaster* were aligned using ClustalX and the tree was constructed using Treeview. Only the Frizzled transmembrane region was considered for the multiple alignment. The *Dictyostelium* gene that is marked with a circle is the object of this study. Bar represents 10% divergence.

1.2.2 Comparison of FrzA protein structure to a typical Frizzled receptor

The full length FrzA cDNA (2487 bp) codes for a polypeptide of ~885 amino acid residues with an estimated molecular weight of ~92 kDa (Figure 3B). The FrzA protein has an unique domain architecture with a seven transmembrane region (Frizzled domain) at the N-terminus and a Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain at the C-terminus (Figure 3C), unlike a typical Frizzled protein (Figure 3A) that has a conserved region of 120 amino acids in the extracellular domain containing a motif of 10 invariantly spaced cysteine residues (the cysteine-rich domain or CRD), a seven-pass transmembrane region and a cytoplasmic tail (Yang-Snyder et al., 1996).

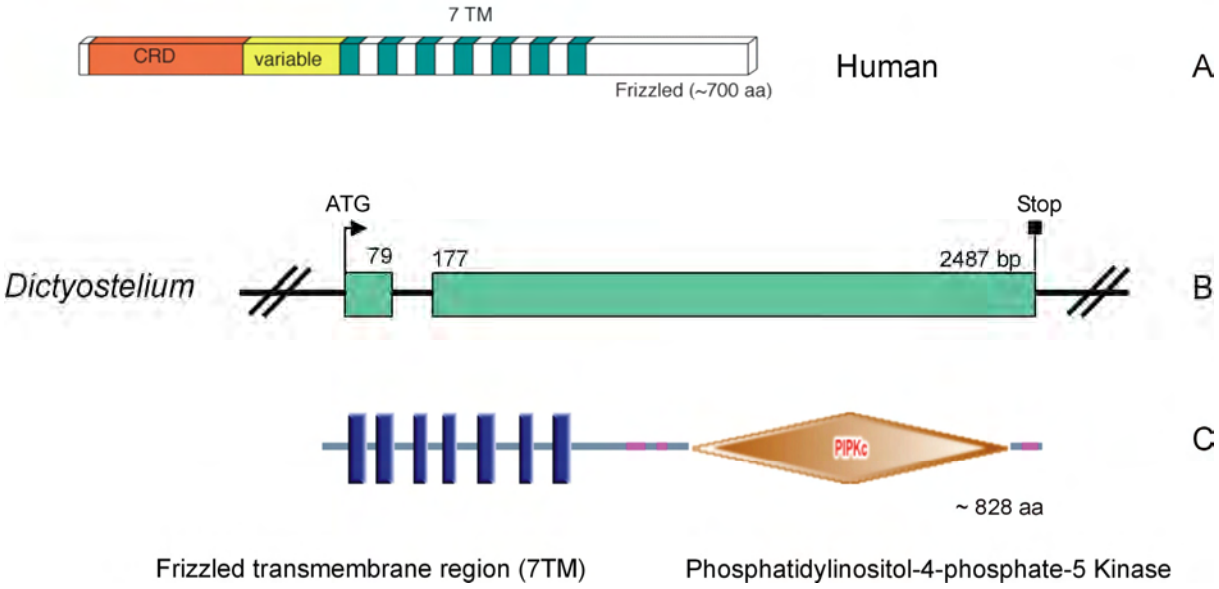


Figure 3. Domain architecture of FrzA. A) A typical Frizzled receptor (human) having a cysteine rich domain (CRD) and seven transmembrane region. B) *Dictyostelium* Frizzled (FrzA) genomic structure. The green box represents the exons and the gap between them represents the intron. C) The protein structure for *Dictyostelium* Frizzled like FrzA was generated using the SMART program available from EMBL showing a seven transmembrane region and PIP5K domain.

1.2.3 Domain analysis of the FrzA protein

Multiple alignment

Transmembrane region of the FrzA

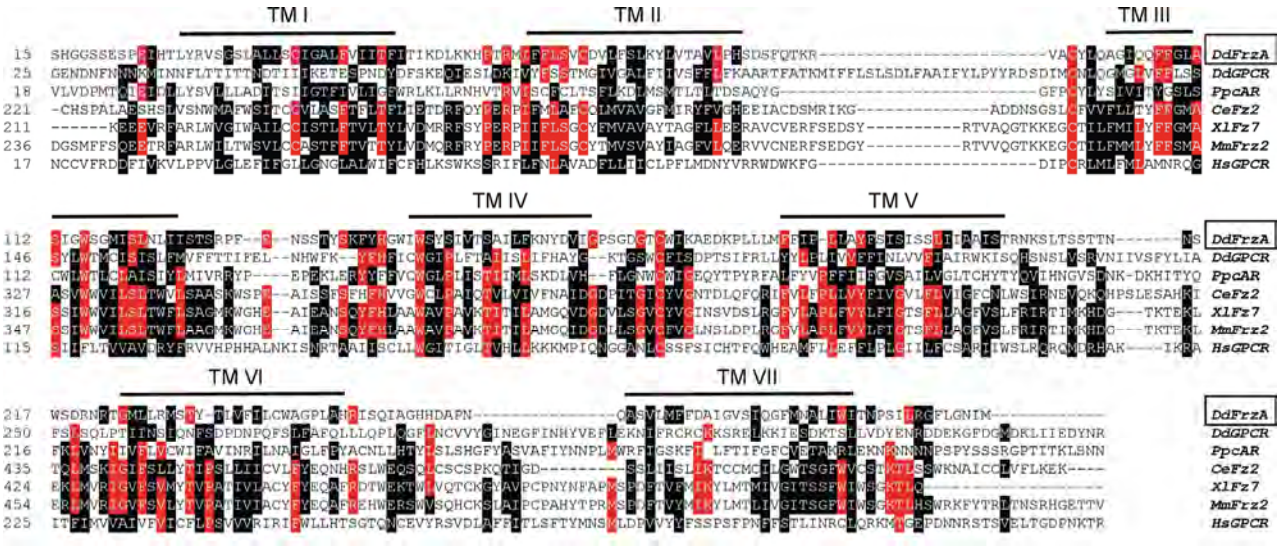


Figure 4. Multiple alignment of *Dictyostelium* and representative Frizzled proteins. Sequences of Frizzled proteins from Dd *Dictyostelium discoideum*, Pp *Polysphondylium pallidum*, Ce *Caenorhabditis elegans*, Xl *Xenopus laevis*, Mm *Mus musculus* and Hs *Homo sapiens* were aligned using ClustalX. Seven transmemrane regions are represented as TM I-VII. Red and black colour represents identity and similarity, respectively. Gene bank accession numbers: FrzA, AY254474; DdGCPR, AY219179; PpcAR; AB045712, CeFz2, T37325; XlFz7, AAH44687; MmFrz2, 10048406; HsGCPR, AAH27965.

With 26% identity and 45% similarity to that of the Frizzled/Smoothened family membrane region of *Caenorhabditis elegans (Ce)*, FrzA has the membrane-spanning region of Frizzled and smoothened receptors. The membrane region is predicted to contain seven transmembrane alpha helices and proteins related to *Drosophila* (Dm) FRIZZLED, which are receptors for the Wnt signaling molecules (Jeffery, 2001).

Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain

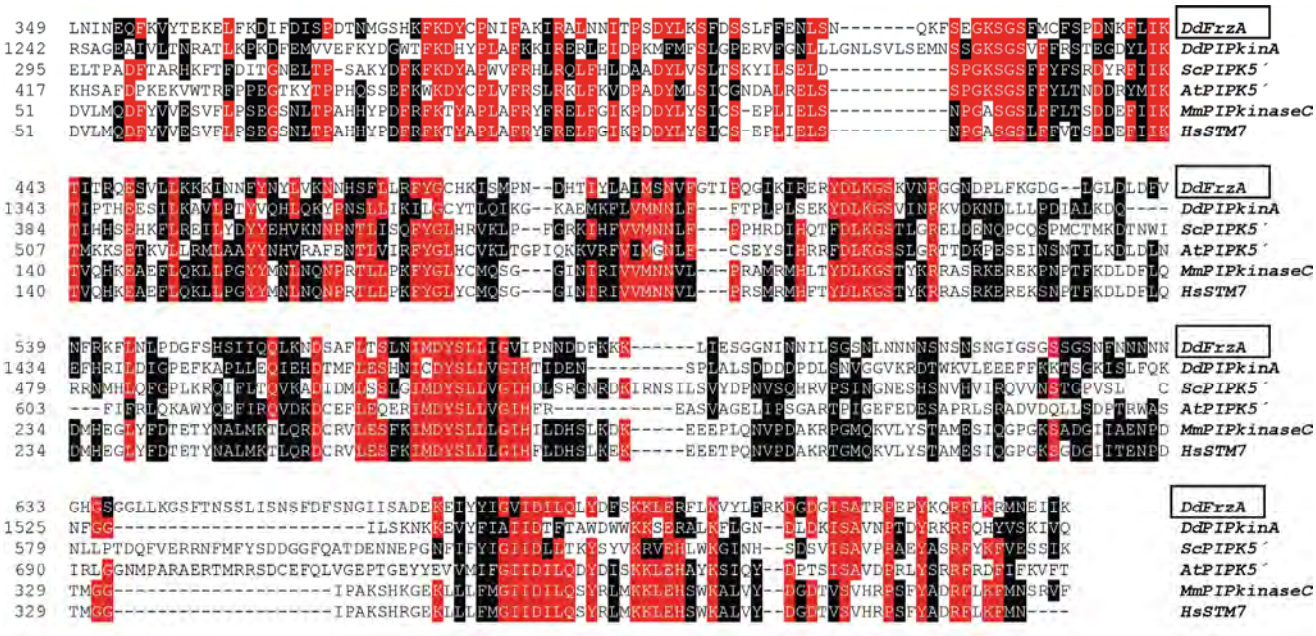


Figure 5. Alignment of the Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain. Multiple alignment was done with PIP5K sequences from Dd *Dictyostelium discoideum*, At *Arabidopsis thaliana*, Mm *Mus musculus*, Hs *Homo sapiens* using ClustalX. Red and black colour represents identity and similarity, respectively. Gene bank accession numbers: FrzA, AY254474; DdPIPkinA, AF339903; AtPIPK5´, CAB72166; MmPIPkinaseC, 6679328; HsSTM7, AAC51327.

The PIP5K domain has 36% identity and 55% similarity to that of the PIP5K domain containing protein STM7 (human) which has been associated to Friedreich's ataxia (Carvajal et al., 1995). PIP5K catalyses the formation of phosphoinositol-4, 5-bisphosphate via the phosphorylation of phosphatidylinositol-4-phosphate, a precursor in the phosphoinositide signaling pathway.

1.3 Northern blot analysis for studying the gene expression pattern of the FrzA

Dictyostelium grow and divide as amoebae and undergo development into multicellular organism when starved (Williams and Harwood, 2003), and this change activates or represses specific genes. Therefore, a northern blot analysis using RNA from different stages of the *Dictyostelium* life cycle was performed to study the expression profile of the FrzA gene. The blot was hybridised with a 450 bp DNA probe derived from the Frizzled domain (40-459 bp) revealing a transcript of ~2.5 kb in size which was present throughout development, although a slightly higher amount of mRNA was detected during aggregation (t8) stage. As a control the same blot after stripping was used for hybridisation with the 1.5 kb CAP cDNA probe that revealed a transcript of nearly 1.7 kb which appears to be upregulated during early aggregation (t4, t8) and is also present throughout development (Noegel et al., 1999).

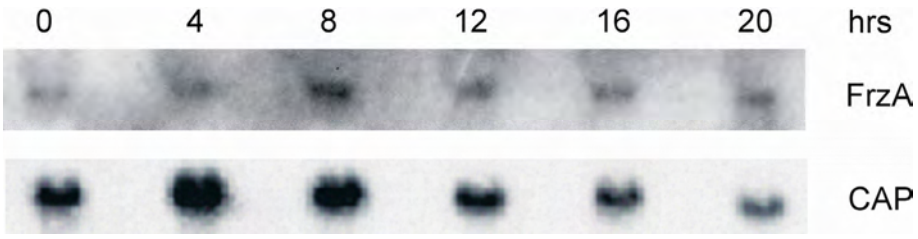


Figure 6. Northern blot analysis shows the presence of the FrzA transcript during the development of *D. discoideum* strain Ax2. 20 µg of total RNA isolated from different time points of development (in hours) as described in Materials and Methods (3.9.1) was loaded in each lane. As a control the same northern blot was used for probing with the cyclase associated protein (CAP) cDNA.

1.4 Expression of GST fusion proteins

The sequences encoding the PIP5K domain of the FrzA protein (480-792 aa) were cloned into the *E. coli* expression vector pGEX-4T3 (glutathione–S-transferase gene fusion system, Pharmacia) in-frame at the C-terminus of GST (Materials and Methods, 5.3) to express it as a GST fusion protein in *E. coli*. Fusion protein expression was done in *E. coli* strain XL1-blue at 37°C with IPTG (1.0 mM) (Materials and Methods, 6.14.1). The GST-PIP5K fusion protein migrated in a SDS-polyacrylamide gel to an expected molecular mass of 75 kDa. Maximum yield was obtained after 5h of induction with 1.0 mM IPTG at 37°C (Figure 7A). The cells were fractionated further for analysing the localisation of the fusion protein and the GST fusion protein was found in the pellet fraction. This was further confirmed by immunoblotting (Figure 7B) using a goat anti-GST antibody (Pharmacia).

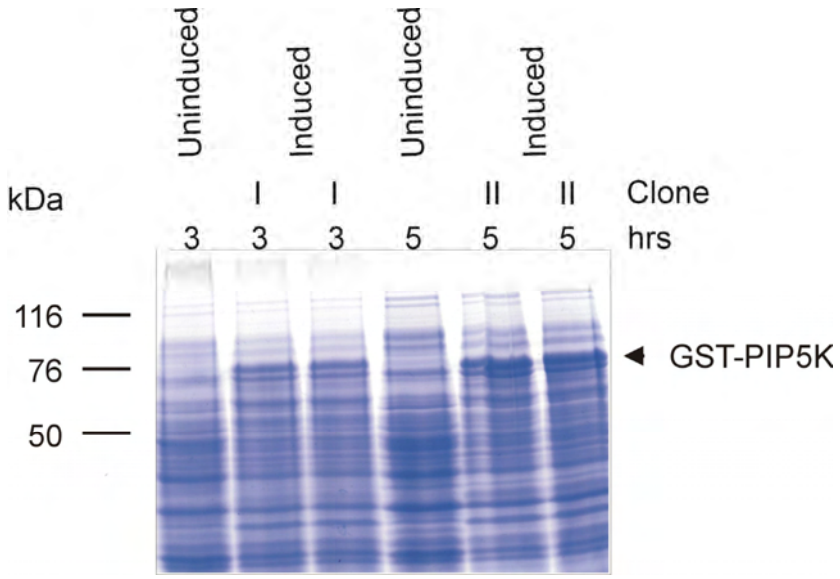


Figure 7A. Expression of GST-PIP5K fusion protein. Coomassie blue stained SDS-polyacrylamide gel (10% acrylamide) showing cell lysates obtained from uninduced and induced (3 and 5 hrs) *E. coli* XL1-blue cultures expressing the GST-PIP5K fusion protein.

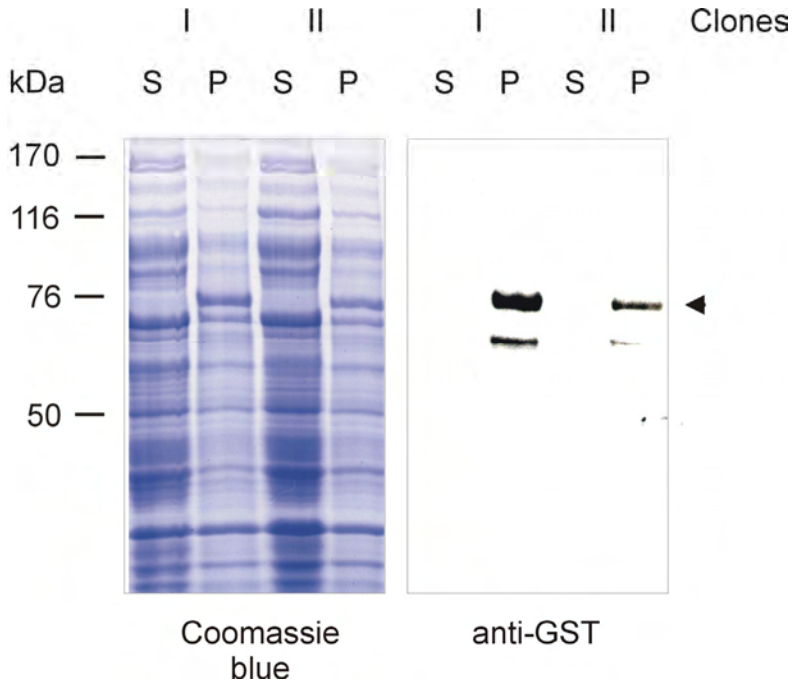


Figure 7B. Analysis of GST-PIP5K fusion protein in cytosol and membrane fractions. Coomassie blue stained SDS-polyacrylamide gel (10% acrylamide) showing supernatant and pellet of induced cells prepared as described in Materials and Methods (6.14.4). GST-PIP5K is found in the pellet fraction and confirmed by western blot analysis with anti-GST antibodies indicated by an arrowhead (◂). Immuno-detection was performed with polyclonal GST-antibodies and secondary peroxidase coupled goat anti rabbit antibodies followed by enhanced chemiluminescence. The lower band of ~66 kDa appears to be due to degradation of the 75 kDa GST-PIP5K fusion protein.

1.5 Purification of GST-PIP5K fusion protein

GST-PIP5K was expressed and extracted from the pellet with increasing amounts of urea (Materials and Methods, 6.14.3). Supernatants obtained at each step were dissolved in 1x SDS sample buffer and separated on 10% SDS-polyacrylamide gel. The GST-PIP5K fusion protein was extracted at higher concentrations of urea (5-7M urea).

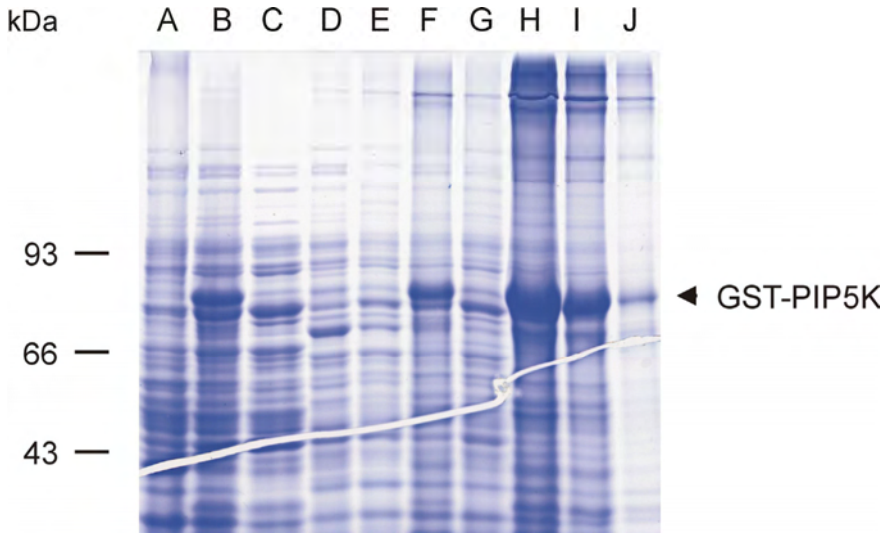


Figure 8. Purification of GST-PIP5K fusion protein. GST-PIP5K expressing *E. coli* XL1- blue cells were prepared as described in Materials and Methods (6.14.3). Urea solubilised proteins (D-J) were separated in a 10% SDS-polyacrylamide gel and stained with Coomassie blue. A, uninduced cell lysate; B, induced cell lysate; C, lysate after lysozyme (1mg/ml) treatment; D-J, 2M, 3M, 5M, 4M, 6M, 7M and 8M urea extracts; respectively.

1.5.1 Characterisation of FrzA polyclonal antibodies

To confirm the identity of the protein expressed, the 8M urea solubilised proteins were separated on a 10% SDS-polyacrylamide gel and the GST fusion protein band was excised for MALDI analysis. To raise polyclonal antibodies specific for the FrzA PIP5K domain, the GST-PIP5K protein was prepared by gel elution (Figure 9A). 100 µg of pure protein was used for immunizing two rabbits to raise polyclonal antibodies (Anti-FrzA). One of the rabbits died after 60 days, therefore, the results given here are for only one rabbit. The rabbit serum after 90 days of immunization was tested for the specificity of the antibody produced. Preliminary screening was done using the recombinant GST-PIP5K. The serum was affinity purified (Materials and Methods, 6.14.4) and tested. It recognised GST-PIP5K but not GST (Figure 9B). To further characterise the polyclonal antibodies, cell lysates from Ax2 control cells and Ax2 and FrzA^{-} transformants expressing GFP-PIP5K were used. Western blot analysis with anti-GFP and Anti-FrzA antibodies showed an appropriate band of ~75 kDa in transformed cells (Figure 9).

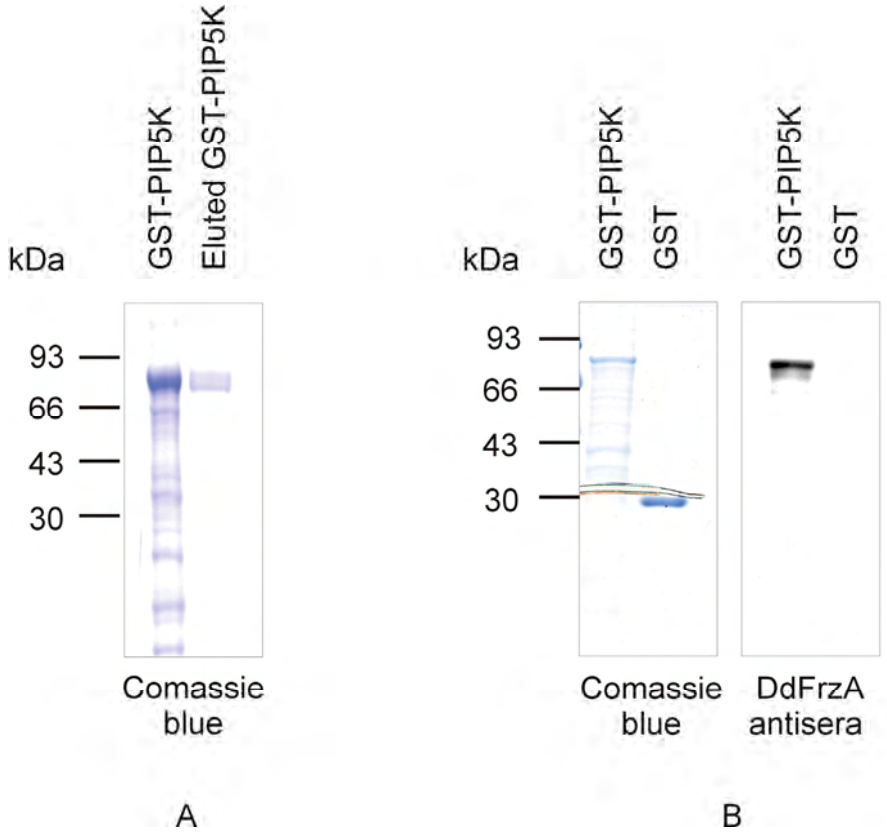


Figure 9. Characterisation of the C-terminus specific FrzA antibodies (Anti-FrzA). A) The pure gel eluted GST-PIP5K protein was run along with the 7M urea solubilised GST fusion protein on a 10% SDS-polyacrylamide gel and stained with Coomassie blue. B) GST and GST-PIP5K were purified from *E. coli* and resolved on a 12% SDS-polyacrylamide gel and transferred to a nitrocellulose membrane by semidry blotting. Immuno-detection was with polyclonal Anti-FrzA and peroxidase coupled goat anti-rabbit antibodies followed by enhanced chemiluminescence.

2.0 Generation of FrzA$^-$ mutant cells

To gain more insight into the function of FrzA *in vivo,* a FrzA$^-$ mutant was generated by homologous recombination (Materials and Methods, 5.4). The knockout construct was generated using the plasmid JC2e123a11.r1 which is 1330 bp in size encompassing four transmembrane regions of the Frizzled domain and also has an additional upstream sequence (-263 nucelotides) to that of the start codon (+1 bp). The neomycin resistance cassette was inserted into the plasmid at the Eco RV site (+530 bp) without loosing any part of the gene (Figure 12A). After homologous recombination the complete FrzA will not be transcribed due to the insertion of Neomycin cassette into the third transmembrane region of the Frizzled domain (Figure 12A). Furthermore, there is no splice site that would allow trascription of the PIP5K domain alone and even if transcribed it cannot be active due to the lack of the seven transmembrane region necessary for membrane localisation. Mutants were initially screened by polymerase chain reaction (PCR), which showed a band of the expected size confirming the insertion of the fragment used as replacement vector.

2.0.1 Southern blot analysis of FrzA$^-$ mutant cells

Information gathered from assembly of genomic contigs (Figure 10A) was used for the screening of the mutants. Southern blot analysis for wild type genomic DNA gave a 5.1 kb and 30 kb fragment for Eco RI and Hind III digestion, respectively (see Results, 1.2). Genomic DNA isolated from Ax2 cells (Materials and Methods, 3.5) and five of the PCR-positive transformants was digested with Eco RI restriction enzyme, which does not have any internal restriction site in the Neomycin resistance cassette. Hybridization analysis with ^{32}P labelled PIP5K cDNA (1642-2553 bp) revealed that a gene replacement event had occurred in the transformants, as the insertion of the 2.2 kb Neomycin resistance cassette causes a shift from 5.1 kb to 7.3 kb (Figure 10B). To confirm the recombination event, another restriction enzyme, Hind III, which has an internal site in the Neomycin resistance cassette was used for Southern blot analysis. The insertion of the 2.2 kb Neomycin resistance cassette introduces a Hind III site into the genomic DNA, which leads to a shift from 30 kb to 8.2 kb (Figure 10B).

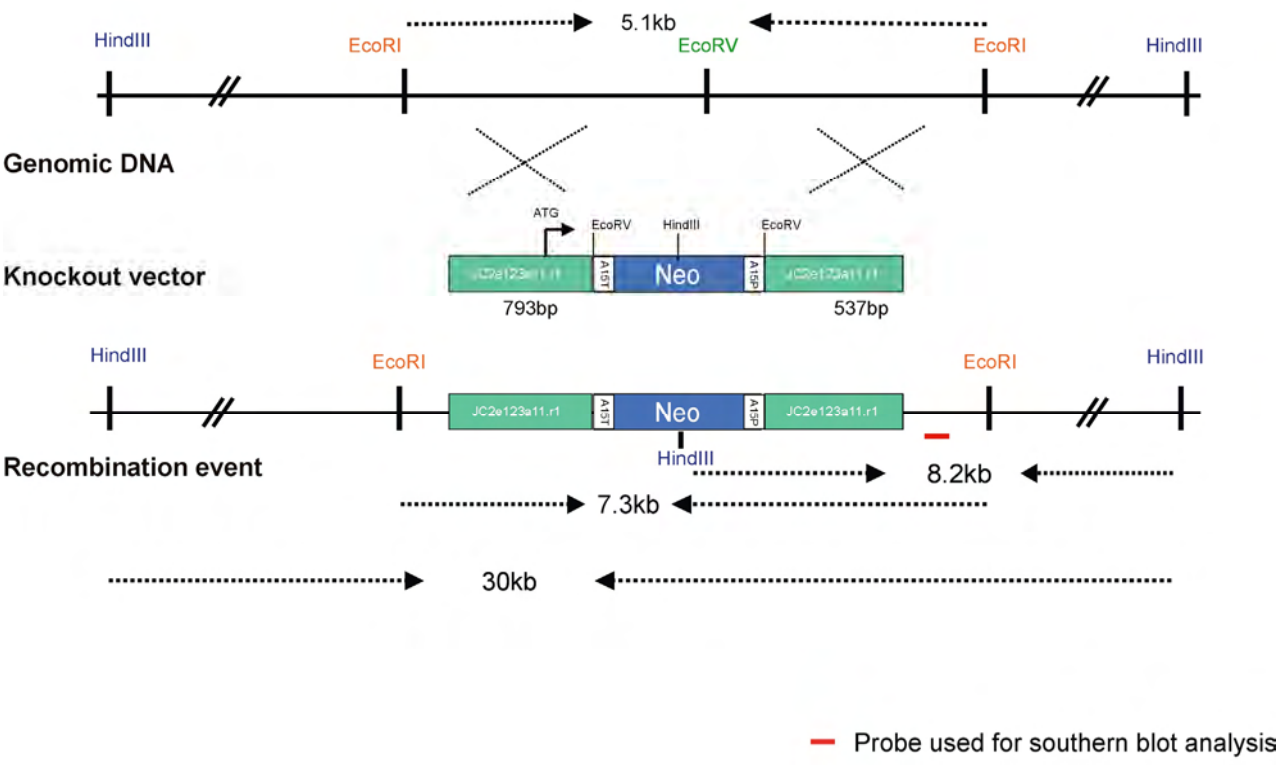


Figure 10A. Illustration of the homologous recombination event. Insertion of the Neomycin resistance cassette introduces a Hind III site that was confirmed by Southern blot analysis. The external probe for Southern blot analysis is shown as a red bar in the recombination event.

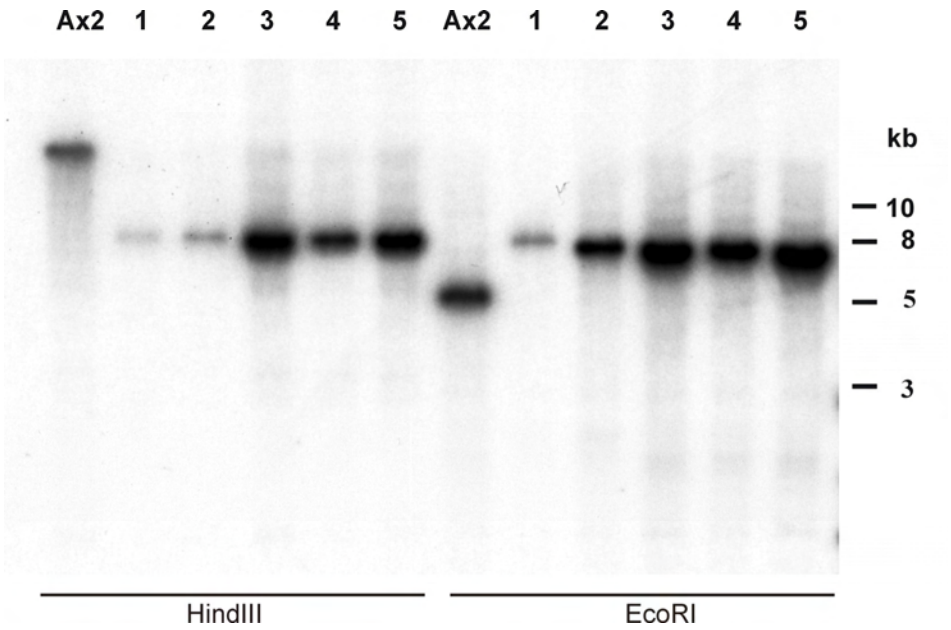


Figure 10B. Disruption of the FrzA gene in wild-type Ax2 cells by homologous recombination. Southern blot analysis of Hind III and Eco RI restricted genomic DNA of Ax2 and mutant (1-5) cells indicates that a gene replacement event has occurred, since insertion of the Neomycin cassette causes the shift of a 5.1 kb band to 7.3 kb in case of Eco RI and 30 kb to 8.2 kb in case of Hind III digestion, respectively. Therefore, this mutant was referred to as FrzA$^-$ mutant and clone 2 was selected for further cell-biological and biochemical characterisation. The probe used for screening the mutants was a ^{32}P labelled PIP5K cDNA.

2.1 Characterization of the FrzA$^-$ mutant

2.1.1 Measurement of cell size of Ax2 and FrzA$^-$

Cells from wild type (Ax2) and FrzA$^-$ were prepared as mentioned in Materials and Methods (7.1) for measurement of cell size. 25% of wild type cells measured were of 10-12 μm in size, whereas 30% of the mutant cells were 9-10 μm (Figure 11). The data showed no significant change in cell size although cells from FrzA$^-$ were slightly smaller in size. Lack of FrzA may not affect cell shape and size.

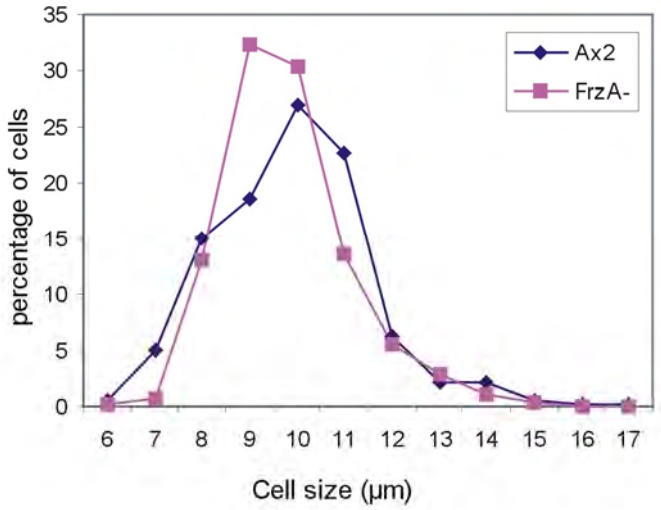


Figure 11. Cell size measurement of Ax2 and FrzA$^-$. Cells were grown at 21ºC to a density of 2 x 10^6 cells/ml with shaking at 160 rpm. Cells were prepared and measured (~650 cells) as mentioned in Materials and Methods (7.1).

2.1.2 Growth in axenic medium

Cell growth involves various cellular processes, and alteration in any of these processes may affect growth rate. Wild-type Ax2 cells attained maximum cell density of 1.1 x 10^7 cells/ml. FrzA$^-$ under similar conditions grew to a density of 1.2 x 10^7 cells/ml and exhibited a doubling point of 53 hrs similar to that of the wild type cells. Therefore, no difference was observed between growth rate and final cell density of Ax2 and FrzA$^-$ cells (Figure 12).

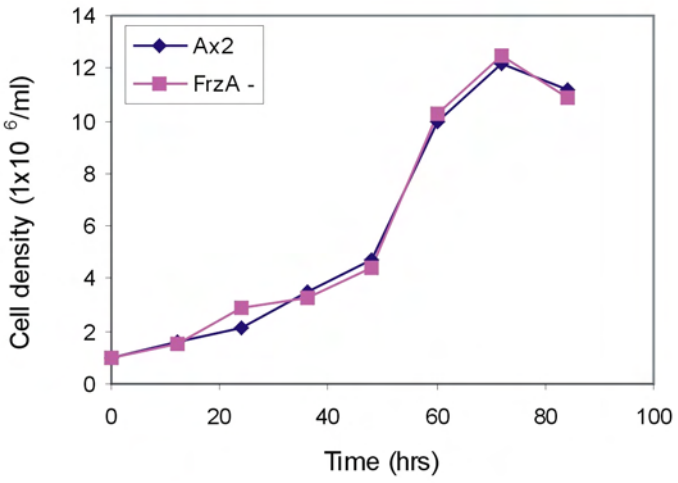


Figure 12. Growth of Ax2 and FrzA$^-$ in axenic medium. Cultures were inoculated in equal volume of medium at a density of 1 x 10^6 cells/ml and grown at 21ºC with shaking at 160 rpm. Cells were counted at the indicated time points using a Neubauer chamber. The graph represents the average of two experiments.

2.1.3 Cytokinesis in FrzA$^-$

Cytokinesis is one of the important processes for cell survival. Therefore, the number of nuclei/cell of FrzA$^-$ mutant cells were quantitated and compared with that of the Ax2 cells employing immunofluorescence studies using the DNA binding dye DAPI (Materials and Methods, 7.8.4). Observation of the DAPI labelled mutant cells under the fluorescence microscope revealed that the FrzA$^-$ mutant cells were mainly mononucleated as are Ax2 cells under these conditions (Figure 13A and B). This suggests that cell division is normal in cells lacking FrzA.

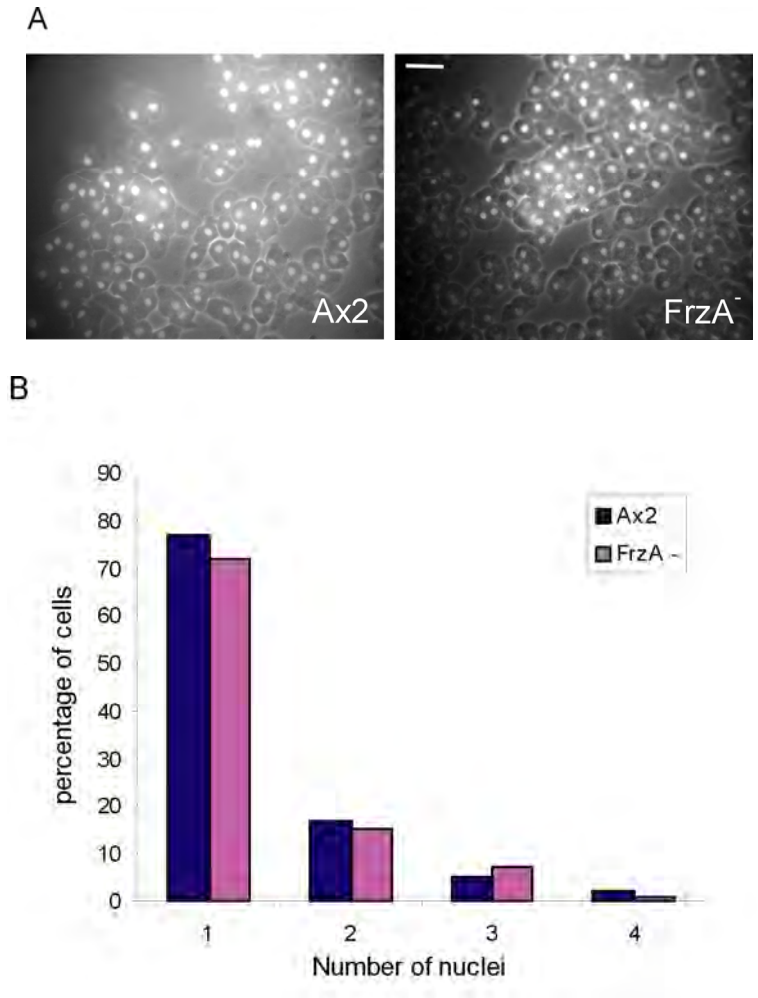


Figure 13. Quantitation of nuclei in Ax2 and FrzA$^-$ cells. A) Overlay of fluorescent images after DAPI staining of the nuclei of the Ax2 and FrzA$^-$ mutant cells (Materials and Methods, 7.8.4). The cells shown are representatives of all the cells in the population. Bar is 10 µm. B) The histogram illustrates quantitation of nuclei of 200 Ax2 and FrzA$^-$ mutant cells.

2.1.4 Fluid uptake in wild type and FrzA$^-$

Dictyostelium cells obtain their nutrients by means of engulfing media via pinocytosis (Maniak, 2001). The kinetics of pinocytosis process is measured using FITC-dextran, which has been previously shown to be an appropriate fluid phase pinocytic marker in *Dictyostelium*

(Hacker et al., 1997). The results obtained for Ax2 and FrzA$^-$ cells show the accumulation of FITC-dextran in a linear fashion over a period of 1 hr. FrzA$^-$ behaved in similar way as the Ax2 cells (Figure 14) indicating that FrzA may not be involved in the process of fluid uptake.

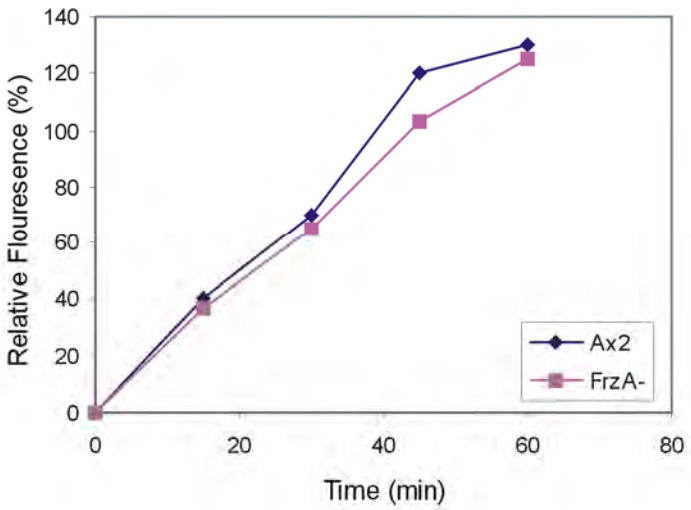


Figure 14. Fluid-phase endocytosis in Ax2 and FrzA$^-$. Cells were resuspended in fresh axenic medium with presence of FITC-dextran and fluorescence was measured at the indicated time points (Materials and Methods, 7.3). All values are the average of at least two independent experiments.

2.1.5 Growth on a lawn of *Klebsiella*

Dictyostelium cells feed on bacteria as a food source and can be propagated on a bacterial lawn on nutrient agar plate. Wild type cells developed fruiting bodies in the region where the food source is depleted (Figure 15), whereas, FrzA$^-$ cells did not produce any fruiting bodies. We can infer that the mutant is able to feed on bacteria for their growth but they are not able to undergo development.

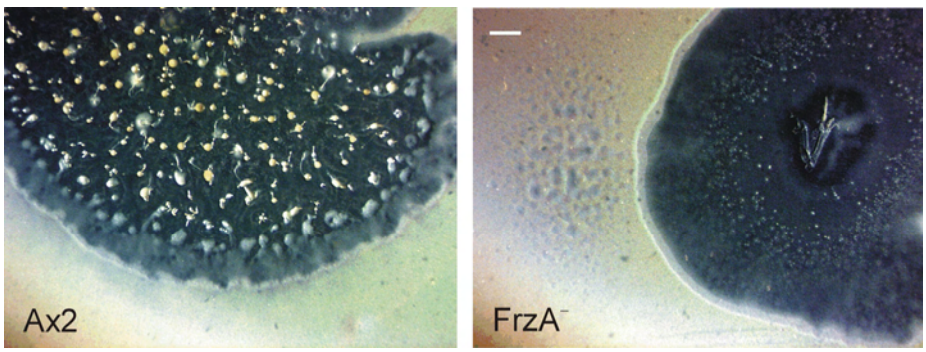


Figure 15. Growth and development of Ax2 and FrzA$^-$ cells on *Klebsiella* plates. Cells were plated on nutrient agar in the presence of *K. aerogenes* and allowed to develop at 21°C. Images were taken using a stereomicroscope. Bar is 1mm.

2.2 Aggregation and development of FrzA$^-$ mutant

2.2.1 Aggregation on a plastic surface

Cells from Ax2 and FrzA$^-$ were starved and analysed for their ability to aggregate under submerged condition (Materials and Methods, 6.1). Ax2 cells were able to form streams towards the aggregation centre (Devreotes, 1982; Janssens and Van Haastert, 1987), whereas, FrzA$^-$ cells formed no streams and remained as single or small group of cells (Figure 16). A similar result was also obtained when starved on phosphate agar plates. This suggests that FrzA$^-$ mutant cells may not recognise the signal from neighbouring cells or may have a motility defect to move towards the aggregation centre or may not develop.

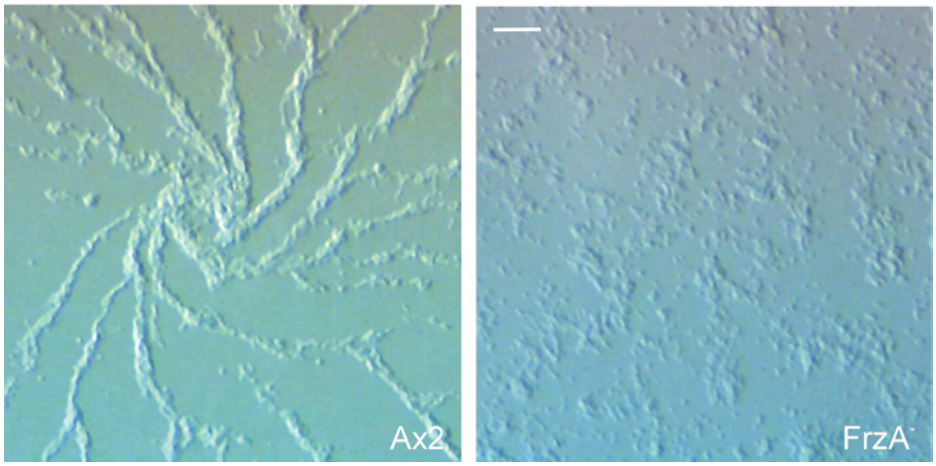


Figure 16. Aggregation on plastic surface. Equal number of cells (2.5 x 10^5 per cm^2) from Ax2 and FrzA$^-$ were analysed for formation of aggregation on plastic surfaces. Images were taken after 6 h of starvation under submerged condition. Bar is 50 µM.

2.2.2 Development on phosphate agar plates

The cells can aggregate when starved under submerged conditions, while post-aggregation development and fruiting require a solid substratum (Coates and Harwood, 2001). Water or phosphate agar is commonly used as substratum to study *Dictyostelium* development. Wild type cells formed tight aggregates after 6 hrs of starvation followed by formation of finger like structures which migrate as slugs after 12 hrs (Kessin, 2001), which then become fruiting bodies by a culmination process after 20-24 hrs of starvation (Figure 17A). But, FrzA$^-$ does not form tight aggregates like wild type cells and remains as loose aggregates even after 24 hrs of starvation. The mutant then undergoes a sudden change in morphology after 48 hrs forming very small fruiting bodies emerging from the loose aggregates. But even after 48 hrs of starvation many of the loose aggregates are still seen (Figure 19B). Therefore, lack of FrzA cause cells to remain predominantly at the loose aggregation stage exhibiting an early developmental defect.

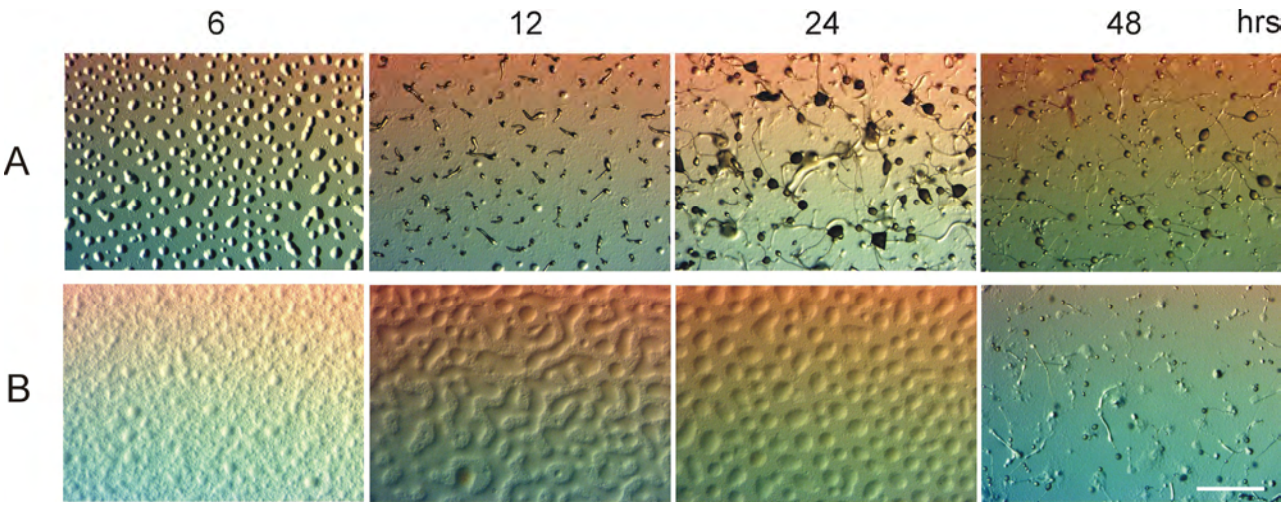


Figure 17. Development of Ax2 and FrzA$^-$ cells on phosphate agar plates. Cells from Ax2 wild type (A) and FrzA$^-$ (B) were plated on phosphate agar plates at a cell density of 5 x 10^7 and allowed to develop at 21°C. A stereomicroscope was used to take images at the indicated time points. Bar is 1 mm.

2.3 Characterisation of FrzA$^-$ development

FrzA$^-$ cells were observed to have a defect at the aggregation stage of development (Figure 19). The genes required for the aggregation process include the cAMP receptor cAR1, adenylyl cyclase gene ACA and the cell adhesion molecule *contact sites A* (csA) (Firtel, 1995; Gerisch, 1968; Noegel et al., 1986a). We compared the expression of the cAMP receptor gene cAR1 (Klein, 1988; Klein et al., 1988), the adenylyl cyclase gene ACA (Pitt et al., 1992) and csA gene (Faix et al., 1992) by Northern blot analysis (Figure 18). The mRNA of cAR1 for the FrzA$^-$ mutant was present at low levels in vegetative cells and did not increase significantly during the first 4 hr of starvation as in wild-type cells. It was notable that in the mutant, the expression of ACA was undetectable during the aggregation stage and found weakly expressed only at 24hrs. By that time the wild type cells had completed the development cycle (Figure 18). The cell adhesion molecule, *contact sites A* was expressed significantly lower in the mutant during the aggregation period (4-8 hrs) and slowly increased after 24 hrs in contrast to wild type cells where *contact sites A* expression decreases after 8 hrs of development. Phosphodiesterase (PDE) controls extracellular and intracellular cAMP concentration in the cell (Riley and Barclay, 1990). Wild type cells express PDE after 4 hrs of intiation of development, which then decreases after 8 hrs. In the FrzA$^-$ mutant PDE was found to be expressed constantly throughout the developmental cycle. A secreted glycoprotein phosphodiesterase inhibitor (PDI) regulates the activity of PDE whose expression is reciprocally regulated by extracellular cAMP levels as PDE (Franke et al., 1991). PDI is expressed significantly lower in the mutant than the wild type cells. Although

the PDI expression in the mutant seems to slowly increase from 12 hrs to 48 hrs of starvation this is in contrast to wild type cells where the expression is completely decreased by that time. While nanomolar pulses of cAMP are necessary for induction of cAMP induced genes, high constant level of cAMP after aggregation is required for expression of postaggregative genes (Landfear and Lodish, 1980). In the FrzA$^-$ mutant prestalk (ecmA) and prespore precursor (pspA) genes are not expressed at similar stages as in wild type (Figure 18), although the expression levels are comparable once they are expressed. On the whole it could be that the periodic signaling which occurs in wild type through an oscillatory circuit (Maeda et al., 2004) may not be present in FrzA$^-$ cells. Proper expression of cAMP regulated early and late developmental genes may therefore not take place.

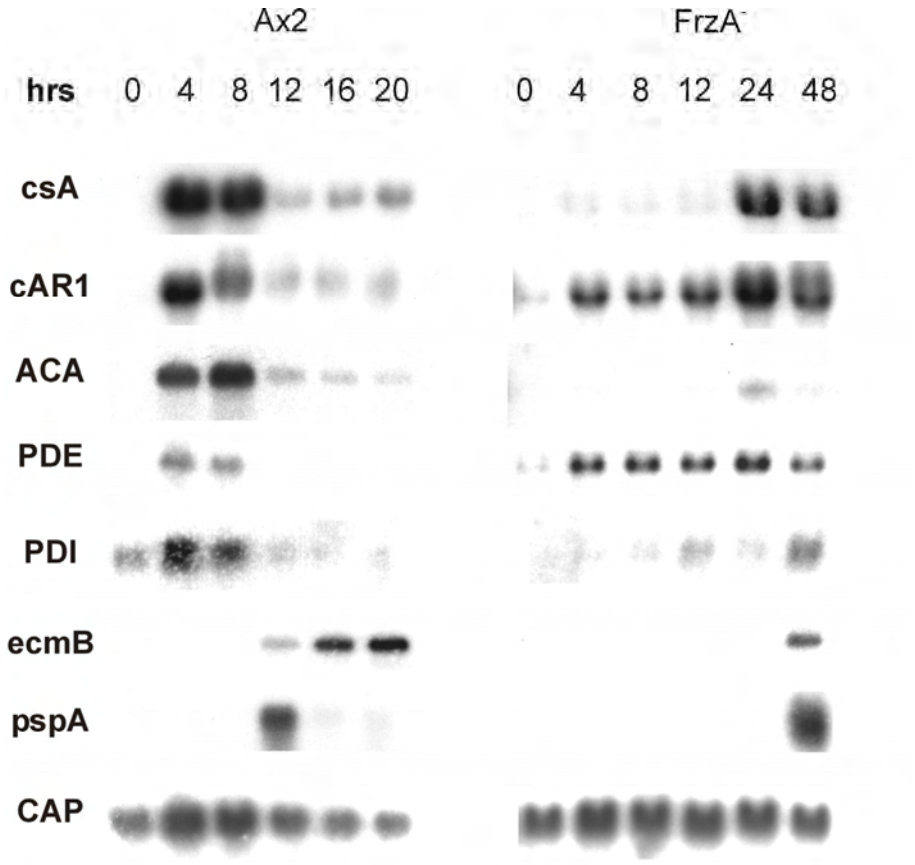
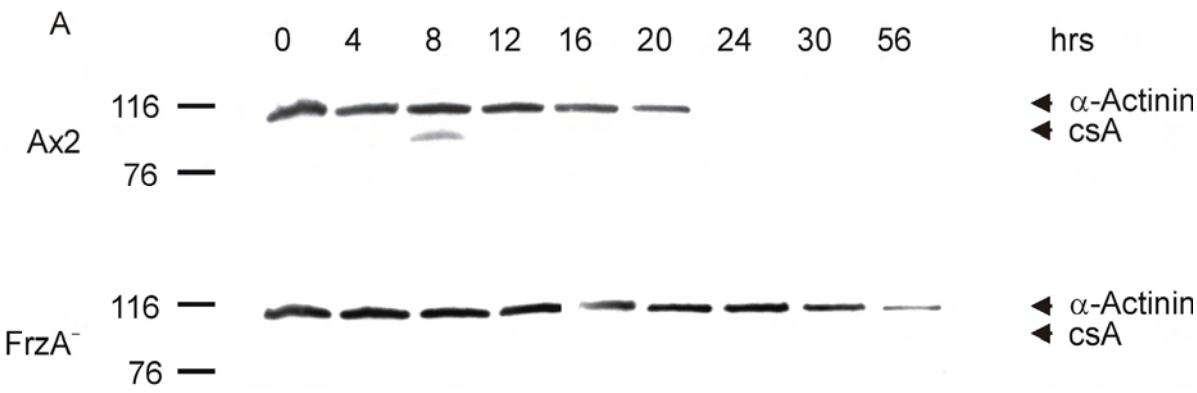


Figure 18. Northern blot analysis of developmentally regulated genes. Total RNA was prepared from wild type and FrzA$^-$ at the indicated the time points. 10 µg of RNA was separated on 1.2 % agarose gels under non-denaturing conditions (6% formaldehyde) and transferred to membranes as described in Material and Methods (3.11). cDNA probes of genes specific for the indicated transcripts were used for hybridizing the blots. A CAP cDNA probe was used as control.

2.4 Role of FrzA in cell adhesion and aggregation

The cell adhesion molecule *contact sites A* is expressed at the aggregation stage between 6 and 10h of development. *Contact sites A* has been implicated in the EDTA-stable (Ca^{2+}-

independent) type of cell adhesion of aggregating cells (Harloff et al., 1989) and is known to be under cAMP regulation (Siu et al., 1986). When FrzA$^-$ cells were analysed for expression of csA at the protein level we found that csA was not produced in the FrzA$^-$ cells either in shaking (Figure 19B) or on agar plate (Figure 19A). This may indicate that FrzA controls csA expression directly or indirectly. Alpha-actinin is used as a positive control, whose expression is unaltered throughout the *Dictyostelium* developmental cycle (Noegel et al., 1986b; Witke et al., 1986). *Contact sites A* is also one of the genes involved in the aggregation process (Noegel et al., 1986a). To test whether the inability of FrzA$^-$ mutant cells to aggregate (Figure 16 and 17B) might be caused by an inability of the cells to respond to cAMP and activate aggregation stage gene expression or whether the cells can be induced to develop if pulsed with exogenous cAMP (Lee et al., 1999), Ax2 and FrzA$^-$ cells were starved in shaking condition with or without exogenous pulses of cAMP. The wild type cells showed csA protein expression at 6 hrs in unpulsed cells and already at 4 hrs when pulsed with cAMP, whereas in FrzA$^-$ mutants csA expression was observed only in cAMP pulsed and not in unpulsed cells (Figure 19B). This suggests that the aggregation defect is not due to an inability to sense the cAMP pulses and induce aggregation stage gene expression.



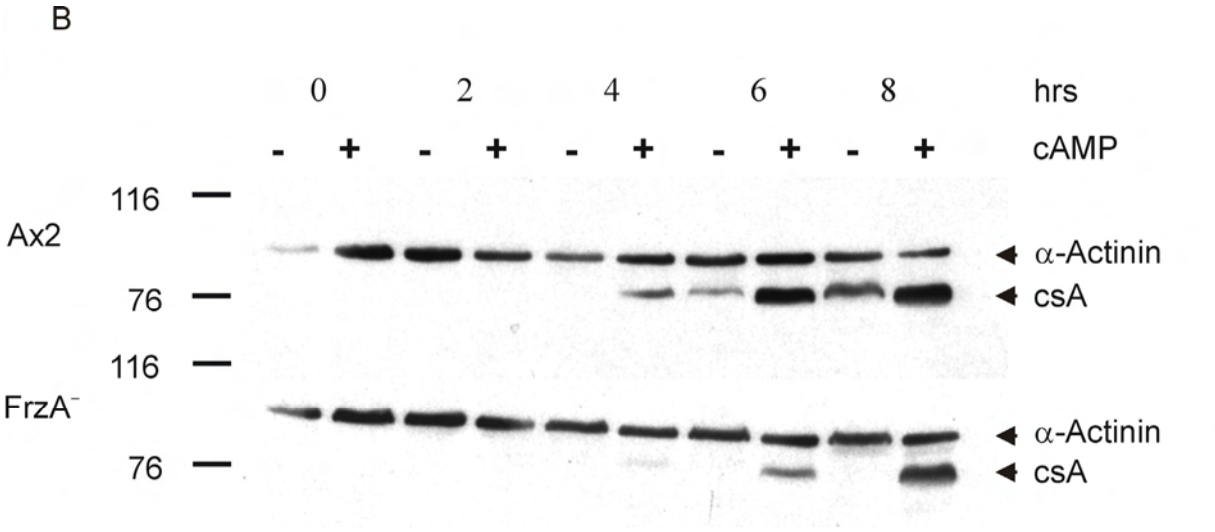


Figure 19. csA expression in Ax2 and FrzA⁻ cells. A) Cells were starved on phosphate agar plate at a density of 5 x 10^7 cells and protein samples were prepared and separated by SDS-polyacrylamide gel (10% acrylamide). B) Wild type and mutant cells at a cell density of 1 x 10^7 cells/ml were developed in suspension with or without cAMP pulses. Cells were collected at indicated time points and resuspended in 1x SDS sample buffer. Equal amount of cells were loaded on the 10% SDS PAGE gel to analyse for csA and alpha-actinin expression by western blot analysis using the monoclonal antibodies for csA (33-294) and for alpha-actinin (47-16-1 or 47-62-1). Alpha actinin was used as a loading control.

2.4.1 Can exogenous cAMP pulses also induce ACA expression?

The FrzA⁻ mutant did not express adenylyl cyclase gene (ACA) during aggregation stage (Figure 18). When FrzA⁻ cells were pulsed with cAMP (Materials and Methods, 6.2) and analysed for ACA transcription in samples collected after 8 hrs of starvation, it was clear that exogenous cAMP pulses were able to induce ACA expression (Figure 20). The data obtained here show that cAMP pulses are able to independently induce ACA transcription even in the absence of FrzA.

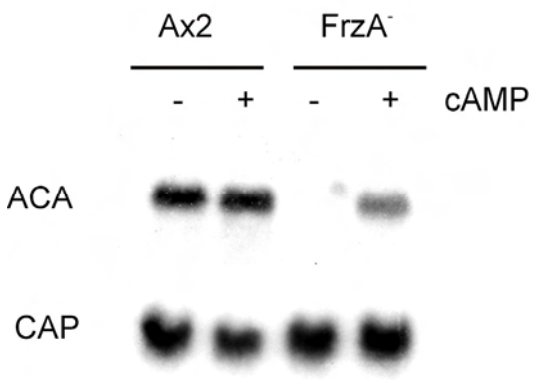


Figure 20. cAMP pulsing of FrzA$^-$ cells restores transcription of ACA. Total RNA from wild type and mutant cells with and without addition of exogenous cAMP was prepared after 8 hrs of starvation (Materials and Methods, 3.9.1). 10 µg of RNA was separated on 1.2 % agarose gel under non-denaturing conditions (6% formaldehyde) and transferred to membranes as described in Material and Methods (3.11). An ACA cDNA probe was used for hybridizing the blot. CAP cDNA probe was used as control.

2.5 Synergy between FrzA$^-$ and wild type cells

The FrzA$^-$ cells were found to be aggregation defective and this phenotype can be suppressed by exogenous pulsing of cAMP. To determine if the developmental defect observed in FrzA$^-$ mutant is cell autonomous or non-cell autonomous, we performed chimeric development in the presence of wild type, which may provide essential secreted morphogens and chemoattractants. The FrzA$^-$ mutant even in chimera with 50% of wild-type cells developed more asynchronously forming few fruiting bodies with many loose aggregates left behind and significantly slower than did wild type alone (Table 1). Relatively, development was improved with an increasing ratio of wild type to FrzA$^-$ mutant cells. The chimeric structures developed normally as the wild type only when the ratio of wild type to mutant is 60:40. This infers that the mutant showed delayed development even in the presence of high percentage of wild type cells. To test this hypothesis we mixed 90% FrzA$^-$ cells with 10% GFP-tagged wild type cells. We found the labelled parental cells to be sorted out leaving the mutant behind, although the chimera exhibited the mutant phenotype (Figure 21). This indicates that only the early developmental process of the wild type was affected in the chimera however they then developed to become fruiting bodies. These results clearly indicate that the delayed development of FrzA$^-$ cells is primarily cell autonomous in nature and not non-cell autonomous in which genotypically mutant cells cause other cells (regardless of their genotype) to exhibit a mutant phenotype.

Ratio of WT: KO (%)	24hrs	48hrs
0:100	-	+
5:95	-	+
10:90	-	+
20:80	-	+
40:60	+	+
50:50	+	+
60:40	++	++
80:20	++	++
100:0	++	++

Table 1. Development of FrzA$^-$ mutants in synergy with wild type cells. Wild type and FrzA$^-$ cells were mixed at different ratio as indicated and allowed to develop on phosphate agar plates as in Materials and Methods (6.1). Images were taken using a stereomicroscope at 24 hrs and 48 hrs and formation of fruiting bodies was checked. Efficiency of development is represented as (–) no fruiting body formation, (+) formed small fruiting bodies with many loose aggregates left behind like in the mutant and (++) most of the tight aggregates developed into fruiting bodies as in wild type.

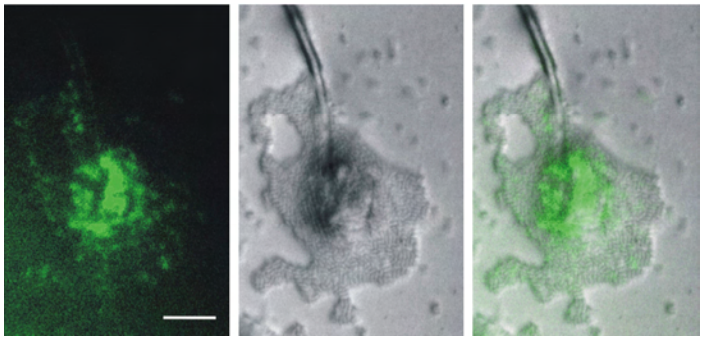


Figure 21. Synergetic behaviour of FrzA$^-$ mutant in mixtures with wild type Ax2 cells. GFP-tagged wild type and untagged mutant cells were mixed (10:90) and developed on phosphate agar plates as in Materials and Methods (6.1). Images were taken after 48 hrs of development using a fluorescent microscope. GFP labelled Ax2 cells were found to form spores and the majority of the FrzA$^-$ mutant cells were left behind. Bar is 50µm.

2.6 Cell motility and chemotaxis of FrzA$^-$ mutant

Development also depends on cell migration towards an external signal such as cAMP, which is produced in a pulsatile fashion during *Dictyostelium* development. Chemotaxis is established by cell polarity and the response to a directional signal (Chung et al., 2001). Since the FrzA$^-$ mutant shows a developmental defect we analysed its ability to perform chemoattractant induced cell migration. We used a chemotaxis assay combined with time-lapse video microscopy. FrzA$^-$ cells were made aggregation competent by developing in suspension for 6 hrs with or without cAMP pulse (Materials and Methods, 6.2). Aggregation competent cells were allowed to migrate toward a micropipette filled with 0.1 mM cAMP and time-lapse image series were taken to generate migration paths and calculate cell motility parameters (Figure 22 and Table 2). Parameters like speed, persistence, directionality and directional change were measured as an indicator of directed migration (Materials and Methods, 7.7). The wild type (Ax2) cells polarized, formed streams and migrated towards the tip at a speed of 14.70 $\pm$ 3.79 µm/min, with high persistence (5.47 $\pm$ 2.9 µm/min), directionality (around 0.83) and lower directional change (around 17°). By contrast FrzA$^-$ cells were not polarized moving at a very low speed of 2.8 $\pm$ 1.03 µm/min or they changed direction frequently at 55.5° and failed to respond to cAMP (Figure 22 and Table 2). As we had found that the aggregation competent FrzA$^-$ pulsed cells were able to sense cAMP and induce aggregation stage specific genes, csA and ACA (Figure 19B and 20) we pulsed FrzÃ

cells to analyse if pulsing can also restore chemotactic motility. The pulsed FrzA$^-$ mutant cells elongated and established polarity with directed movement towards the tip exhibiting low directional change of 27° but at a significantly lower speed (7.5 $\pm$ 1.2 µm/min) and persistence (2.55 $\pm$ 1.15 µm/min). These results indicate that FrzA$^-$ cells are impaired in their ability to chemotax up a concentration gradient of chemoattractants and exogenous pulsing of cAMP of the FrzA$^-$ cells was able to restore the chemotactic defect but not the speed and persistence of the directional movement.

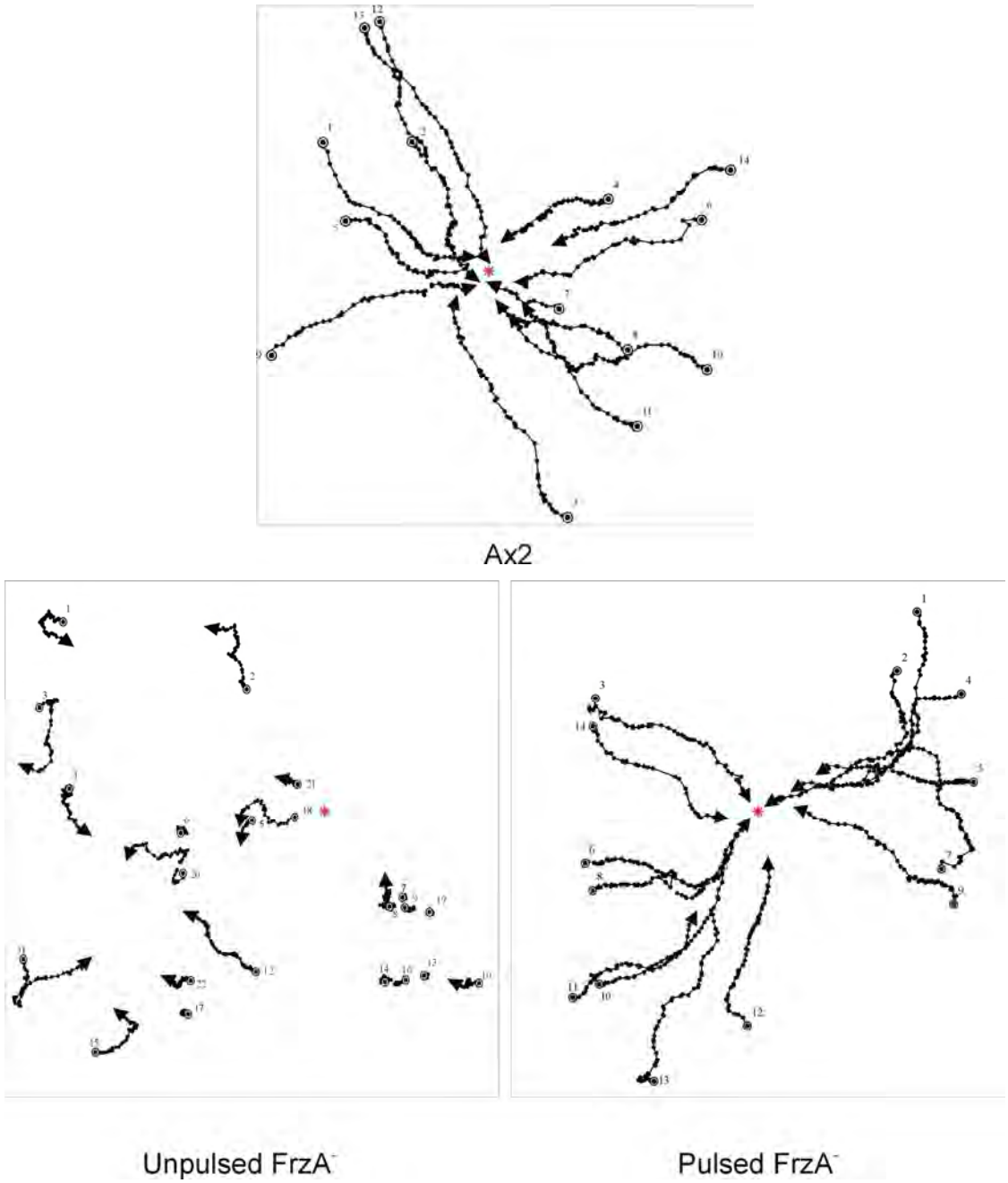


Figure 22. Chemotaxis of wild type and FrzA$^-$ cells. Cells were washed and developed in suspension for 6 hrs before they are stimulated with a micropipette filled with 0.1 mM cAMP (Materials and Methods, 6.2). Wild-type cells polarize, migrate fast and orientate properly towards the tip of the micropipette. FrzA$^-$ cells did not migrate towards the source of cAMP when unpulsed.

Upon pulsing with cAMP the mutants were able to move in a directed fashion towards the micropipette. Time-lapse images of FrzA$^-$ cells when subjected to a spatial temporal gradient of cAMP chemoattractant were taken using a microscope and the images were processed using DIAS software as described in Materials and Methods (7.7). The cell paths shown for wild type and mutant are representative of two independent experiments and the asterix in red colour represents the tip of the micropipette.

Strain	**Speed (µm/min)**	**Persistence (µm/min x deg)**	**Directionality (deg)**	**Directionality change (deg)**
Ax2	14.70 ± 3.79	5.47 ± 2.9	0.834 ± 0.11	16.04 ± 6.50
Unpulsed FrzA$^-$	2.8 ± 1.03	0.76 ± 0.50	0.3 ± 0.21	55.5 ± 17.7
Pulsed FrzA$^-$	7.5 ± 1.2	2.55 ± 1.15	0.69 ± 0.20	26.93 ± 12.5

Table 2. Analysis of cell motility towards a cAMP filled capillary for wild type Ax2 and FrzA$^-$ mutant. Cells were prepared as described in legend Figure 22. Time-lapse image series were captured and stored on a computer hard drive at 30 seconds intervals. The DIAS software was used to trace individual cells along image series and calculate motility parameters. Persistence is an estimation of movement in the direction of the path. Directionality is calculated as the net path length divided by the total path length, and gives 1.0 for a straight path. Directional change represents the average change of angle between frames in the direction of movement. Values are mean ± standard deviation of 40 to 50 cells from at least two independent experiments.

2.7 Does FrzA play an important role even before aggregation?

When the *Dictyostelium* cells starve, they sense the local density of other starving cells by simultaneously secreting and sensing conditioned medium factor (CMF) that binds to a G protein coupled receptor (Brazill et al., 1998) and allows further aggregation by chemotaxis towards cAMP when CMF reaches a threshold concentration (Van Haastert et al., 1996). Since the FrzA$^-$ cells were not able to aggregate and behaved like the CMF$^-$ cells we tested whether FrzA has a role in controlling the cell density during starvation.

2.7.1 Cell density factor in FrzA$^-$ mutant cells

Cell density is an important criteria for development in *Dictyostelium* (Jain et al., 1992). Therefore, FrzA$^-$ cells were analysed for aggregation and development at varying cell density. Wild type cells can aggregate and develop fruiting bodies at high cell density of 5 x 10^6 per cm^2 or even at a low cell density of 10^5 per cm^2 at 24 hrs from intiation of starvation, but for comparison 48 hrs images are shown here in Figure (23A-C). FrzA$^-$ cells formed loose aggregates at high cell density of 5 x 10^6 per cm^2 and developed into small fruiting bodies emerging from loose aggregates after 48 hrs, but most of the cells remained at the loose aggregate stage (Figure 23F). The mutant phenotype at high cell density may be due to random collision of cells. In contrast, FrzA$^-$ cells at a low cell density of 10^6 per cm^2 or 10^5

per cm^2 remained single even after 48 hrs without forming aggregates and fruiting bodies (Figure 23E). This may indicate that FrzA$^-$ does not produce or sense the cell density factor in order to determine the cell density necessary for aggregation and development.

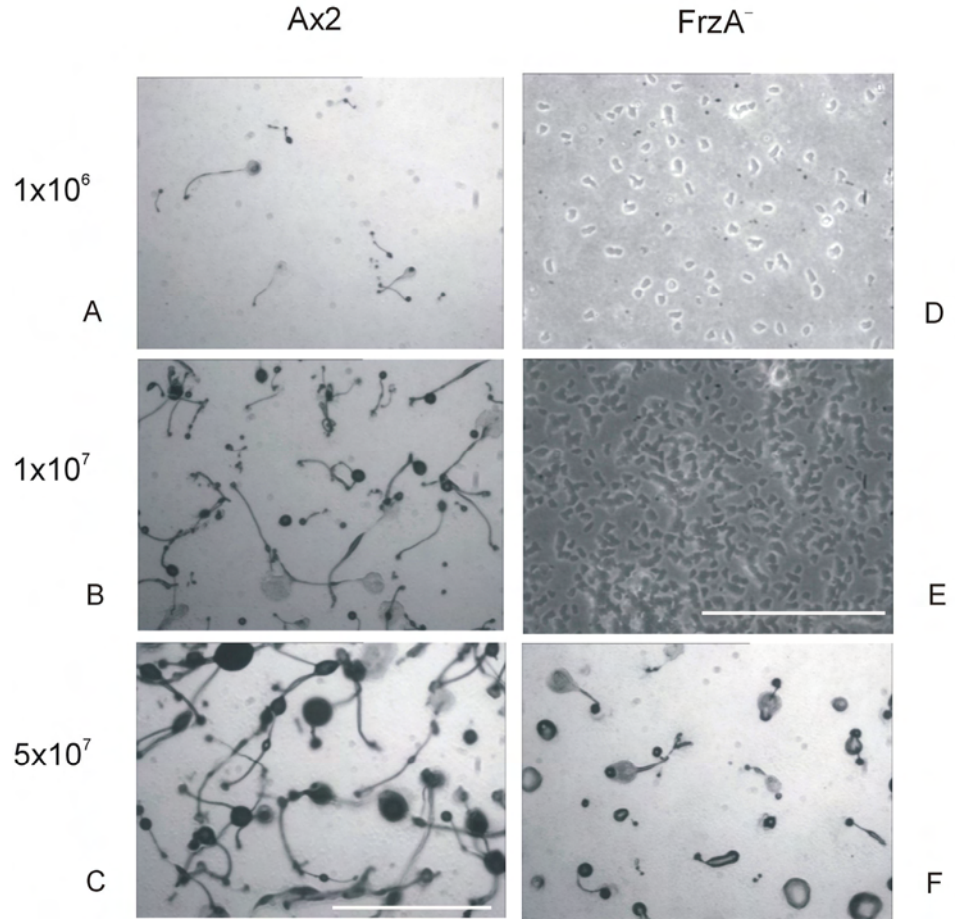


Figure 23. Cell density dependent development of Ax2 and FrzA$^-$. Cells from wild type and FrzA$^-$ were developed (Materials and Methods, 6.1) at varying cell densities as indicated. Pictures were taken after 48 hrs of starvation on a phosphate agar plate using a stereomicroscope. Bar is 1 mm (A-C and F) and 0.6 mm (D-E).

2.7.2 Do FrzA$^-$ cells produce and/or sense cell density factors?

Cells when starved secrete factors, which are called conditioned medium factors (Mehdy and Firtel, 1985). Conditioned medium containing secreted factors can induce aggregation when added to other cells (Nakagawa et al., 1999). As we know from the previous experiment, FrzA$^-$ starving cells may not be able to sense and/or secrete CMF. We analysed if FrzA$^-$ cells can produce conditioned medium factors by starving the wild type cells in the presence of CM prepared from the mutant (koCM) and found that wild type cells had formed streams towards aggregation centre at 5 hrs after initiation of starvation (Figure 24), whereas, wild type cells starved in Soerensen buffer begin to form streams at 5 hrs, indicating that the koCM contains

the necessary factors that can induce wild type cells to aggregate as early as in the CM from wild type (WtCM). We then analysed if the FrzA$^-$ cells can sense the secreted factors by starving in wtCM that contains the necessary factors for aggregation. The mutant was not able to form aggregation streams in presence of wtCM and behaved similar to starvation in buffer (Figure 24). This suggests that FrzA$^-$ can produce the necessary factors for aggregation but cannot sense these factors when starved. Furthermore we can conclude that FrzA is involved in sensing these factors.

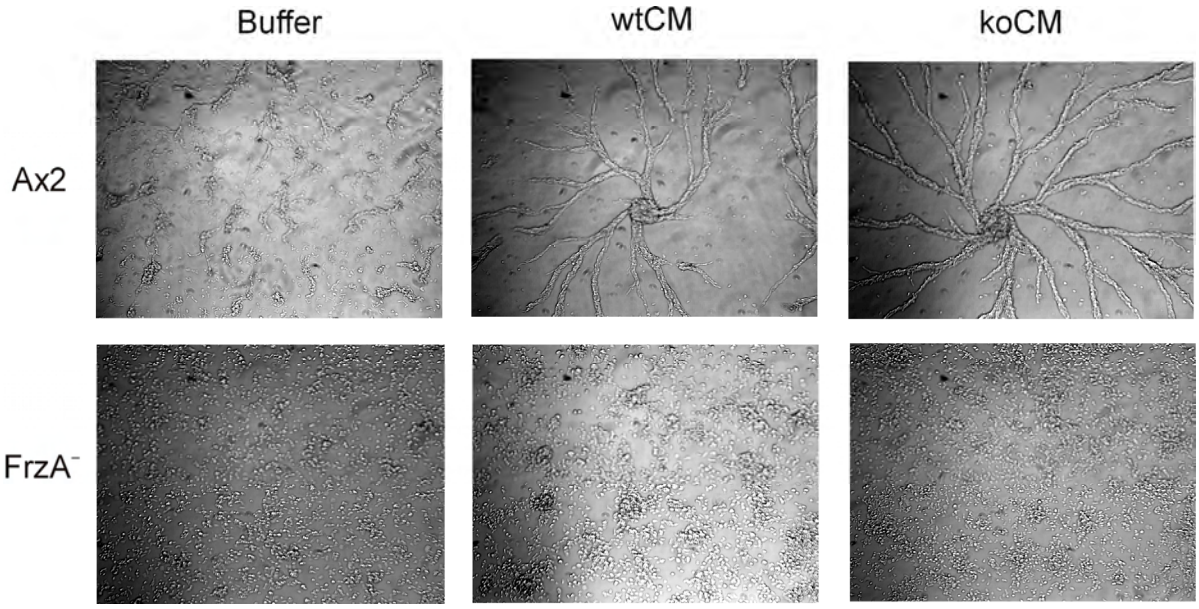


Figure 24. Analysis of the conditioned medium of FrzA$^-$. Exponentially growing cells from Ax2 and FrzA$^-$ were collected and starved under submerged condition at a density of 10^5 per cm^2 in the presence of 2 ml of buffer or CM from wild type or FrzA$^-$ cells. CM from Ax2 and FrzA$^-$ was prepared by starving 10^7 cell/ml in Soerensen buffer for 16-20 hrs at 21ºC with shaking at 160 rpm. Images were taken after 5 hrs of starvation using a stereomicroscope.

2.7.3 Expression of CMF and CMFR1 in FrzA$^-$

Cell density factor (CMF) is a secreted glycoprotein which allows the cells to sense the density of the surrounding starving cells (Brazill et al., 1998). As the FrzA$^-$ mutant was able to produce conditioned medium factors but not able to sense these factors, we compared at the trascriptional level the expression of CMF and receptor for cell density factor (CMFR1) between the wild type and mutant at different developmental time points. Here, the time points 0, 4, 8, 12, 16 and 20 hrs cDNA was used for wild type as the development was achieved by 20 hrs, whereas, for the mutant 24 and 48 hrs cDNA was also used in addition to 0-20 hrs cDNA due to prolonged development (Figure 17). Using forward (rtCMFfp, rtCMFRfp) and reverse (rtCMFrp, rtCMFRrp) primers specific to CMF and CMFR1,

expression was analysed by real time PCR. In wild type cells CMF and CMFR1 expression was high at the vegetative stage, which is consistent with results shown in (Deery and Gomer, 1999; Jain et al., 1992). But, during progression of development the expression of CMF and CMFR1 in wild type decreases (Figure 25), whereas in FrzA$^-$ cells high expression of CMF and CMFR1 was not observed at 0 hrs. This indicates that expression of these molecules was not induced with the onset of starvation unlike in wild type cells (Figure 25). However, the expression of CMF and CMFR1 in the mutant was detectable although at low levels when compared to the wild type remained constant after 4 hrs suggesting that in FrzA$^-$ mutant expression of CMF and CMFR1 is maintained at basal level throughout the developmental cycle.

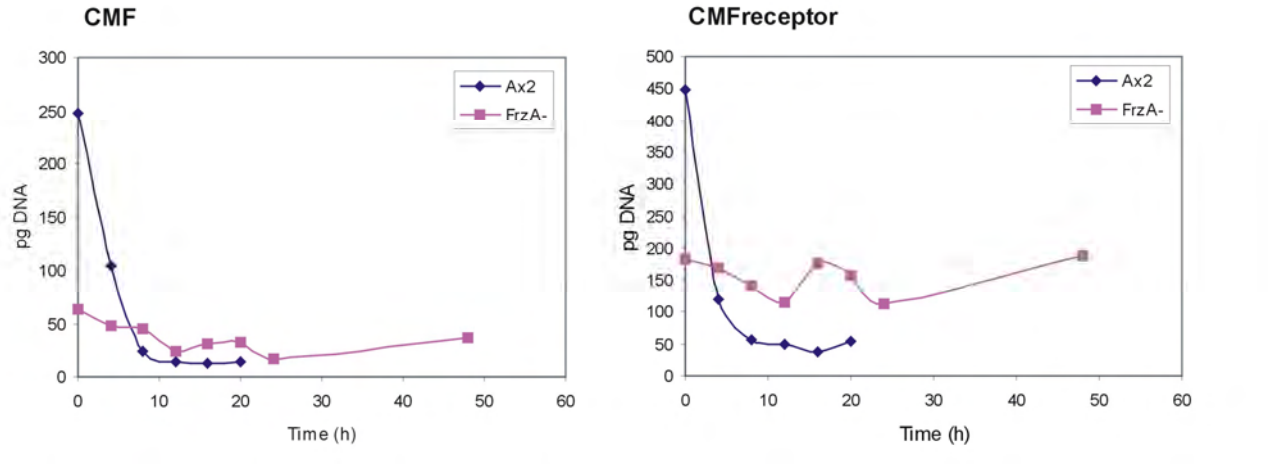


Figure 25. Transcript levels of cell density factor and its receptor. Cells from Ax2 and FrzA$^-$ were developed on phosphate agar plates and cDNA was prepared (Materials and Methods, 3.9.2) for different developmental time points indicated in the text. Real time PCR analysis for CMF and CMFR1 in wild type and FrzA$^-$ cells was carried out using primers specific for CMF and CMFR1. Values are average of two independent experiments.

2.7.4 Is CMF mediated signaling in FrzA$^-$ mutant cells affected?

When cells are starved in a given area the extracellular level of CMF rises and permits aggregation using relayed pulses of cAMP as the chemoattractant (Yuen et al., 1995). From previous experiments we understand that FrzA$^-$ cells do produce the cell density factor (CMF), but the mutant is not able to respond to it. To test this hypothesis we subjected wild type (Ax2) and FrzA$^-$ cells to recombinant CMF (rCMF) at low cell density (10^4 or 10^5 cells per cm^2), as the effect of CMF can be appreciated when cells are starved at low cell density (Clay et al., 1995). Figure 26 shows that Ax2 cells when starved at a cell density of 10^5 per cm^2 in buffer they show cell polarization. In the presence of rCMF (1ng/ml) the cells make

aggregation streams. At a cell density of 10^4 per cm^2 many of the Ax2 cells are rounded and some having few protrusions when starved in buffer, whereas in the presence of rCMF the Ax2 cells become elongated and polarize. In contrast, the FrzA$^-$ cells remain rounded in the presence of rCMF at both cell densities, but when starved in buffer the mutant cells have small protrusions at a cell density of 10^5 per cm^2 and remain rounded at 10^4 per cm^2 (Figure 26). This indicates that the FrzA$^-$ mutant does not respond to rCMF and could be a potential G-protein coupled receptor for CMF as described by (Brazill et al., 1998).

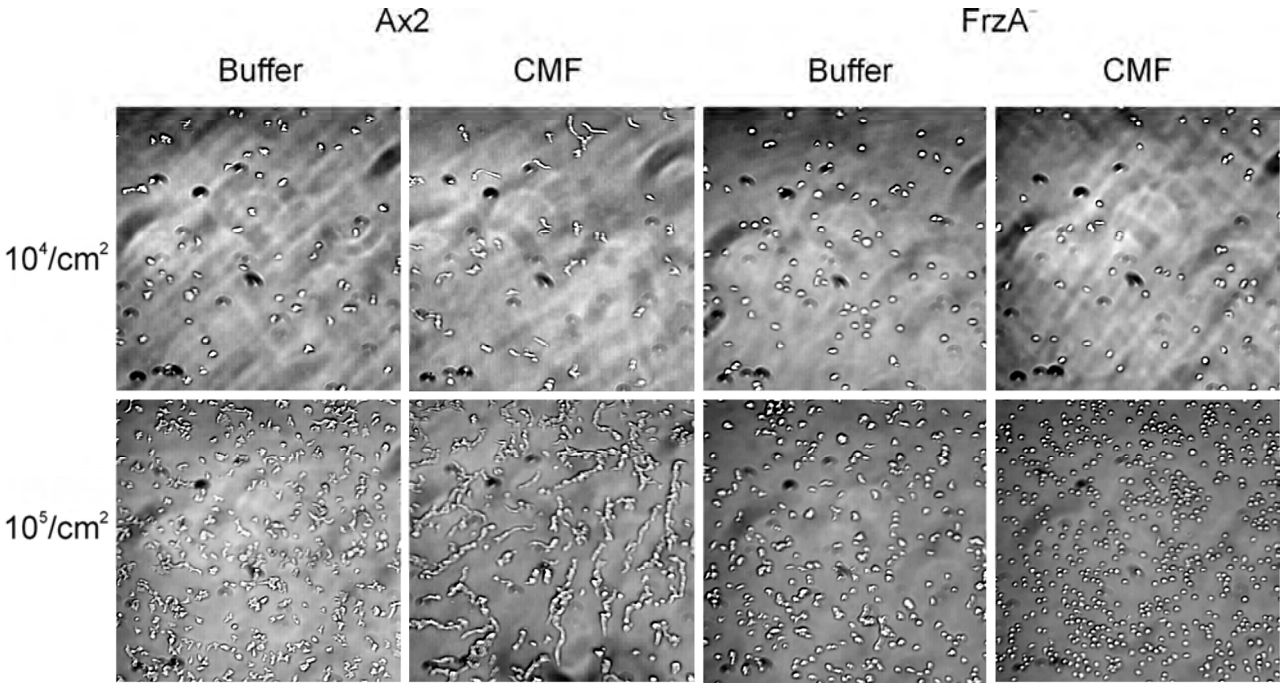


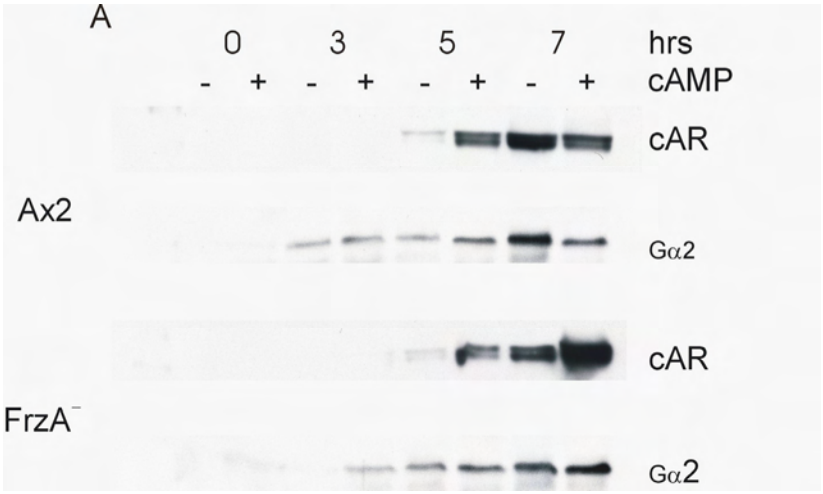
Figure 26. Effect of recombinant CMF on FrzA$^-$ cells. Ax2 and FrzA$^-$ cells were starved in the presence and absence of rCMF (Materials and Methods, 7.4) and phase contrast images were taken after 6 hrs of starvation.

3.0 cAMP signal transduction in FrzA$^-$ cells

CMF controls cAMP receptor (cAR1) mediated activation of adenylyl cyclase and thus regulates cAMP signal transduction (Yuen et al., 1995). As the FrzA$^-$ mutant was not able to respond to CMF we examined the expression of cAR1 in the FrzA$^-$ mutant and its parental strain Ax2. Cells were developed in the presence or absence of 100 nM cAMP pulses for up to 7 h (Materials and Methods, 6.4). Aliquots of cells were removed at 2 or 3 hrs intervals for preparation of the membrane-enriched cell fraction. cARl was detected by a specific polyclonal antibody. In the absence of cAMP pulses, cARl could be detected in the Ax2 cells at 5 hrs of development and its expression increased at 8hrs (Figure 27A). cAMP pulsed Ax2 cells were found to have an increased cAR1 expression at 5 hrs of starvation. In FrzA$^-$ the

cAR1 expression profile was comparable to the wild type Ax2 which is in agreement with the results from Northern blot analysis (2.3). These results further demonstrate that cARl expression is not dependent on FrzA. The G alpha 2 (Gα2) gene, like cARl, is required for aggregation and its expression can be induced by cAMP pulses (Kumagai et al., 1991). The expression of Gα2 protein was compared by western blot analysis in Ax2 and FrzA$^-$ mutant cells. In pulsed and unpulsed Ax2 cells Gα2 expression was detected at 3 h of development and increased upto 7 h of development as described by (Kumagai et al., 1991). The pattern of Gα2 expression in the FrzA$^-$ mutant was not significantly different from Ax2, although the Gα2 expression was found at 3 hrs only in cAMP pulsed cells (Figure 27A). Therefore, Gα2 expression is not dependent on FrzA.

As FrzA$^-$ mutant did not develop at low cell densities (Figure 23) we analysed the cAR1 expression when cells were developed at high (5 x 10^6 cells per cm^2) and low (1 x 10^6 cells per cm^2) cell density conditions. Cells were harvested at 4 hrs intervals. Figure 27B shows Ax2 expressing cAR1 at 8 hrs of starvation in both high (HCD) and low cell density conditions (LCD). The phosphorylation of cARl is required for the ligand induced reduction of receptor affinity that is a hallmark of G-protein-coupled receptors (Caterina et al., 1995). The phosphorylated form of cARl was observed as the slower mobility form in the Ax2 cells developed at high cell density (Figure 27B, asterix), at LCD Ax2 cells did not show the phosphorylated form of cARl. The FrzA$^-$ mutant developed at LCD did not show cARl expression, although when developed at HCD cAR1 expression was observed, the phosphorylated cARl was not visible in the membrane-enriched cell fraction. (Figure 27B). This indicates that cAR1 expression is dependent on FrzA when the cell density is low.



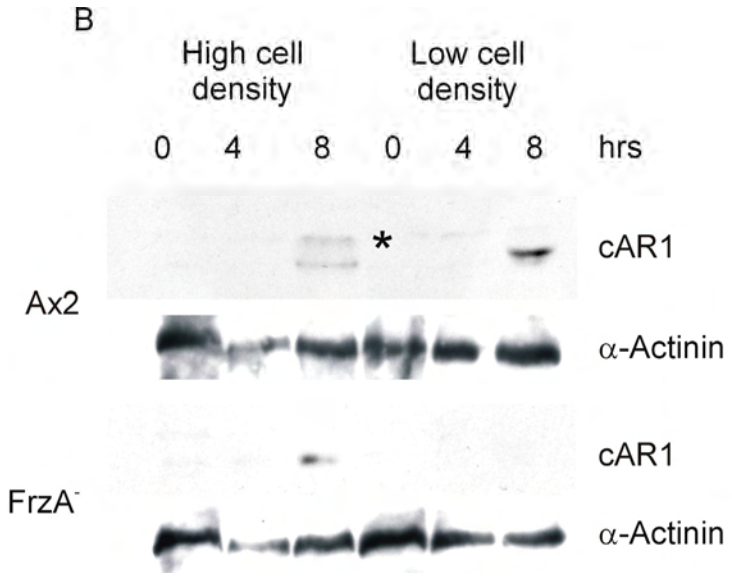


Figure 27. Suspension assay for cAR1 and Gα2 expression. A) Wild type (Ax2) and FrzA$^-$ cells were prepared as described (Materials ad Methods, 6.4). Samples prepared were run on 12 % SDS PAGE gels and blotted onto nitrocellulose membrane for western blot analysis. B) Cells were developed on phosphate agar plates at different cell densities for protein sample preparation as given in Materials and Methods (6.1). Immunodetection was done for cAR1, Gα2 and alpha-actinin, using cAR1 and Gα2 polyclonal antibodies and alpha-actinin monoclonal antibodies (47-16-1 or 47-62-1), respectively. The phosphorylated form of cAR1 is marked as ✱. Alpha-actinin was used as a control.

3.1 Function of FrzA in cell differentiation at low cell density

Dictyostelium cells can differentiate even without cell-cell contact (Kay and Trevan, 1981) and CMFR1 can mediate CMF induced prestalk and prespore expression in the absence of cell-cell contact (Deery and Gomer, 1999). We observed that FrzA$^-$ mutant cells produced only a basal level of CMF and CMFR1 with a little response to rCMF (Figure 25 and 26). We analysed if FrzA$^-$ mutants can also mediate CMF induced prespore differentiation. We found that wild type cells were able to respond to rCMF and differentiate into prespores cells (Figure 28), which was observed by staining the prespore cells with Mud-1 monoclonal antibody specific for D19, a prespore marker. The mutant cells were not able to differentiate into prespore cells which correlates with the result that FrzA$^-$ do not respond to rCMF (Figure 28). This indicates that FrzA may be directly or indirectly involved in the CMF induced cell differentiation.

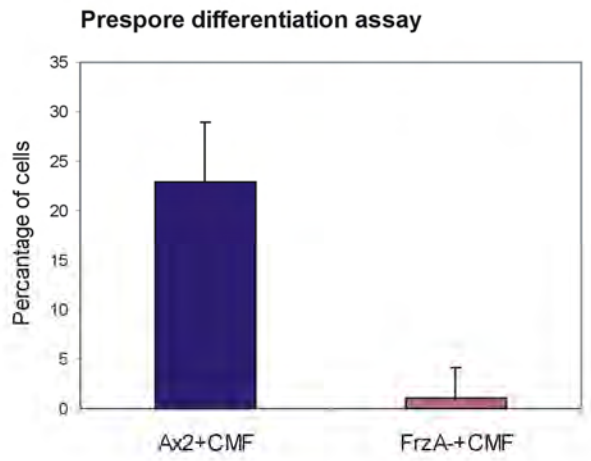


Figure 28. Differentiation of cells starved at low cell density. Cells from Ax2 and FrzA$^-$ were starved in presence of buffer or rCMF (1ng/ml) for 6 hrs and then subjected to continuous cAMP treatment (300µM) for 16-18 hrs followed by immunostaining using Mud-1 monoclonal antibody for D-19, a prespore marker. For each condition, approximately 700 cells were examined, and the number of D19-positive cells was counted. The percentage of positive cells was then calculated. The graph is representative of two separate experiments and the error bar represents the standard deviation between two experiments.

3.2 Production of Inositol Phosphate-3 (IP3) and cGMP in wild type and FrzA$^-$ cells

CMF was found to activate Phospholipase-C (PLC) in wild type cells causing an increase in the IP3 levels which occurs within ~30 sec of stimulation (Brazill et al., 1998). To test the hypothesis that FrzA$^-$ cells are not able to respond to rCMF, we analysed the mutant for CMF induced activation of PLC. When FrzA$^-$ cells were stimulated with rCMF there was no increased IP3 production (Figure 29) whereas when the Ax2 cells were induced by rCMF IP3 production increased by two fold at 60 sec in comparison to 0 sec. This indicates that FrzA could be involved in the process of activating PLC via CMF to produce IP3. The estimation of IP3 production in response to rCMF was done by Dr.Derrick Brazill, Hunter College, USA: cGMP accumulates in response to cAMP stimulation and is important for cytoskeletal rearrangement during chemotaxis (Goldberg et al., 2002). As the FrzA$^-$ cells exhibited a chemotaxis defect we analysed the mutant for accumulation of cGMP in response to cAMP stimulation. Wild type cells were stimulated by cAMP and a 2.25 fold increase in cGMP was observed at 10 sec after induction, which correlates with the result from (Brazill et al., 1998), whereas the mutant cells stimulated by cAMP showed only a 0.9 fold cGMP accumulation at 10 sec. This result is consistent with the chemotaxis defect in the mutant and indicates FrzA could be a G protein coupled CMF receptor that mediates CMF induced PLC activation.

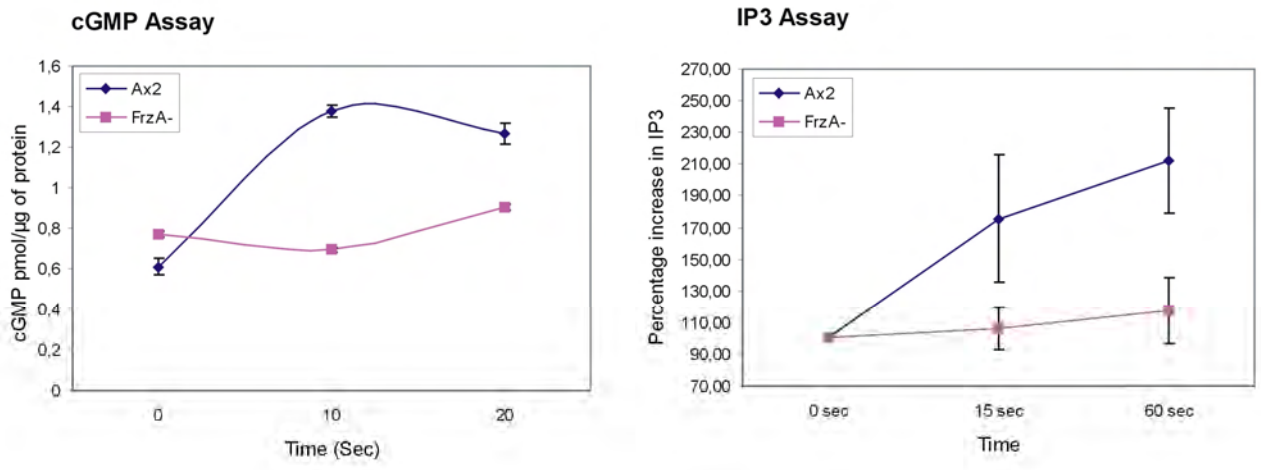


Figure 29. cGMP and IP3 production in FrzA⁻ cells. Cells were prepared (Materials and Methods, 6.5 and 6.6) and production of cGMP and IP3 was measured, using cGMP and IP3 assay kit (Amersham), respectively. Error bars represent mean ± standard deviation of at least two independent experiments.

3.3 Calcium oscillation in FrzA⁻ cells

The light scattering oscillations coincide with cAMP relay and extracellular Ca^{2+}-oscillations (Bumann et al., 1986). Wild type cells in the presence of the calmodulin antagonist W-7 interfere with an internal Ca^{2+}oscillation by inhibiting Ca^{2+}-influx and enhancing light scattering as well as cAMP relay (Malchow et al., 2004). In contrast to wild type, in FrzA⁻ suspension there is only one large peak followed by very few or no oscillations (Figure 30). This observation indicates that in FrzA⁻ the regulation of oscillations/production of cAMP relay might not be as tight as in wild type. Dr. Christina Schlatterer, University of Konstanz, Germany, carried out the analysis for calcium oscillation by light scattering method in the FrzA⁻ mutant.

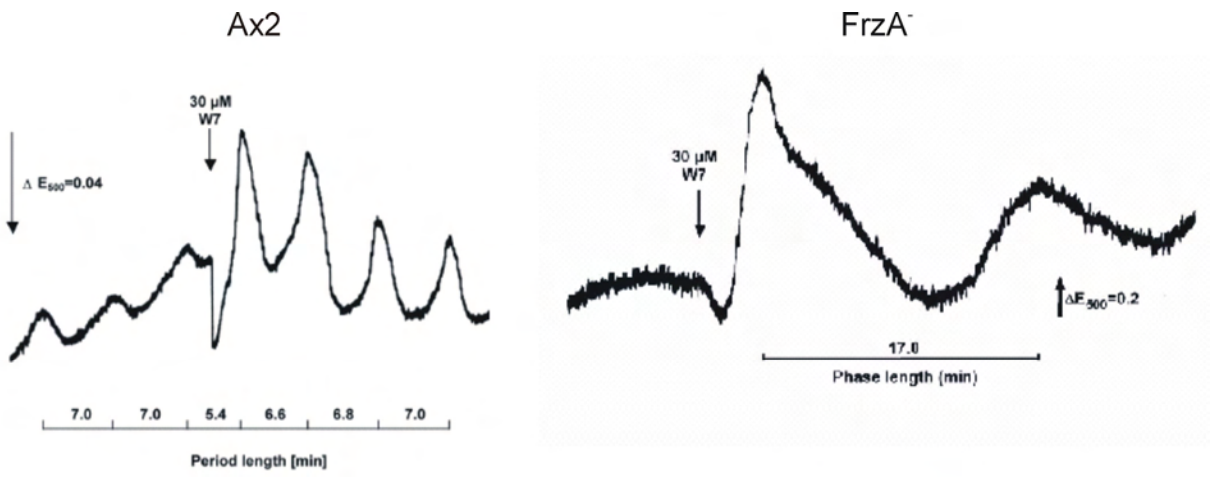


Figure 30. Measurement of $[Ca^{2+}]_i$. Early light scattering oscillations are enhanced by W-7. Free running oscillations of a cell suspension at 2×10^7 cells/ml were recorded using the light scattering technique as described in Materials and Methods (7.5). The result shown here is representative of at least two independent experiments.

3.4 Complementation analysis for FrzA$^-$

To understand whether a part of the gene or the complete gene is responsible for the aggregation negative phenotype of the FrzA$^-$ mutant, we tried to rescue the phenotype by overexpressing different domains (Frizzled transmembrane and PIP5K) or the full length FrzA as a GFP fusion protein driven by a constitutive promotor. In the following we discuss only the results of overexpression of the PIP5K domain in Ax2 and FrzA$^-$ mutant.

3.4.1 Expression of PIP5K-GFP fusion proteins

GFP fusion proteins are ideal for the analysis of proteins *in vivo*. The PIP5K-GFP fusion construct was generated as given in Materials and Methods (5.2) and the recombinant vector was introduced into *D. discoideum* Ax2 and FrzA$^-$ cells using the electroporation method (Materials and Methods, 2.3). Stable transformants were isolated and the appropriate sizes of the fusion proteins were confirmed by western blot analysis using GFP-specific monoclonal antibodies (K3-184-29) and affinity purified Anti-FrzA polyclonal antibodies.

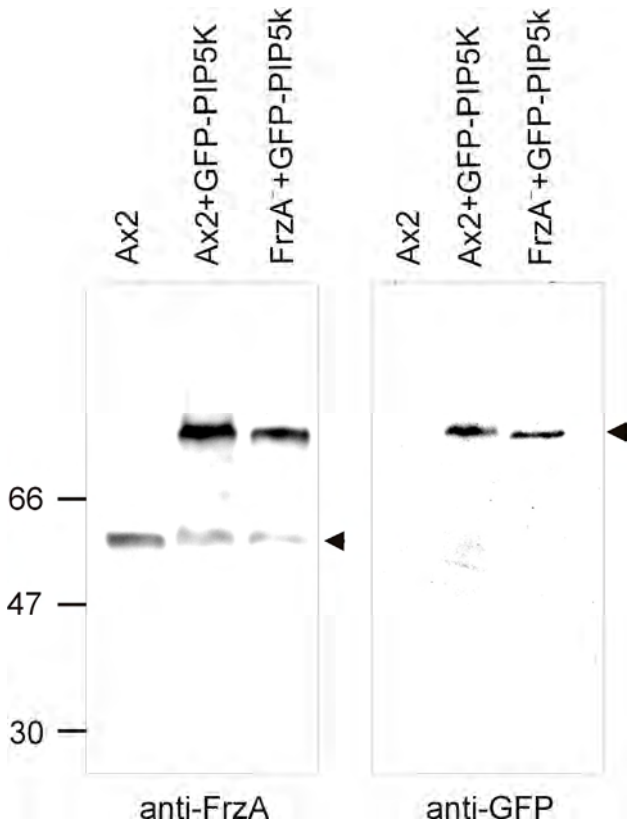


Figure 31. Immunoblot showing the expression of GFP-PIP5K proteins in Ax2 wild type and FrzA$^-$ cells. Whole cell homogenates of Ax2 and FrzA$^-$ cells expressing the GFP-PIP5K fusion protein were resolved on a SDS-polyacrylamide gel (12% acrylamide), blotted onto a nitrocellulose membrane and immunolabelled with anti-GFP monoclonal antibodies (K3-184-2) and anti-FrzA polyclonal antibodies. Equal amounts of total cellular protein (equivalent to 2 x 10^5 cells) were loaded in each lane. The immunoblots were processed after incubation with an appropriate HRP-conjugated secondary antibody using the ECL-detection system for visualization of the specific immunolabelled band of ~75 kDa for the GFP fusion protein. Wild type Ax2 cells were used as negative control. The band at ~60 kDa (◂) appears to be due to non-specific binding of the polyclonal antibodies.

The purified polyclonal antisera specific for PIP5K recognised proteins of ~75 kDa in wild type and mutant cells expressing a GFP-PIP5K polypeptide. A GFP specific antibody recognised this protein as well. The fainter band at approximately 60 kDa that is labelled by the purified polyclonal antisera (Anti-FrzA) is probably a non-specific signal. The anti-FrzA was not able to recognise the endogenous protein of ~92 kDa in the wild type.

3.4.2 Subcellular localization of GFP-PIP5K in vegetative cells

Wild type Ax2 and FrzA$^-$ mutant expressing GFP-PIP5K were grown axenically and prepared for imaging under a confocal laser scan microscope (Leica DM/IRBE) as described in Materials and Methods (7.8). GFP-PIP5K expressing cells were fixed with cold methanol and immunostained with anti-actin monoclonal antibodies (Act 1-7) followed by staining with Cy3 conjugated goat-anti mouse IgG as the secondary antibody as described in Materials and Methods (7.8.3). Localisation of GPF-PIP5K is not homogenous and the protein appears to be present in the internal membranes, whereas the actin antibody shows mainly a cortical staining (Figure 31A). This indicates that the GFP-PIP5K fusion protein is not associated with the cortical actin. To test this hypothesis we performed Triton X-100 insoluble fractionation on the cells expressing GFP-PIP5K fusion protein (Materials and Methods, 6.3), where a substantial proportion of intracellular proteins are solublised with Triton-X 100 containing buffer, whereas the insoluble fraction is enriched in cytoskeletal proteins (Prassler et al., 1998). Figure 31B shows an immunoblot of the Triton X-100 soluble and insoluble fractions of GFP-PIP5K expressing *Dictyostelium* cells labelled with anti-GFP antibodies, where the GFP-PIP5K fusion protein is observed in the Triton X-100 soluble fraction of both Ax2 and the mutant cells. This clearly supports our findings that the GFP-PIP5K fusion protein in both Ax2 and FrzA$^-$ cells is not associated with the actin cortex.

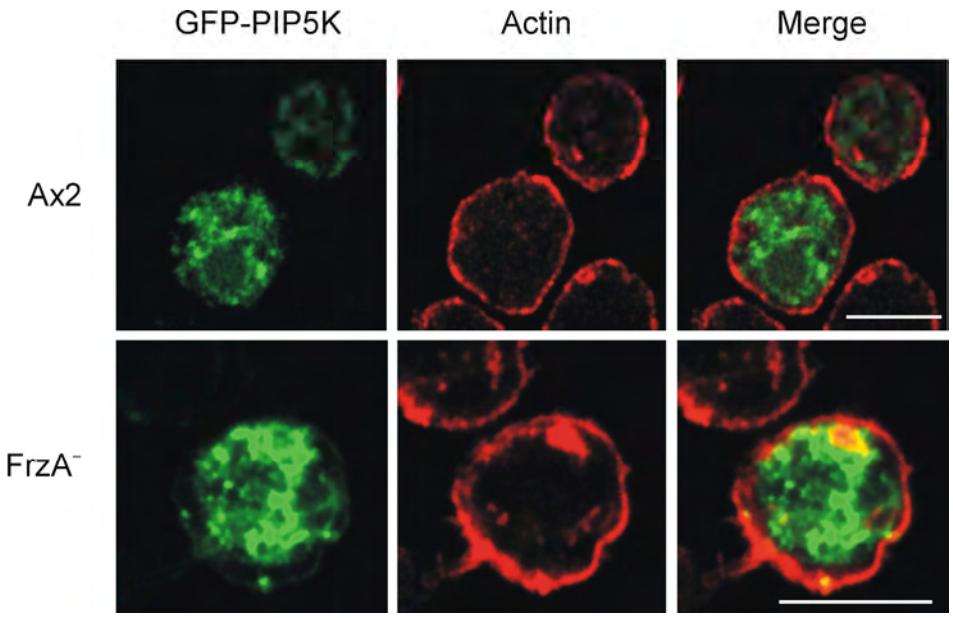


Figure 31A. Localization of GFP-PIP5K fusion protein in Ax2 and FrzA⁻ cells. Immunofluorescence studies performed with GFP-PIP5K (GFP-PIP5K) expressing cells exhibit a fluorescence pattern that does not coincide with the actin staining in the cortex and predominantly remains cytoplasmic. The cells were fixed with methanol and labelled with anti-actin monoclonal antibodies (Act 1-7). Bar is 8 µm.

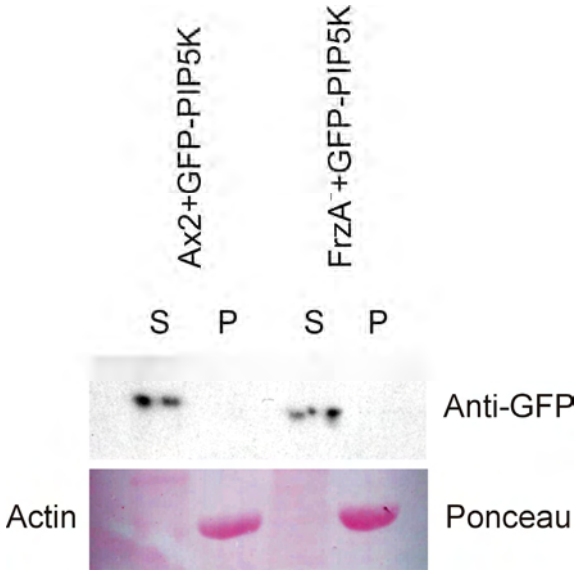


Figure 31B. Distribution of GFP-PIP5K fusion protein in Ax2 and FrzA⁻ cells. Cells expressing GFP-PIP5K fusion protein were treated with 0.1% Triton-X 100 containing buffer and cytosolic and membrane fractions was prepared (Materials and Methods, 6.3). The GFP-PIP5K fusion protein was found only in the Triton X-100 soluble fraction (S) fraction. The fusion protein was recognized with GFP specific monoclonal antibodies (K-184-3), whereas actin is enriched in the insoluble fraction (P) as visualized by ponceau staining.

3.4.3 Development of Ax2 and FrzA$^-$ mutant expressing the PIP5K domain

Wild type and FrzA$^-$ cells expressing the GFP-PIP5K protein were analysed for development. When Ax2 cells expressing GFP-PIP5K fusion protein were developed on phosphate agar plates, they aggregated and developed fruiting bodies at 24 hrs similar to that of the untransformed Ax2 cells (Figure 32A and B). FrzA$^-$ cells expressing GFP-PIP5K did not aggregate at 12 hrs unlike the wild type cells and remained as loose aggregates like the untransformed FrzA$^-$ cells (Figure 32C and 17B) suggesting that the PIP5K domain may not rescue the aggregation defect in FrzA$^-$ mutant. However, we cannot exclude the possibility of the PIP5K function in rescuing the mutant phenotype. Since the SMART program predicted that FrzA has a signal peptide (1-30 aa), probably PIP5K exists as membrane bound protein *in vivo*. Therefore, a myristoylated consensus sequence from the c-Src was added to the PIP5K domain as described in (Chung and Firtel, 1999) to analyse the function of membrane bound myrPIP5K in wild type and FrzA$^-$ cells.

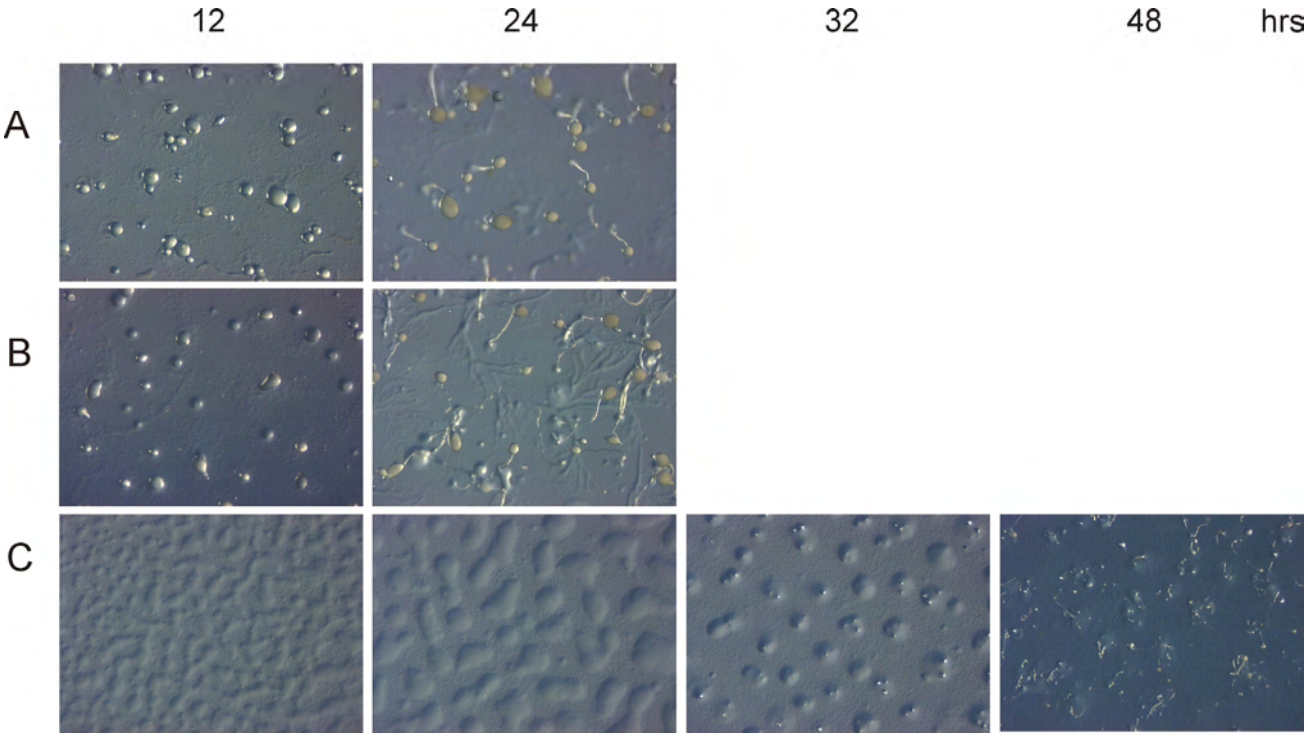


Figure 32. **Development of cells expressing the GFP-PIP5K fusion protein**. Cells were starved at a cell density of 5 x 10^7 on phosphate agar plates and images were taken at indicated time points using a stereomicroscope. A - wild type cells, B - wild type cells expressing GFP-PIP5K, and C - FrzA$^-$ expressing GFP-PIP5K.

3.5 Microarray analysis

3.5.1 Experimental design

DNA microarrays can be used to investigate the transcriptional changes between wild type and mutant cells on a large scale. For these experiments we used the *Dictyostelium* DNA microarray (Materials and Methods, 4.0) that represents approximately 50% of the *Dictyostelium* genome. Northern blot analysis showed that several genes of the FrzA$^-$ mutant exhibited an altered expression pattern during early development when compared to the Ax2 wild type (Figure 18). The developmental defect in the FrzA$^-$ mutant was more pronounced when the mutant cells were developed at low cell density (Figure 23). Therefore, we compared the expression pattern of the mutant and wild type cells during early development (0-8 hrs) to identify those genes whose expression is significantly changed.

Developmental stage	Cell density (cells per cm^2)	Microarray	Wild type		Mutant	
			Total RNA	dye	Total RNA	dye
T0 or vegetative	(cells/ml)	12874460	Culture A	Cy3	Culture A	Cy5
		12945001	Culture B	Cy3	Culture B	Cy5
		12874461	Culture A	Cy5	Culture A	Cy3
		12945002	Culture B	Cy5	Culture B	Cy3
T4	5 x 10^6	12874463	Culture A	Cy3	Culture A	Cy5
	5 x 10^6	12945003	Culture B	Cy3	Culture B	Cy5
	5 x 10^6	12874464	Culture A	Cy5	Culture A	Cy3
	5 x 10^6	12945004	Culture B	Cy5	Culture B	Cy3
	1 x 10^6	12880811	Culture A	Cy3	Culture A	Cy5
	1 x 10^6	12944986	Culture B	Cy3	Culture B	Cy5
	1 x 10^6	12882231	Culture A	Cy5	Culture A	Cy3
	1 x 10^6	12953217	Culture B	Cy5	Culture B	Cy3
T8	5 x 10^6	12882232	Culture A	Cy3	Culture A	Cy5
	5 x 10^6	12953218	Culture B	Cy3	Culture B	Cy5
	5 x 10^6	12882233	Culture A	Cy5	Culture A	Cy3
	5 x 10^6	12953220	Culture B	Cy5	Culture B	Cy3
	1 x 10^6	12882234	Culture A	Cy3	Culture A	Cy5
	1 x 10^6	12953221	Culture B	Cy3	Culture B	Cy5
	1 x 10^6	12882235	Culture A	Cy5	Culture A	Cy3
	1 x 10^6	12953222	Culture B	Cy5	Culture B	Cy3

Table 3. Microarray analysis for the FrzA$^-$ mutant. Ax2 wild type and FrzA$^-$ mutant cells were developed on phosphate agar plates at the indicated cell densities (5 x 10^6 cells per cm^2 = HCD; 1 x 10^6 cells per cm^2 = LCD). T0 cells were collected before plating, the other samples at 4 and 8 hrs after initiation of development. Total RNA was prepared as described in Materials and Methods (4.2.2). Cy3 and Cy5 are green and red fluorescent dyes, respectively, and were used to label the cDNA generated from total RNA of wild type and mutant cells. Two independent experiments were performed and are represented as culture A and B. The numbers in the third column are the bar code numbers of the microarray slides used.

Ax2 cells and the FrzA$^-$ mutant were developed on phosphate agar plates at either high cell density (HCD, 5 x 10^6 cells per cm^2) or low cell density (LCD, 1 x 10^6 cells per cm^2). Samples were collected at 0, 4 and 8 hrs of development and total RNA isolated. Fluorescently labeled cDNA of the mutant was generated from RNA of each time point and compared to labeled cDNA from Ax2 wild type RNA (Table 3). Dye-swap correction was done to minimize the effects of dye bias on fluorescent hybridization signals and to maximize the experimental design efficiency (Rosenzweig et al., 2004). Data from each time point were normalized (Materials and Methods, 4.7) and differentially expressed genes were identified using the program "Significance Analysis for Microarray" (SAM).

3.5.2 Comparison of the expression profile of vegetatively growing cells.

We compared the expression profile of vegetatively growing Ax2 and FrzA$^-$ cells and found only 54 genes that were at least 1.5 fold up- or downregulated. This finding shows that the expression of the majority of the genes that are expressed during vegetative growth was unaltered in the mutant. Only approximately 1 % of all genes appear to be differentially regulated. Table 4 lists the 15 most significantly upregulated and 12 most significantly downregulated genes. Notable among these are three up-regulated genes that appear to be involved in glucose metabolism and the downregulated V4a and V4b genes whose expression is attributed to transition from growth to development in response to lack of nutrients (McPherson and Singleton, 1992; Morita et al., 2000). This finding indicates that the mutant might not sense the depletion of food for the initiation of the preaggregation program as in the wild type cells. This is in good agreement with the early developmental defects exhibited by the mutant.

Gene ID	Score	Factor	BLAST result	p-value
SSD175	7.45	2.68	Q94465 *Dictyostelium discoideum* (slime mold). gtp cyclohydrolase	1.00E-126
VSA360	11.02	2.13	-	-
SSC656	18.63	1.99	P14326 *Dictyostelium discoideum* (slime mold). vegetative specific protein H5	1.00E-148
X15387	9.60	1.98	*Dictyostelium discoideum* H5 gene	NA
SLC679	17.56	1.97	Aas38798 *Dictyostelium discoideum* (slime mold) cutinase negative acting protein	3.00E-76
SLC335	17.19	1.93	Q8ye76 *Brucella melitensis* chaperone protein dnak (hsp 70)	0
SLB403	9.34	1.78	O04308 A*rabidopsis thaliana* probable mitochondrial processing peptidase alpha subunit 2	5.00E-42
SLA176	11.16	1.76	P08113 *Mus musculus* 94 kda glucose-regulated protein	1.00E-167
SSH169	13.12	1.72	Q8t690 *Dictyostelium discoideum* (slime mold). abc transporter	1.00E-54
M64282	8.85	1.70	*Dictyostelium discoideum* GMP synthetase mRNA	NA
SLD415	23.41	1.69	O60037 *Cunninghamella elegans*. 6-phosphogluconate dehydrogenase	1.00E-151
SLG272	10.30	1.67	Q7rvs9 *Neurospora crassa*. phosphoenolpyruvate carboxykinase [atp]	0
SLE355	9.37	1.65	Q9ur07 *Schizosaccharomyces pombe* (fission yeast). Retrotransposable element tf2	5.00E-17
SLE817	22.86	1.62	P54659 *Dictyostelium discoideum* major vault protein beta (mvp-beta)	0
VSH185	10.46	1.60	Q869w9 *Dictyostelium discoideum* (slime mold) similar to anabaena sp polyketide synthase	0
SLC380	-19.73	0.27	-	-
VSI664	-10.63	0.28	-	-
X15380	-15.77	0.29	*Dictyostelium discoideum* V4b gene	NA
SLJ376	-8.25	0.37	Q9uag7 *Dictyostelium discoideum* (slime mold). Flavohemoglobin	0
VSB701	-20.58	0.38	-	-
X15381	-10.23	0.42	*Dictyostelium discoideum* V4a gene	NA
SSM789	-8.50	0.42	O77257 *Dictyostelium discoideum* (slime mold). 17 kda protein precursor	5.00E-23
SLI122	-18.75	0.43	-	-
SSD184	-13.04	0.46	O00780 *Dictyostelium discoideum* vacuolar atp synthase subunit e	1.00E-112
VSJ323	-9.15	0.47	-	-
AB025583	-8.04	0.49	*Dictyostelium discoideum* DdFHa mRNA for flavohemoglobin	NA
SSL817	-22.41	0.49	-	-

Table 4. Significantly up- or downregulated genes of vegetatively growing FrzA cells in comparison to Ax2. Differentially expressed genes were identified by the SAM program. Those genes are listed that are in addition more than 1.5 fold up- or downregulated in comparison to wild type. **Gene ID** is the sequence identification number, **Score** represents the SAM value for reproducibility of the result between independent experiments, **Factor** is the averaged value for up- or downregulation for four independent experiments, **BLAST result** provides the description of the best hit obtained from searches against the SwissPort/TrEMBL database (http://ch.expasy.org/cgi-bin/niceprot) and the **p-value** indicates the statistical significance of the search result. For up-regulated genes score values are shown in red and for downregulated genes in green, respectively. The absence of a significant BLAST result is indicated by '-'. NA = not applicable

3.5.3 Comparison of the expression profile during early development

The FrzA$^-$ mutant exhibited a cell density dependent developmental defect. The phenotype of cells that were allowed to develop at low cell density (LCD) was much more severe than for those that developed at high cell density (HCD). To identify the genes that are differentially expressed during early development in the mutant in comparison to wild type we studied the expression profile at 4 and 8 hrs of development for two cell densities, HCD and LCD.

3.5.3.1 Differential gene regulation after 4 hrs of starvation

We compared the expression profile of Ax2 and FrzA$^-$ cells that were developed for 4 hrs (t_4) under HCD and LCD conditions and found that nearly twice as many genes were differentially regulated under HCD conditions in comparison to LCD conditions. In total 63 genes were at least 1.5 fold up- or downregulated under HCD conditions and 36 genes under LCD conditions. This corresponds to approximately 1.2% of all genes under HCD conditions and 0.7% of all genes under LCD conditions. Table 5 and 6 shows those genes for HCD and LCD conditions, respectively, were also found at 4 hrs HCD development that were found by SAM and whose expression deviated strongly from wild type.

Gene ID	Score	Factor	BLAST result	p-value
SLE355	15.44	2.87	Q9ur07 *Schizosaccharomyces pombe* retrotransposable element tf2	5.00E-17
J05457	13.98	2.25	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
AF025951	8.19	2.03	*Dictyostelium discoideum* heat shock cognate protein 70 (hsc70) mRNA	NA
A32505	8.71	1.92	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
SLA176	9.44	1.89	P08113 *Mus musculus* 94 kda glucose-regulated protein	1.00E-167
SLI421	9.81	1.83	-	-
SLC470	9.01	1.74	Q8t869 *Dictyostelium discoideum* 78 kda glucose-regulated protein homolog	0
VSI585	19.91	1.74	-	-
SSE777	10.68	1.73	Q8mpa5 *Dictyostelium discoideum* hypothetical protein	1.00E-77
SLJ671	8.69	1.72	Q02860 *Dictyostelium discoideum* retrotransposable element dre, chain b	8.00E-39
SLD225	6.72	1.72	P36415 *Dictyostelium discoideum* heat shock cognate protein 70 (hsc70) mRNA	0
SSH524	11.76	1.70	P34112 *Dictyostelium discoideum* cell division control protein 2 homolog	0
SLB742	12.82	1.68	P43280 *Lycopersicon esculentum* s-adenosylmethionine synthetase 1	1.00E-124
VSB688	11.71	1.65	P05165 *Homo sapiens* propionyl-coa carboxylase alpha chain, mitochondrial precursor	4.00E-85
SLG566	16.70	1.65	Q86k01 *Dictyostelium discoideum* 60s ribosomal protein	1.00E-94
SSK802	-17.48	0.62	Aas38648 *Dictyostelium discoideum* similar to homo sapiens (human). piwi-like	1.00E-113
SLB737	-11.00	0.63	Q09840 *Schizosaccharomyces pombe* probable alpha-amylase c23d3.14c	2.00E-70
X56297	-7.19	0.63	*Dictyostelium discoideum* LLRep3 Mrna	NA
SSB743	-9.43	0.63	Q96a08 *Homo sapiens* testis-specific histone h2b	2.00E-14
SSB153	-10.79	0.63	-	-
SSM847	-12.82	0.64	-	-
SLE621	-13.14	0.64	DDB0191456 delta 5 fatty acid desaturase (Dictybase curated)	-
X61797	-7.31	0.65	*Dictyostelium discoideum* mRNA for p17, purified from actin-myosin complex	NA
SLB406	-17.01	0.65	-	-
SLB425	-15.00	0.65	P54670 *Dictyostelium discoideum* calfumirin-1	3.00E-12
SLB350	-14.54	0.65	-	-
SSG712	-14.15	0.65	-	-
SSK687	-16.06	0.65	-	-
SSM830	-9.57	0.66	O15819 *Dictyostelium discoideum* histone h3	3.00E-65

Table 5. Differentially up- or downregulated genes at HCD after 4 hrs of development. Cells were starved on phosphate agar plates for 4 hrs at HCD. Please see the legend of Table 4 for the description of the table parameters.

Most of the differentially regulated genes that were found under LCD conditions were also found at 4 hrs HCD development. However, there are exceptions and those genes that were only found under LCD conditions are interesting because they might help to explain the more severe developmental phenotype of the FrzA$^-$ mutant under LCD conditions. While FrzA$^-$ cells formed very small fruiting bodies when developed under HCD conditions, the mutant did not aggregate or develop fruiting bodies under LCD conditions. Interestingly, several genes like ACA, smlA and discoidin that are important during early development were found to be downregulated only under LCD conditions. It is also noteworthy that under HCD conditions none of the differentially regulated genes was downregulated more than 1.6-fold while under LCD conditions significantly larger factors were observed e.g ACA was down regulated 3.3-fold.

Gene ID	Score	Factor	BLAST result	p-value
SLE355	26.88	4.26	Q9ur07 *Schizosaccharomyces pombe* retrotransposable element tf2	5.00E-17
SLD225	28.72	2.62	P36415 *Dictyostelium discoideum* heat shock cognate protein 70 (hsc70) mRNA	0
SLC470	5.81	2.29	P24067 *Zea mays* luminal binding protein 2	1.00E-180
AF025951	10.17	2.16	*Dictyostelium discoideum* heat shock cognate protein 70 (hsc70) mRNA	NA
SSE777	13.90	2.11	Q869n5 *Dictyostelium discoideum* phosphatidylinositol phosphate kinase 6	1.00E-43
SLJ671	12.54	1.94	Q9gq49 *Dictyostelium discoideum* group--specific antigen	2.00E-36
SLA176	16.35	1.79	P08113 *Mus musculus* 94 kda glucose-regulated protein	1.00E-167
J05457	6.00	1.69	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
A32505	5.57	1.66	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
SLB742	9.62	1.60	P31153 *Homo sapiens* s-adenosylmethionine synthetase gamma form	1.00E-124
SSH524	7.57	1.58	P34112 *Dictyostelium discoideum* cell division control protein 2 homolog	1.00E-140
SLI455	8.66	1.56	O15901 *Dictyostelium discoideum* putative transposase	0.00E+00
SSG654	8.63	1.51	Q9um47 *Homo sapiens* neurogenic locus notch homolog protein 3 precursor (notch 3)	5.00E-11
SSM424	-13.69	0.30	-	-
SSL845	-9.52	0.31	Q86au2 *Dictyostelium discoideum* adenylyl cyclase, ACA	3.00E-49
SSJ758	-11.17	0.34	-	-
SSL878	-13.34	0.34	P11491 *Schizosaccharomyces cerevisiae* repressible alkaline phosphatase precursor	3.00E-47
VSJ403	-14.53	0.35	P13021 *Dictyostelium discoideum* f-actin capping protein beta subunit (cap32)	1.00E-153
SSJ693	-11.54	0.41	-	-
VSI401	-11.07	0.44	Q8t690 *Dictyostelium discoideum* abc transporter	0
SSL818	-12.35	0.45	Q9gye0 *Dictyostelium discoideum* calcium-binding protein 4a	3.00E-78
VSB592	-15.79	0.47	P54661 *Dictyostelium discoideum* small aggregate formation protein, smlA	4.00E-72
SSM847	-13.21	0.55	-	-
VSK348	-13.28	0.56	-	-
SSH269	-10.10	0.60	Q869k6 *Dictyostelium discoideum* similar to anabaena sp. bacterioferritin comigratory protein	5.00E-12
SSK802	-20.84	0.60	Q9h9g7 *Homo sapiens* eukaryotic translation initiation factor 2c	4.00E-34
VSD289	-11.29	0.65	P42530 *Dictyostelium discoideum* discoidin ii	1.00E-149
VSJ578	-13.41	0.66	P27685 *Dictyostelium discoideum* 40s ribosomal protein s2	1.00E-123

Table 6. Differentially up- or downregulated genes at LCD after 4 hrs of development. Cells were starved on phosphate agar plates for 4 hrs at LCD. Those genes that are highlighted in the table were only found to be differentially regulated under LCD conditions. Please see the legend of Table 4 for the description of the table parameters.

3.5.3.2 Differential gene regulation after 8 hrs of starvation

The microarray results of the FrzA$^-$ mutant in comparison to Ax2 wild type cells after 8 hrs of development showed a similar percentage of differentially regulated genes under HCD and LCD conditions. Approximately 3.7% of all genes were found to be differentially regulated under both conditions.

Gene ID	Score	Factor	BLAST result	p-value
SLE355	7.67	4.35	Q9ur07 *Schizosaccharomyces pombe* retrotransposable element tf2	5.00E-17
SLI421	8.31	3.91	-	-
SLD225	27.39	2.84	P36415 *Dictyostelium discoideum* heat shock cognate protein	0
SSF869	19.36	2.72	P62242 *Mus musculus* 40s ribosomal protein s8	4.00E-51
AF140780	10.39	2.68	*Dictyostelium discoideum* countin gene, complete cds	NA
SLB758	15.45	2.66	P15112 *Dictyostelium discoideum* elongation factor 2	0
SLD607	8.42	2.62	P15112 *Dictyostelium discoideum* elongation factor 2	0
SSM731	15.77	2.59	P21147 *Schizosaccharomyces cerevisiae* acyl-coa desaturase 1	1.00E-107
SLH759	13.40	2.59	P35679 *Schizosaccharomyces cerevisiae* 60s ribosomal protein l4-a	2.00E-96
SSI266	17.93	2.58	Q86ae0 *Dictyostelium discoideum* phosphoinositide phosphatase sac1	5.00E-97
SLA374	20.26	2.56	O15706 *Dictyostelium discoideum* vacuolin a	0.00E+00
SSA863	7.77	2.45	P57728 *Schizosaccharomyces cerevisiae* 60s ribosomal protein	6.00E-48
VSC841	9.43	2.43	Q86kq6 *Dictyostelium discoideum* hypothetical protein	1.00E-132
X56192	22.27	2.39	*Dictyostelium discoideum* mRNA for ribosomal acidic phosphoprotein P2	NA
VSG377	7.62	2.36	P36241 *Drosophila melanogaster* 60s ribosomal protein	2.00E-21
X82784	-11.01	0.20	*Dictyostelium discoideum* mRNA for calcium binding protein	NA
VSI401	-10.17	0.34	Q8t690 *Dictyostelium discoideum* abc transporter	0
VSD859	-7.33	0.37	-	-
X52465	-7.72	0.47	*Dictyostelium discoideum* D2 gene	NA
X61581	-8.37	0.47	*Dictyostelium discoideum* mRNA for profilin I	NA
X15430	-13.52	0.51	*Dictyostelium discoideum* mRNA for gelation factor	NA
X04004	-13.72	0.53	*Dictyostelium discoideum* mRNA for *contact sites A* (csA) protein	NA
VSH187	-10.40	0.55	Q9erg0 *Mus musculus* epithelial protein lost in neoplasm (meplin)	2.00E-13
X66483	-12.85	0.57	*Dictyostelium discoideum contact sites A* (csA) protein	NA
VSJ349	-9.68	0.58	Q86l09 *Dictyostelium discoideum* hypothetical protein	0
VSK466	-9.39	0.59	-	-
VSJ403	-8.00	0.59	P13021 *Dictyostelium discoideum* f-actin capping protein beta subunit (cap32)	1.00E-153
VSJ758	-16.46	0.59	Q86ac9 *Dictyostelium discoideum* similar to kaposi's sarcoma-associated herpesvirus	0
VSC385	-7.62	0.60	O15254 *Homo sapiens* acyl-coenzyme a oxidase 3	6.00E-72
VSG463	-7.52	0.63	Q60715 *Mus musculus* prolyl 4-hydroxylase alpha-1 subunit precursor	1.00E-20
VSG756	-7.89	0.64	Aap35881 *Homo sapiens* proteasome (prosome, macropain) 26s subunit	1.00E-100

Table 7. Differentially up- and downregulated genes at 8 hrs for HCD. Cells were starved for 8 hrs at LCD and cDNA was prepared as described in Materials and Methods (4.2.2). Genes highlighted are mentioned in the discussion. Please see the legend of Table 4 for the description of the table parameters.

This number is considerably higher than for vegetative and t_4 cells. It probably reflects the increasing delay in the development of the FrzA$^-$ mutant in comparison to wild type cells. Interestingly, although the total number of differentially regulated genes was similar for HCD

and LCD development, the proportion of up- and downregulated genes was different for both conditions (see Figure 33).

Gene ID	Score	Factor	BLAST result	p-value
SLE355	21.87	6.00	Q9ur07 *Schizosaccharomyces pombe* retrotransposable element	5.00E-17
SLG566	12.73	5.59	Q96s84 *Homo sapiens* ribosomal protein l15	9.00E-58
SLI421	16.53	4.11	-	-
AF140780	11.70	3.76	*Dictyostelium discoideum* countin gene	NA
J05457	12.68	3.41	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
SLA374	16.18	3.06	O15706 *Dictyostelium discoideum* vacuolin a	0
AF025951	7.12	2.97	*Dictyostelium discoideum* heat-shock cognate protein 70 (hsc70) mRNA	NA
SSI266	23.95	2.96	P61358 *Mus musculus* 60s ribosomal protein l27	4.00E-32
SSM731	7.62	2.93	O00767 *Homo sapiens* acyl-coa desaturase	1.00E-47
SSL471	11.04	2.80	-	-
A32505	10.72	2.75	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
SSH336	25.79	2.73	Q10344 *Schizosaccharomyces pombe* translationally controlled tumor protein p32 homolog p32	3.00E-36
SSE777	7.20	2.72	Q8mmn1 *Dictyostelium discoideum* hypothetical protein	2.00E-74
SLH759	19.95	2.68	P08429 *Xenopus laevis* 60s ribosomal protein l4a	7.00E-97
SSF869	13.73	2.64	P48156 *Caenorhabditis elegans* 40s ribosomal protein s8	2.00E-50
SSB469	-7.17	0.57	putative calmodulin-binding protein CaM-BP15	-
SLB640	-11.29	0.58	Q9tx40 *Dictyostelium discoideum* tat-binding protein alpha	0
SSF841	-7.70	0.59	Q86uw6 *Homo sapiens* nedd4-binding protein 2	9.00E-12
SSC210	-8.13	0.60	-	-
SLF270	-7.05	0.60	O00231 *Homo sapiens* 26s proteasome non-atpase regulatory subunit 11	3.00E-86
VSJ758	-8.07	0.60	Q86ac9 *Dictyostelium discoideum* similar to kaposi's sarcoma-associated herpesvirus	0
Y07497	-8.64	0.60	*Dictyostelium discoideum* putative alpha-L-fucosidase gene	NA
SSI530	-7.14	0.62	Q7x2a0 *Pseudomonas aeruginosa*. tetracycline inactivating enzyme	2.00E-26
VSG756	-9.99	0.62	O43242 *Homo sapiens* 26s proteasome non-atpase regulatory subunit 3	1.00E-101
SSK505	-8.44	0.63	-	-
SLE486	-10.95	0.64	P49598 *Arabidopsis thaliana* protein phosphatase 2c	3.00E-15
VSK466	-7.25	0.64	-	-
SSK819	-8.36	0.65	O74850 *Schizosaccharomyces pombe* diacylglycerol o-acyltransferase 1	5.00E-67
VSE243	-7.44	0.66	O43242 *Homo sapiens* 26s proteasome non-atpase regulatory subunit 3	1.00E-101
VSG496	-7.71	0.66	P34117 *Dictyostelium discoideum* cdc2-like serine/threonine-protein kinase crp	1.00E-162

Table 8. Differentially up- and downregulated genes at 8 hrs for LCD. Cells were starved for 8 hrs at LCD and cDNA was prepared as described in Materials and Methods (4.2.2). Genes highlighted in the table are differentially regulated only at this condition and also mentioned in the discussion. Please see the legend of Table 4 for the description of the table parameters.

Table 7 and 8 lists the 15 most significantly up- or downregulated genes under HCD and LCD conditions, respectively. It also shows that there are considerable number of downregulated genes that are unique for the LCD development. Among these are five genes (highlighted in Table 8), which are associated with the proteasome-mediated degradation pathway. This suggests that the proteasome regulated degradation which is necessary for multicellular development (Lindsey et al., 1998) is somehow reduced in the FrzA$^-$ mutant. This could be

one of the reasons why the mutant cells were not able to transit from growth phase to developmental phase when starved under LCD conditions.

3.5.3.3 Comparison of differentially regulated genes under HCD and LCD conditions

During an analysis of the FrzA$^-$ mutant we found that the developmental defect depends on the cell density. Cells starved on phosphate agar plates at HCD developed considerably slower than wild type cells and only produced small fruiting bodies. However, when starved at LCD the mutant did neither form aggregates nor did it develop into fruiting bodies. This difference in the developmental defect can be analysed by looking at those genes which are only up- or downregulated under HCD or LCD conditions respectively. Since most transcriptional changes occur between 4-10 hrs of development (Van Driessche et al., 2002), we analysed the data from 4 and 8 hrs. Figure 33 summarises our findings for up- or downregulated genes under LCD and HCD conditions at 4 and 8 hrs of development. With exception of the downregulated genes at 4 hrs of development under HCD conditions, the percentage of common genes between the two conditions was generally higher than 50%. Those genes that are common might be responsible for the delay in development when compared to wild type while those genes that are only differentially regulated under LCD conditions might be responsible for the more severe phenotype under this condition. It is also noteworthy that 3 to 6 fold more genes were differentially regulated at 8 hrs of development as compared to 4 hrs. This probably mirrors the increasing delay in the development of the mutant.

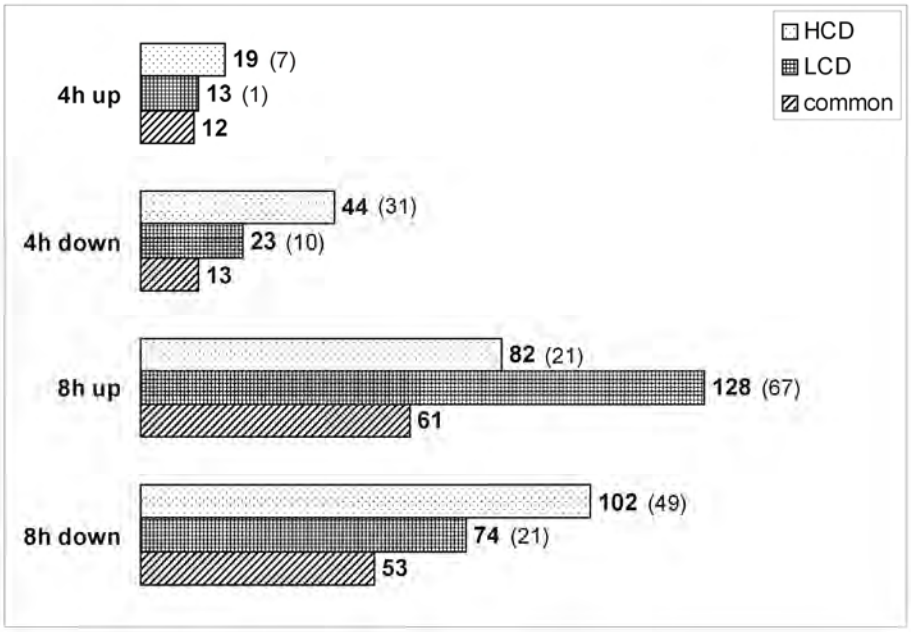


Figure 33. Bar diagram showing the genes that are common and unique between HCD and LCD conditions. All genes that are up- or down regulated at the 4 and 8 hrs time points and that are common between the HCD and LCD condition are represented as bar graph. The size of the bars corresponds to the number of differentially regulated genes and their absolute number is stated. Genes that are unique to the HCD or LCD condition are given in brackets.

3.5.3.4 Cluster analysis

To simplify the results we applied different clustering methods. We subjected those genes to clustering methods that were found to be at least 1.5-fold up- or downregulated at either 0, 4 or 8 hrs. A set of 257 and 271 differentially regulated genes for HCD and LCD development, respectively, was grouped into five clusters using the K-means clustering method (Figure 34). We compared the cluster profile of HCD and LCD conditions and found that the expression of genes in clusters 1 and 5 went down with starvation whereas it went up in clusters 3 and 4. Most informative was cluster 2. For this cluster the expression profile was completely opposite for HCD and LCD. It contained genes that were predominantly downregulated in HCD and upregulated in LCD development. Table 9 shows that at least 50% of the genes were common between the clusters 1, 3, 4 and 5 of HCD and LCD development, while cluster 2 showed a very low number of common genes. Since the strongest developmental defect is exhibited during LCD condition we looked more closely at the unique genes under HCD and LCD conditions of cluster 2. Table 10 shows up- and downregulated genes of cluster 2 at 8 hrs of development for HCD and LCD conditions, respectively. Only the ten highest upregulated genes for the LCD and the nine most downregulated genes for the HCD condition are listed.

An interesting aspect of the list of unique genes under HCD conditions was the presence of four genes encoding protein disulfide isomerases. The protein disulfide isomerase is an ER resident protein and deletion of this protein in yeast was found to be lethal (LaMantia et al., 1991). Another gene that was found to be downregulated was Calreticulin, which interacts with protein disulfide isomerase and found to be important for normal heart development in mouse (Baksh et al., 1995; Michalak et al., 2004). It is at present not clear why these genes are downregulated at 8 hrs of HCD development. In addition a cell division cycle protein (cdc48) that is important for M to G1 transition during the cell cycle was also found to be downregulated (Cao and Zheng, 2004). The list of uniquely upregulated genes of cluster 2 under LCD conditions was not very telling. To find out more about the molecular basis of the developmental defect under LCD and HCD conditions it will be necessary to analyse all genes that are unique to LCD or HCD, respectively, in more detail.

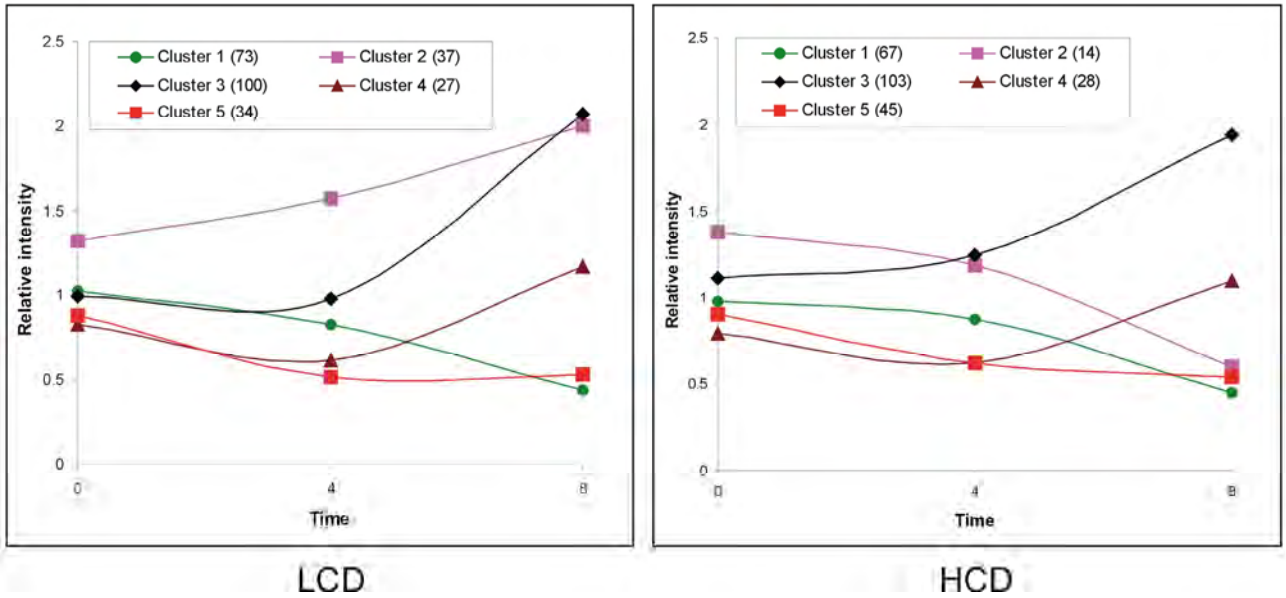


Figure 34. Cluster analysis. Genes that were at least 1.5 fold up- or downregulated at either 0, 4 or 8 hrs of development were subjected to cluster analysis. For LCD (left) and HCD (right) K- means clustering resulted in each case in five groups. Symbols represent the average up- or downregulation of all members of the corresponding cluster. The number of genes in each cluster ranged from approximately 15 to 100. The clusters of LCD and HCD are indicated by consistent colors.

Cluster	LCD	HCD	Common
1	73	67	39
2	37	14	3
3	100	103	54
4	27	28	17
5	34	45	27

Table 9. Comparison of genes between their respective clusters. The numbers given under LCD and HCD are the total number of genes in the respective clusters that have a similar expression profile. The genes between similar cluster profiles were compared and the number of genes in common is presented.

Cluster 2 HCD

Gene ID	Score	0	4	8	BLAST result	p-value
SSD437	-10.11	1.68	0.82	0.26	P54653 *Dictyostelium discoideum* calcium-binding protein 2	2.00E-92
SLC177	-11.30	1.41	1.29	0.27	Aao52220 *Dictyostelium discoideum* protein disulfide isomerase	1.00E-129
U83085	-15.34	1.34	1.13	0.50	*Dictyostelium discoideum* cell division cycle protein 48 (cdcD)	NA
AF019112	-8.43	1.11	1.15	0.50	*Dictyostelium discoideum* protein disulfide isomerase	NA
U36937	-12.68	1.47	1.33	0.55	*Dictyostelium discoideum* calreticulin mRNA	NA
VSC385	-7.62	1.07	1.09	0.60	P05335 *Candida maltosa* acyl-coenzyme a oxidase pox4 (acyl-coa oxidase)	4.00E-65
SLC694	-8.21	1.10	1.05	0.61	P35573 *Homo sapiens* glycogen debranching enzyme (glycogen debrancher)	1.00E-117
SLH760	-10.53	1.09	1.16	0.62	Q12730 *Aspergillus niger*. protein disulfide-isomerase precursor	4.00E-93
SLC492	-16.18	1.11	1.09	0.63	Q12730 *Aspergillus niger*. protein disulfide-isomerase precursor (pdi)	4.00E-93

Cluster 2 LCD

SLE355	21.87	1.65	4.26	6.00	Q9ur07 *Schizosaccharomyces pombe* retrotransposable element tf2	5.00E-17
SLI421	16.53	1.56	1.70	4.11	-	-
J05457	12.68	1.23	1.69	3.41	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
AF025951	7.12	1.56	2.16	2.97	*Dictyostelium discoideum* heat-shock cognate protein 70 (hsc70) mRNA	NA
SSM731	7.62	1.08	1.60	2.93	Q86ak4 *Dictyostelium discoideum* similar to *Mortierella alpina*. stearoyl-coa desaturase	0
A32505	10.72	1.37	1.66	2.75	*Dictyostelium discoideum* nucleoside diphosphate kinase mRNA	NA
SSE777	7.20	1.00	2.11	2.72	Q8mpa5 *Dictyostelium discoideum* hypothetical protein	1.00E-77
SLH759	19.95	1.37	1.23	2.68	Q86kt2 *Dictyostelium discoideum* similar to arabidopsis thaliana (mouse-ear cress)	0
SSL485	6.91	1.29	1.76	2.60	-	-
SLB742	10.12	1.20	1.60	2.13	P31153 *Homo sapiens* s-adenosylmethionine synthetase gamma form	1.00E-124

Table 10. Unique genes in cluster 2. The factors for up- or down regulation at 0, 4 or 8 hrs of development of the mutant in comparison to wild type is given under 0, 4 and 8. The score values represent only the values for the 8 hrs time point. Highlighted genes are mentioned in the text. Please see the legend of Table 4 for the description of the other table parameters.

3.5.3.5 Developmental regulation of genes in cluster 2 in comparison to Ax2 and the ACA$^-$ mutant

Those genes which are found upregulated in the mutant might only appear upregulated because they are actually downregulated during development in the wild type cells; correspondingly genes that are found downregulated in the mutant might be upregulated in the wild type during development. Therefore, to understand the profile of cluster 2 obtained from the cluster analysis of the FrzA$^-$ mutant we compared the expression of genes at the 8 hrs time point with that of the parental strain Ax2 (Van Driessche et al., 2002) and ACA$^-$, an aggregation defective strain (Iranfar et al., 2003).

At HCD conditions only values for six of the genes were available from published results (Iranfar et al., 2003; Van Driessche et al., 2002). Three of these, SSD437, SLC177 and SLH760 confirmed our theory that some of those genes that appear downregulated in the FrzA$^-$ mutant are actually upregulated during wild type development. One of these genes, AF019112, was also found to be downregulated during wild type development by (Van

Driessche et al., 2002) and it is not clear why this gene appeared to be downregulated in the FrzA$^-$ mutant at 8 hrs of development. For two of the genes no data were available from wild type development. It is interesting that five of the genes are very similarly regulated in the FrzA$^-$ and the ACA$^-$ mutant.

For development under LCD conditions there are 34 unique upregulated genes in cluster 2 at 8 hrs of development (Table 9). For 25 of these there were values available for either the Ax2 or for ACA null development and Figure 35B shows only the ten most significantly upregulated genes of cluster 2 of the FrzA$^-$ mutant. Only two of these, SSM731 and SLB742, are downregulated during Ax2 development. These two confirm our theory that some of the genes only appear upregulated during mutant development because they are infact downregulated during wild type development but not so in the mutant. Most other genes, with exception of SLH759, appear truly upregulated at 8 hrs of mutant development in comparison to wild type. It is interesting that, in contrast to development under HCD conditions, most of these genes are similarly regulated in wild type and ACA null cells. It appears therefore that the expression profile of the FrzA$^-$ mutant is only very similar to the ACA null mutant when the cells are allowed to develop under HCD conditions.

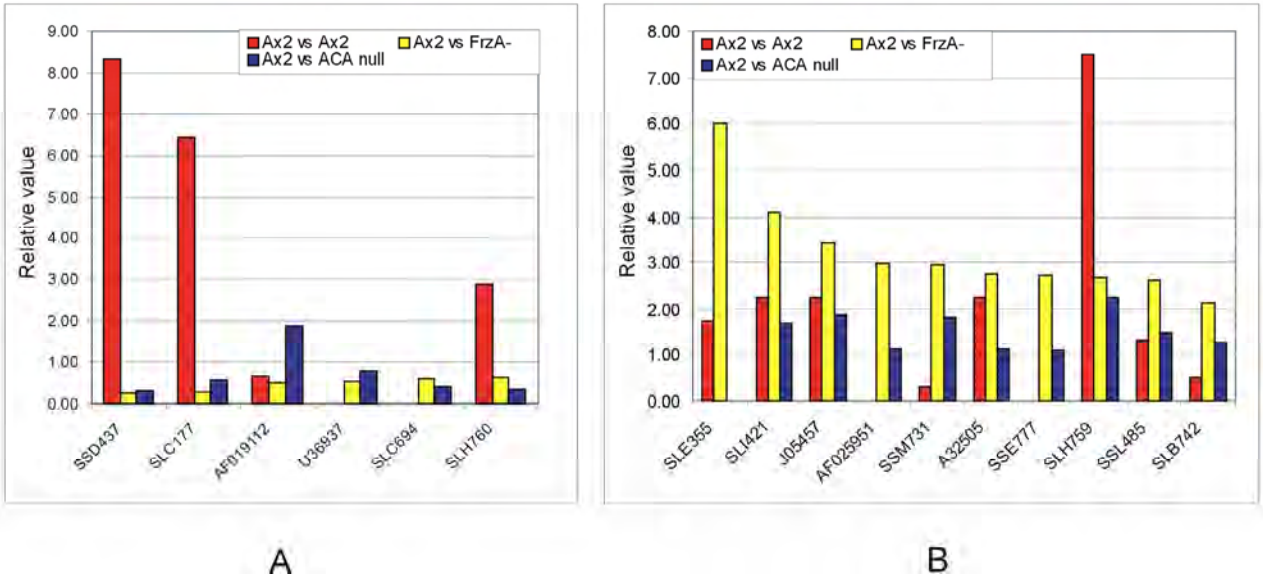


Figure 35. Expressions of unique genes in cluster 2 in comparison to Ax2 and ACA null strain at 8 hrs of development. A shows unique genes for HCD conditions, B for LCD conditions**.** Only those genes identified in the FrzA$^-$ mutant are shown for which corresponding values were available in either Ax2 and/or the ACA null strain. Gene identity numbers are given below the X-axis. Relative value is the fold up- or downregulation in the corresponding comparison.

1. Discussion

The Frizzled gene was first identified in *Drosophila* in a screen for mutations that disrupt the polarity of epidermal cells in the adult fly (Gubb and Garcia-Bellido, 1982). Subsequently, Frizzleds have been found in diverse metazoans (Wang et al., 1996), including at least ten in vertebrates, four in *Drosophila*, and three in *Caenorhabditis elegans*. Frizzleds have also been identified in primitive metazoans, including the sponge *Suberites domuncula* (Adell et al., 2003) and in *Hydra vulgaris* (Minobe et al., 2000). The Frizzled (Fz) proteins are 7-pass transmembrane proteins characterized by an extracellular N-terminal, cysteine-rich domain that might constitute part or all of the ligand-binding domain of the Wnt proteins (Hsieh et al., 1999). When we searched the *Dictyostelium* database using the Frizzled transmembrane domain we identified 25 Frizzled like receptors (Figure 2). *Dictyostelium* FrzA was unique among them, with a N-terminal Frizzled transmembrane and C-terminal Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain. The sequence homologies of Frizzled transmembrane region clearly places FrzA in the smoothened Frizzled receptor family. Frizzled in *Dictyostelium* is reported for the first time and the function of FrzA is studied here.

In general, Frizzleds are widely and dynamically expressed in almost all cell types. Expression pattern and function of Frizzleds in different model organisms are well described (Strutt, 2003). Frizzleds are essential for cell fate determination during embryonic development and various other Frizzled family members are associated with cell proliferation and activating stimuli for specific signal transduction. However, Frizzled functions are as varied as the number of cell types that express them. Our object of interest, FrzA, a novel Frizzled like protein in *Dictyostelium*, was found to be expressed throughout the development although the expression was slightly higher after 8 hrs of development (Figure 6).

We generated a mutant (FrzA$^-$) lacking the functional FrzA gene to understand the *in vivo* function of this novel Frizzled like protein, FrzA in *Dictyostelium*. The FrzA$^-$ mutant was able to grow normal in axenic medium or feed on bacteria, but was not able to form fruiting bodies with the depletion of the food source when grown on a bacterial lawn. Therefore, absence of FrzA in *Dictyostelium* is not lethal unlike the homozygous deletion of Fzd5 in mouse that causes embryonic death at around 10.5 days due to a defect in yolk sac and placental angiogenesis (Ishikawa et al., 2001). However the FrzA$^-$ null cells exhibit developmental defects such as lack of aggregation and delayed development of small and few fruiting bodies.

1.1 FrzA role in aggregation and development of *Dictyostelium*

Dictyostelium discoideum grows vegetatively as individual amoebae and on depletion of food, a multicellular developmental program is initiated. When cells begin to starve, a 10 hrs aggregation process is initiated where a cell functioning as an aggregation center begins to emit pulses of cAMP at 6 to 9 min intervals. Wild type cells form streams at ~6 hrs of starvation on a plastic surface in a submerged culture assay (Thiery et al., 1992). But we found that the FrzA$^-$ null cells were not able to form streams (Figure 16). Aggregation is stimulated by pulse-induced genes in a two-phase process (Mann and Firtel, 1989). The initial phase of starvation requires the synthesis of a secreted soluble protein factor, CMF, and coordinates development (Mehdy and Firtel, 1985). When a majority of the cells in a given area starve, the extracellular level of CMF rises above a threshold value, and cells aggregate using relayed pulses of cAMP as the chemoattractant. With wild type cells when starved on phosphate agar plate, approximately 10^5 cells stream together to form tight aggregates between 6-10 hrs that proceed through a series of morphogenetic changes, culminating into a fruiting body at around 24 hrs. While the FrzA$^-$ mutant exhibits an aberrant developmental pattern, lacking formation of streams or aggregates at 6 hrs after initiation of starvation, they form a loose group of cells from which very few and small fruiting bodies emerge. But 85% of the loose aggregates do not undergo further development (Figure 17). And when wild type cells are starved at a density below a particular threshold, they do not develop. Under these conditions, the concentration of CMF in the extracellular environment is insufficient to allow gene expression necessary for aggregation (Mehdy and Firtel, 1985). *Dictyostelium* can successfully aggregate only if there are enough cells within a given space secreting CMF that monitors the cell density prior to inducing genes that are required for aggregation (Deery and Gomer, 1999) and CMF functions *in vivo* as a cell density-determining factor (Yuen et al., 1995). Wild type cells when starved on phosphate agar plates at different cell densities, even at 1 x 10^5 cells per cm^2 aggregated followed by formation of fruiting bodies after 20-24 hrs of starvation. When performing this analysis with the FrzA$^-$ mutant, we observed that they were polarized with many lateral pseudopods but did not aggregate and develop into fruiting bodies at a density less than 5 x 10^6 cells per cm^2 (Figure 23). The mutant did however form few fruiting bodies at 5 x 10^6 cells per cm^2 but after 48 hrs of development, which may be due to random collision of cells at high cell density. Thus FrzA might play a role in determining cell density necessary at early stages of development by functioning as a receptor for the secreted cell density factors.

Molecular evidence for aggregation defect in the mutant was coming from northern blot analysis. Aggregation specific genes csA and cAR1 (Firtel, 1995; Gerisch, 1968; Noegel et al., 1986a) were found to be significantly lower in the FrzA$^-$ mutant when compared to the wild type Ax2. During aggregation cAMP pulses are synthesized by activation of adenylyl cyclase (ACA), which is mediated by cAR1 (Anjard et al., 2001). In comparison to the wild type Ax2 the FrzA$^-$ mutant did not produce any ACA transcript during 6-10 hrs of starvation and this is consistent with the aggregation defect (Figure 18) indicating FrzA might not produce the cAMP pulse to induce pulse-dependent genes necessary for aggregation.

During the second phase of induction of pulse-induced genes, the cell-surface receptors bind cAMP and change to an inactive state (Devreotes, 1982). As the extracellular cAMP is degraded by an extracellular cAMP phosphodiesterase (Gerisch, 1987; Kessin, 1988), the receptors return to an active state, making the cells ready to receive the next signal. cAMP pulse produced as signal is amplified and propagated outward from the aggregation center in form of waves. This signal relay system serves to establish a gradient of cAMP, which functions as the chemoattractant during aggregation. Cells move up the cAMP gradient toward the aggregation center, forming streams as they establish cell-cell contacts and move together (Devreotes, 1982). A number of developmentally regulated genes have been identified in *Dictyostelium* whose expression is regulated by cAMP activated signal transduction processes (Mehdy et al., 1983). PDE that may contribute to the negative feedback loop of oscillatory cAMP signaling (Meima et al., 2003), was found to be stimulated by cAMP at the aggregation stage in wild type. But in FrzA$^-$ mutant the PDE expression was high and constant throughout the development process. A secreted glycoprotein phosphodiesterase inhibitor (PDI) that regulates the activity of PDE (Franke et al., 1991) is induced in response to cAMP secretion in the wild type cells. In the FrzA$^-$ mutant transcription of PDI was observed to be significantly low. Development followed by aggregation regulates genes that are stimulated by moderate and high concentration of cAMP. Prestalk and prespore specific late genes are induced at 10 and 15 hrs of development, respectively, and require moderate levels of cAMP for their expression (Mehdy et al., 1983; Schaap and van Driel, 1985). Changing levels of cAMP within the population of cells is used to regulate gene expression throughout *Dictyostelium* development. In the FrzA$^-$ mutant Prestalk (ecmA) and prespore precursor (pspA) genes were expressed but delayed by 36 hrs in comparison to the wild type Ax2 (Figure 18). These observations infer that FrzA may have a role even before the aggregation stage with a defect in generating cAMP pulses necessary for induction of other genes to continue development.

1.2 csA mediated cell adhesion is controlled by FrzA

During *Dictyostelium* development the signal transduction pathways induced by cAMP are involved in the regulation of gene expression. At the early phase of development, several genes become responsive to pulses of low levels of cAMP upon the initiation of the cAMP signal-relay (Mann et al., 1988). One gene whose expression is highly augmented by pulses of cAMP is gp80/csA. Transcription of the csA gene is first turned on at a low basal level at the preaggregation stage and then greatly enhanced by low levels of cAMP at the aggregation stage. Expression of csA is under developmental regulation and accumulates rapidly on the cell surface between 6 and 10 hrs of development, corresponding to the aggregation stage (Siu et al., 1987). csA is a cell adhesion molecule with an apparent molecular weight of 80 kDa (Siu et al., 1985). Northern blot analysis for the FrzA$^-$ mutant shows that the csA expression level was not comparable to the wild type Ax2 at the aggregation stage (Figure 18), while csA protein expression was not observed either when developed in phosphate agar plates or when developed in suspension (Figure 19A and B). When Ax2 development was carried out in the presence of exogenous pulses of nanomolar amounts of cAMP, cells express csA in an enhanced and precocious manner (Siu et al., 1988), while in the FrzA$^-$ mutant csA expression could be induced by exogenous pulsing with cAMP (Figure 19B). This clearly indicates that csA mediated cell adhesion in *Dictyostelium* requires direct or indirect role of FrzA.

1.3 FrzA plays a major role in chemotaxis

Directed movement of the cells toward or away from a chemoattractant, mediates the inflammatory and immune responses, neuronal outgrowth, embryonic cell movements, fertilization, angiogenesis and metastasis. Chemotaxis in *Dictyostelium* is better studied than in any other cell type (Malchow et al., 1996a). During growth, cells feed on bacteria by orienting to folic acid (Pan et al., 1972) and in the absence of food changes in gene expression are observed with onset of cAMP secretion and responsiveness towards cAMP initiating development (Firtel, 1991). Chemotaxis is also a critical determinant in tissue organization of *Dictyostelium* (Durston and Vork, 1979). Determining the ability of the FrzA$^-$ mutant to chemotax up a concentration gradient of chemoattractant was necessary due to the developmental defect at low cell density, where motility of cells towards the signal is important. FrzA$^-$ cells when challenged with cAMP through a micropipette showed poor directed movement towards the cAMP which coincides with low level of cAR1 expression. Exogenous cAMP pulses were able to rescue the chemotaxis defect in DdPIPkinA$^-$ cells (Guo et al., 2001). In FrzA$^-$ mutant exogenous cAMP pulsing was also able to restore the

chemotaxis defect, but speed and persistence during directed migration was not rescued (Table 2) suggesting that FrzA could be involved in a distinct pathway for directed movement of the cells towards the chemoattractant.

1.4 Function of FrzA in cAMP signaling

cAMP mediated chemotaxis is regulated by gene products that are dependent on cAMP signaling and Gα2 protein (Sun and Firtel, 2003). cAMP outside the cell acts as chemoattractant responsible for conversion of a unicellular into a multicellular organism (Konijn et al., 1967) and regulates genes that are required for chemotactic aggregation (Gerisch et al., 1975). Expression of many receptors is controlled by their own ligands. cAR1, receptor for cAMP is expressed at undetectable level when cells growing in low density and expressed at low level with increase in cell density. When cells starve and start to develop, cAR1 expression increases more than 20 fold (Kimmel, 1987; Kimmel and Firtel, 1991). Starving cells secrete factors to allow cells to aggregate and cells aggregate by sensing and secreting cAMP in a pulsatile fashion. The phosphorylated cAR1 is transiently expressed upon cAMP stimulation and increases the extracellular cAMP level (Klein et al., 1985). The PDE degrades cAMP and resulting in partial deactivation of cAR1 inducing a cycle of responsiveness and desensitization of cAR1 leading to low level oscillations in extracellular cAMP concentration. In the FrzA$^-$ mutant the cAR1 and Gα2 protein level was normal when developed in suspension, where random collision can induce gene expression, whereas during development on phosphate agar plates the FrzA$^-$ mutant was not able to produce normal levels of cAR1 like cAR1$^-$ null cells that do not initiate cAMP singaling (Saxe et al., 1991). Thus the mutant may have abnormal cAMP signaling. Since cell density determines development we analysed if cell density also affects cAR1 expression during development. When wild type cells were developed at low and high cell density, normal cycle of activation and deactivation of cAR1 was observed at high cell density, but at low cell density they mainly expressed the deactivated form of cAR1 but underwent development (Figure 27B). And FrzA$^-$ mutant under similar conditions showed expression of cAR1 at high cell density but with abnormal development, whereas cAR1 expression was completely absent at low cell density. The phenotype exhibited by the mutant at low cell density is similar to that of the cAR1$^-$ mutant (Sun and Devreotes, 1991). Thus FrzA controls expression of cAR1 and is indispensable for induction of cAMP signaling to initiate the process of development when developed at low cell density.

1.5 FrzA affects calcium oscillation

Dictyostelium cells exhibit a rapid activation and delayed inhibition of ACA causing a periodic oscillation of cAMP production, that controls the chemotactic signaling mechanism during the aggregation stage of development (Maeda et al., 2004). Cytosolic Ca^{2+} was also found to play an important role during aggregation and development (Newell et al., 1995), where Ca^{2+} influx elevates cytosolic calcium level shortly after cAMP synthesis and Ca^{2+} efflux occurs when cAMP level comes to a basal level (Bumann et al., 1984). However, a direct link has not been shown between Ca^{2+} and cAMP oscillation. This was shown with W7, an antagonist of calmodulin that was found to enhance the light scattering and cAMP oscillation linking Ca^{2+} to cAMP oscillation. In the wild type cells, W7 increases the light scattering and cAMP relay by Ca^{2+} efflux from intact cells, whereas in the $FrzA^-$ mutant W7 only one large peak is elicited and no oscillation occurs (Figure 30) like in the PLC null cells (Malchow et al., 2004). Therefore, FrzA may be important for *Dictyostelium* cells to induce an oscillatory circuit by producing periodic cAMP pulses and this is consistent with the aggregation and chemotaxis defect in the $FrzA^-$ mutant.

1.6 The FrzA acts as a GPCR like CMF Receptor

The secreted factors in conditioned medium promotes expression of genes induced early in the developmental process (Mehdy and Firtel, 1985). We found that the $FrzA^-$ mutant to be able to produce these factors but is not able to sense them (Figure 24). A putative receptor that mediates cell density sensing in *Dictyostelium* was identified and characterized (Deery and Gomer, 1999). Disruption of CMFR1 does not affect the CMF mediated binding of cAMP to its receptor cAR1 or induction of IP3 synthesis. But, (Brazill et al., 1998) showed that CMF regulates cAMP binding and IP3 synthesis by activating a G protein. Therefore, Deery and Gomer (1999) assumed the existence of an unknown G protein-coupled CMF receptor and that the absence of CMFR1 did not affect this receptor. However absence of FrzA affected both CMF and CMFR1 expression (Figure 25), which correlated with the cell differentiation assay at low cell density (Figure 28). The $FrzA^-$ mutant did not induce prespore (D19) differentiation in response to rCMF like in the CMFR1 or CMF null cells indicating that FrzA may affect a G-protein independent pathway by controlling the expression of CMF and its receptor CMFR1. $CMFR1^-$ null cells are aggregation negative and show a defect in expression of pulse-induced genes, csA and cAR1 as in the $FrzA^-$ mutant. Therefore we examined the $FrzA^-$ cells ability to sense the rCMF and found the cells not

responding to the rCMF by remaining unpolarised (Figure 26) providing a clue that FrzA could be a receptor specific to CMF.

The model shows the extracellular protein CMF binding to a G protein coupled receptor, which is associated with Gα1βγ. Binding of CMF allows Gβγ to dissociate from Gα1 activating phospholipase (PLC). The activation of PLC either directly or indirectly causes the cAMP stimulated GTPase activity of Gα2 to decrease by prolonging the lifetime of the Gα2-GTP configuration. This prolongation could be reduced in the absence of CMF causing rapid hydrolysis of Gα2-GTP to GDP form and not allowing the cells to aggregate (Brazill et al., 1998). A similar response was observed in the FrzA$^-$ mutants, where the presence of CMF did not stimulate the activation of PLC to increase the IP3 production. Therefore Gα1 appears to be a component downstream of the FrzA, a GPCR like CMF receptor in a pathway mediating CMF signaling to Gα2 which is consistent with the hypothesis put forth by Brazill et al., (1998).

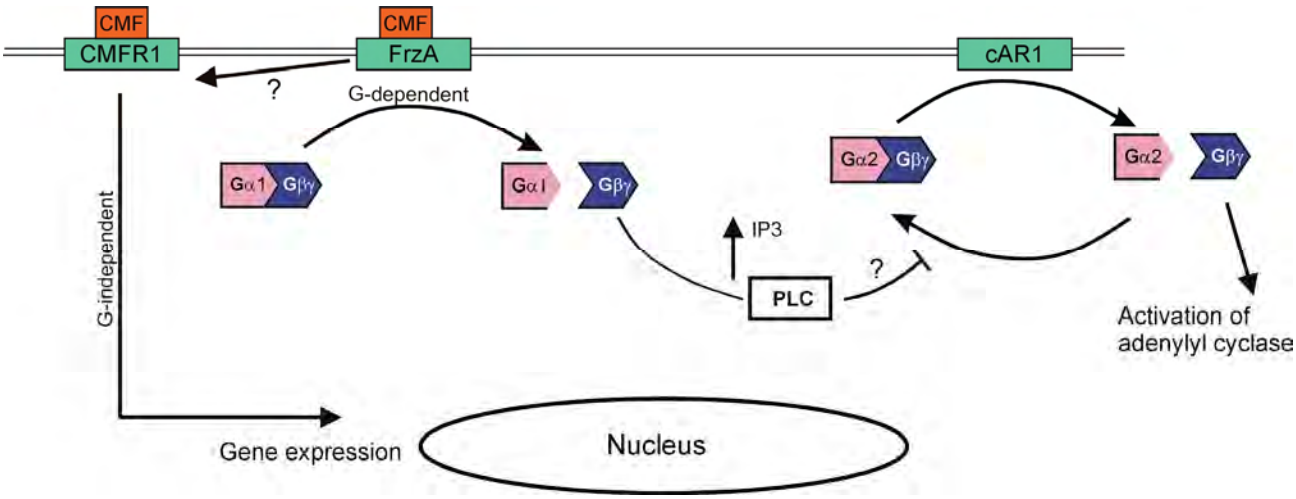


Figure 36. Hypothetical function of FrzA in *Dictyostelium*

1.7 Microarray analysis of FrzA$^-$ mutant

Northern blot analysis showed that the FrzA$^-$ mutant exhibited poor expression of some of the genes that are involved in the aggregation stage during *Dictyostelium* development (Figure 18). In addition, the mutant showed a cell density dependent phenotype where cells at HCD condition underwent delayed development forming few and small fruiting bodies, whereas under LCD condition they did not aggregate or develop into fruiting bodies at all. To identify the genes that were differentially regulated under these two different conditions we performed a large scale expression study using the *Dictyostelium* DNA microarray.

When *Dictyostelium* Ax2 cells are depleted of nutrients they arrest proliferation to initiate a developmental program during which genes necessary for differentiation are differentially regulated. At the onset of starvation genes necessary for the initiation of the aggregation process are induced. One of these is the contact sites A (csA), gene which encodes a cell adhesion molecule whose expression is induced by cAMP pulses produced during aggregation (Desbarats et al., 1992). Our biochemical studies showed that the FrzA$^-$ mutant might not be able to secrete or sense the pulses of cAMP and we also found that expression of csA is delayed and also reduced (Figure 18 and 19). This result was confirmed by our microarray studies which showed that csA was significantly downregulated under HCD and LCD conditions. In addition the analysis revealed changes in the expression pattern of several more genes like ACA, cAR1, smlA and vegetative specific proteins which either play a role during aggregation or are important for transition from growth to development. This suggests that the results that were obtained by the microarray analysis are trustworthy and mirror the observed changes during development of the FrzA$^-$ mutant.

We performed a detailed analysis of all the differentially regulated genes under HCD and LCD conditions at 0, 4 and 8hrs of development (see Table 4-8). We found that most of the upregulated genes were common between HCD and LCD development and thus gave no clear explanation for the difference in development. However, we observed some interesting differences for the downregulated genes of which some were unique for LCD conditions. Most interesting among these were at 4 hrs of development, ACA, smlA and discoidin and at 8 hrs of development five genes enconding components of the 26S proteasome. In *Drosophila* the 26S proteasome machinery is found to play a major role in degrading cell cycle regulatory proteins to initiate early embryonic mitosis and development (Klein et al., 1990). This finding suggests that proteins that need to be degraded during wild type development might not be efficiently degraded under LCD conditions in the FrzA$^-$ mutant. It

is also interesting that the heat shock protein 70 was found to be upregulated under most conditions. Gao et al., (2000) showed that in human endothelial cell line ECV304 the heat shock proteins 70 (Hsp 70) class of protein is overexpressed with inhibition of the 26S proteasome.

We performed a cluster analysis to simplify the comparison of the microarray results under HCD and LCD conditions. K-means clustering resulted in five clusters for HCD and LCD development (Figure 34). Cluster 1, 3, 4 and 5 showed similar expression patterns for HCD and LCD development and contained mainly genes in common with both conditions. In contrast cluster 2 was markedly different between the two conditions. At LCD development the genes of this cluster were upregulated while they were downregulated at HCD development. In addition, only few genes were common between the two conditions. To gain further insight we compared the expression levels of the genes in cluster 2 at 8 hrs of development to the respective expression levels in wild type and ACA null cells (Iranfar et al., 2003; Van Driessche et al., 2002). It turned out that most of the genes of cluster 2 at HCD had similar expression levels as the ACA null cells (Figure 35A), consistent with the similar development phenotypes of the FrzA$^-$ and the ACA$^-$ mutant (Anjard et al., 2001). Unfortunately, the list of genes found in cluster 2 at LCD development was not very telling. It will be necessary to study all the genes that are differentially regulated and unique to LCD development in more detail. This might give more clues for the cell density dependent phenotype exhibited by the FrzA$^-$ mutant.

Summary

Frizzled genes encode integral membrane proteins that function in multiple signal transduction pathways. They have been identified in diverse animals, from sponges to humans. Frizzled genes are well characterized for their functions in development and diseases. In this study we report the identification and characterisation of a Frizzled like protein in *Dictyostelium*. We identified a novel Frizzled like protein (FrzA) in the *Dictyostelium* database, which has a seven transmembrane region at the N-terminus but unlike other Frizzleds it lacks the CRD domain and instead has a Phosphatidylinositol-4-phosphate 5-kinase (PIP5K) domain at the C-terminus.

The FrzA gene is present on the chromosome 4 of the *Dictyostelium* genome. The expression pattern of FrzA revealed a single transcript of ~2.5kb in size in the northern blot analysis. To characterize the function of FrzA protein *in vivo*, cells carrying an inactivated FrzA gene were generated by homologous recombination. The FrzA$^-$ cells grew normal on a bacterial lawn or in axenic medium indicating normal pinocytosis as well as intact cytokinesis. However the cell size was slightly smaller when compared to the Ax2 wild type cells. Notable was the defect in development on bacterial plates. When developed on phosphate agar plates the FrzA$^-$ cells did not form tight aggregates during early development. However the loose aggregates gave rise to very few and small fruiting bodies, which were formed with a delay of 24hrs. Also, the FrzA$^-$ mutant exhibited a pronounced developmental defect when developed at low cell density, where the mutant cells did not aggregate or develop fruiting bodies. Northern blot analysis to characterise the developmental defect in the FrzA$^-$ mutant showed expression of genes expressed early in aggregation such as csA; cAR1, ACA, PDE and PDI. However, the pattern was strongly altered and showed prolonged expression in case of CAR1 nad PDE and delayed expression for ACA and csA. The mutant cells did not exhibit a directed migration towards cAMP and thus showing a defect in chemotaxis. However, application of the exogenous cAMP pulses restored the expression of contact sites A and adenylyl cyclase (ACA) transcripts necessary for aggregation and also rescued the defect in chemotaxis. Calcium oscillation, which is important for initiating aggregation and further development, was disturbed in the mutant. We could show that the FrzA$^-$ mutant did not sense the cell density factor (CMF) and produce IP3 in response to the CMF stimulation. We therefore presume that FrzA is involved in the early steps of aggregation during *Dictyostelium* development.

Our results indicate that FrzA is a potential G protein coupled receptor for the ligand CMF to induce the CMF signaling during the early development of *Dictyostelium*.

Zusammenfassung

Frizzled Gene kodieren für integrale Membranproteine, die in verschiedenen Signaltransduktionswegen beteiligt sind. Sie sind in Entwicklungsprozesse involviert und sind auch als Krankheitsgene identifiziert worden. In dieser Arbeit wird ein Frizzled Gen, FrzA, aus *Dictyostelium* beschrieben. Das Vorkommen von Frizzled in niederen Eukaryonten war unerwartet. FrzA besitzt sieben für Frizzled Proteine charakteristische Transmembrandomänen. Der N-Terminus ist ungewöhnlich kurz und enthält nicht die übliche CRD-Domäne, eine Cysteinreiche Aminosäuresequenz. Ungewöhnlich ist auch der C-Terminus. Hier wird die Transmembrandomäne von einer C-terminalen Phosphatidylinositol-4-Phosphat-5-Kinase Domäne gefolgt. Die FrzA mRNA ist während des gesamten Entwicklungszyklus nachweisbar.

Um die Funktion von FrzA zu analysieren, wurde eine Mutante durch homologe Rekombination erzeugt. Die Wachstumseigenschaften der Mutante waren gegenüber dem Wildtyp nicht verändert. Verändert war dagegen die Entwicklung. Normalerweise aggregieren *Dictyostelium* Zellen unter Nahrungsentzug und formen einen vielzelligen Organismus, der sich in einen Fruchtkörper umbildet. In der *FrzA*$^-$ ist die Entwicklung stark beeinträchtigt. Sowohl auf einem Bakterienrasen als auch auch Phosphatagarplatten ist die Mutante grösstenteils nicht in der Lage, den Entwicklungszyklus normal zu durchlaufen und mit der Ausbildung von Fruchtkörpern abzuschliessen. Eine Genexpressionsanalyse hat gezeigt, dass das Muster der Expression von Genen, die normalerweise in der frühen Entwicklungsphase angeschaltet werden, stark verändert ist. So werden z. B. das Adenylatzyklase A Gen und das Contact site A Gen verspätet exprimiert, während die Transkriptmengen für den cAMP Rezeptor CAR1 und für die Phosphodiesterase durchgehend exprimiert sind. Entsprechend ist auch die chemotaktische Migration auf cAMP hin gestört. Ebenfalls veändert sind Calcium Oszillationen, die normalerweise während der Entwicklung beobachtet werden. Die Gabe von exogenem cAMP in Pulsen stellt die gestörte Entwicklung wieder her.

In einem Test für Ereignisse, die d

er Entwicklung vorausgehen, konnte gezeigt werden, dass *FrzA*$^-$ Zellen nicht in der Lage waren, das CMF-Signal aufzunehmen und darauf zu antworten. CMF ist ein Faktor, mit dem die Zellen die Zelldichte und damit den Nahrungsvorrat messen. Aufgrund der Eigenschaften der Mutante wurde FrzA als Rezeptor für CMF vorgeschlagen.

References

Adell, T., Nefkens, I., and Muller, W. E. (2003). Polarity factor 'Frizzled' in the demosponge Suberites domuncula: identification, expression and localization of the receptor in the epithelium/pinacoderm(1). FEBS Lett *554*, 363-368.

Adler, P. N. (2002). Planar signaling and morphogenesis in *Drosophila*. Dev Cell *2*, 525-535.

Ahumada, A., Slusarski, D. C., Liu, X., Moon, R. T., Malbon, C. C., and Wang, H. Y. (2002). Signaling of rat Frizzled-2 through phosphodiesterase and cyclic GMP. Science *298*, 2006-2010.

Alcantara, F., and Monk, M. (1974). Signal propagation during aggregation in the slime mould *Dictyostelium discoideum*. J Gen Microbiol *85*, 321-334.

Anjard, C., Soderbom, F., and Loomis, W. F. (2001). Requirements for the adenylyl cyclases in the development of *Dictyostelium*. Development *128*, 3649-3654.

Baksh, S., Burns, K., Andrin, C., and Michalak, M. (1995). Interaction of calreticulin with protein disulfide isomerase. J Biol Chem *270*, 31338-31344.

Bhanot, P., Brink, M., Samos, C. H., Hsieh, J. C., Wang, Y., Macke, J. P., Andrew, D., Nathans, J., and Nusse, R. (1996). A new member of the Frizzled family from *Drosophila* functions as a Wingless receptor. Nature *382*, 225-230.

Bockaert, J., and Pin, J. P. (1999). Molecular tinkering of G protein-coupled receptors: an evolutionary success. Embo J *18*, 1723-1729.

Bonner, J. T. (1947). Evidence for the formation of aggregates by chemotaxis in the development of the slime mold *Dictyostelium discoideum*. J Exp Zool *106*, 1-26.

Bonner, J. T. (1967). The cellular slime molds. Second edition. (Princeton, NJ, Princeton Univ. Press).

Brandon, M. A., and Podgorski, G. J. (1997). Galpha3 regulates the cAMP signaling system in *Dictyostelium*. Mol Biol Cell *8*, 1677-1685.

Brazill, D. T., Lindsey, D. F., Bishop, J. D., and Gomer, R. H. (1998). Cell density sensing mediated by a G protein-coupled receptor activating phospholipase C. J Biol Chem *273*, 8161-8168.

Brown, J. M., and Firtel, R. A. (1999). Regulation of cell-fate determination in *Dictyostelium*. Dev Biol *216*, 426-441.

Bumann, J., Malchow, D., and Wurster, B. (1986). Oscillations of Ca^{2+} concentration during the cell differentiation of *Dictyostelium discoideum*. Differentiation *31*, 85-91.

Bumann, J., Wurster, B., and Malchow, D. (1984). Attractant-induced changes and oscillations of the extracellular Ca^{2+} concentration in suspensions of differentiating *Dictyostelium* cells. J Cell Biol *98*, 173-178.

Cao, K., and Zheng, Y. (2004). The Cdc48/p97-Ufd1-Npl4 complex: its potential role in coordinating cellular morphogenesis during the M-G1 transition. Cell Cycle *3*, 422-424.

Carvajal, J. J., Pook, M. A., Doudney, K., Hillermann, R., Wilkes, D., al-Mahdawi, S., Williamson, R., and Chamberlain, S. (1995). Friedreich's ataxia: a defect in signal transduction? Hum Mol Genet *4*, 1411-1419.

Caterina, M. J., Hereld, D., and Devreotes, P. N. (1995). Occupancy of the *Dictyostelium* cAMP receptor, cAR1, induces a reduction in affinity which depends upon COOH- terminal serine residues. J Biol Chem *270*, 4418-4423.

Chen, C. M., and Struhl, G. (1999). Wingless transduction by the Frizzled and Frizzled2 proteins of *Drosophila*. Development *126*, 5441-5452.

Chen, W., ten Berge, D., Brown, J., Ahn, S., Hu, L. A., Miller, W. E., Caron, M. G., Barak, L. S., Nusse, R., and Lefkowitz, R. J. (2003). Dishevelled 2 recruits beta-arrestin 2 to mediate Wnt5A-stimulated endocytosis of Frizzled 4. Science *301*, 1391-1394.

Chung, C. Y., and Firtel, R. A. (1999). PAKa, a putative PAK family member, is required for cytokinesis and the regulation of the cytoskeleton in *Dictyostelium discoideum* cells during chemotaxis. J Cell Biol *147*, 559-575.

Chung, C. Y., Funamoto, S., and Firtel, R. A. (2001). Signaling pathways controlling cell polarity and chemotaxis. TIBS *26*, 557-566.

Claviez, M., Pagh, K., Maruta, H., Baltes, W., Fisher, P., and Gerisch, G. (1982). Electron microscopic mapping of monoclonal antibodies on the tail region of *Dictyostelium* myosin. EMBO J *1*, 1017-1022.

Clay, J. L., Ammann, R. R., and Gomer, R. H. (1995). Initial cell-type choice in a simple eukaryote: Cell- autonomous or morphogen-gradient dependent? Dev Biol *172*, 665-674.

Coates, J. C., and Harwood, A. J. (2001). Cell-cell adhesion and signal transduction during *Dictyostelium* development. J Cell Sci *114*, 4349-4358.

Crowley, T. E., Nellen, W., Gomer, R. H., and Firtel, R. A. (1985). Phenocopy of discoidin I-minus mutants by antisense transformation in *Dictyostelium*. Cell *43*, 633-641.

Deardorff, M. A., Tan, C., Saint-Jeannet, J. P., and Klein, P. S. (2001). A role for Frizzled 3 in neural crest development. Development *128*, 3655-3663.

Deery, W. J., and Gomer, R. H. (1999). A putative receptor mediating cell-density sensing in *Dictyostelium*. J Biol Chem *274*, 34476-34482.

Desbarats, L., Lam, T. Y., Wong, L. M., and Siu, C. H. (1992). Identification of a unique cAMP-response element in the gene encoding the cell adhesion molecule gp80 in *Dictyostelium discoideum*. J Biol Chem *267*, 19655-19664.

Devreotes, P. N. (1982). Chemotaxis. In The development of *Dictyostelium discoideum*, W. F. Loomis, ed. (New York, Ac. Press), pp. 117-168.

Devreotes, P. N. (1994). G protein-linked signaling pathways control the developmental program of *Dictyostelium*. Neuron *12*, 235-241.

Dohlman, H. G., Thorner, J., Caron, M. G., and Lefkowitz, R. J. (1991). Model systems for the study of seven-transmembrane-segment receptors. Annu Rev Biochem *60*, 653-688.

Durston, A. J., and Vork, F. (1979). A cinamatographical study of the development of vitally stained *Dictyostelium discoideum*. J Cell Sci *36*, 261-279.

Faix, J., Gerisch, G., and Noegel, A. A. (1992). Overexpression of the csA cell adhesion molecule under its own cAMP-regulated promoter impairs morphogenesis in *Dictyostelium*. J Cell Sci *102*, 203-214.

Firtel, R. A. (1991). Signal transduction pathways controlling multicellular development in *Dictyostelium*. Trends Genet (TIG) *7*, 381-388.

Firtel, R. A. (1995). Integration of signaling information in controlling cell- fate decisions in *Dictyostelium*. Genes Devel *9*, 1427-1444.

Firtel, R. A., and Chung, C. Y. (2000). The molecular genetics of chemotaxis: sensing and responding to chemoattractant gradients. BioEssays *22*, 603-615.

Fontana, D. R., Price, P. L., and Phillips, J. C. (1991). Cell-cell contact mediates cAMP secretion in *Dictyostelium discoideum*. Dev Genet *12*, 54-62.

Franke, J., Faure, M., Wu, L., Hall, A. L., Podgorski, G. J., and Kessin, R. H. (1991). Cyclic nucleotide phosphodiesterase of *Dictyostelium discoideum* and its glycoprotein inhibitor: structure and expression of their genes. Dev Genet *12*, 104-112.

Franke, J., and Kessin, R. H. (1992). The cyclic nucleotide phosphodiesterases of *Dictyostelium discoideum*: molecular genetics and biochemistry. Cell Signal *4*, 471-478.

Gao, Y., Lecker, S., Post, M. J., Hietaranta, A. J., Li, J., Volk, R., Li, M., Sato, K., Saluja, A. K., Steer, M. L., *et al.* (2000). Inhibition of ubiquitin-proteasome pathway-mediated I kappa B alpha degradation by a naturally occurring antibacterial peptide. J Clin Invest *106*, 439-448.

Gerisch, G. (1968). Cell aggregation and differentiation in *Dictyostelium*. In Current Topics in Developmental Biology., A. A. Moscona, and A. Monroy, eds. (New York, Ac. Press), pp. 157-197.

Gerisch, G. (1987). Cyclic AMP and other signals controlling cell development and differentiation in *Dictyostelium*. Annu Rev Biochem *56*, 853-879.

Gerisch, G., Fromm, H., Huesgen, A., and Wick, U. (1975). Control of cell-contact sites by cyclic AMP pulses in differentiating *Dictyostelium* cells. Nature *255*, 547-549.

Gerisch, G., and Hess, B. (1974). Cyclic-AMP-controlled oscillations in suspended *Dictyostelium* cells: Their relation to morphogenetic cell interactions. Proc Natl Acad Sci USA *71*, 2118-2122.

Gerisch, G., and Malchow, D. (1976). Cyclic AMP receptors and the control of cell aggregation in *Dictyostelium*. Adv Cycl Nucl Res *7*, 49-68.

Goldberg, J. M., Bosgraaf, L., Van Haastert, P. J., and Smith, J. L. (2002). Identification of four candidate cGMP targets in *Dictyostelium*. Proc Natl Acad Sci U S A *99*, 6749-6754.

Gomer, R. H., Yuen, I. S., and Firtel, R. A. (1991). A secreted 80x10(3)Mr protein mediates sensing of cell density and the onset of development in *Dictyostelium*. Development *112*, 269-278.

Gubb, D., and Garcia-Bellido, A. (1982). A genetic analysis of the determination of cuticular polarity during development in *Drosophila* melanogaster. J Embryol Exp Morphol *68*, 37-57.

Guo, K., Nichol, R., Skehel, P., Dormann, D., Weijer, C. J., Williams, J. G., and Pears, C. (2001). A *Dictyostelium* nuclear phosphatidylinositol phosphate kinase required for developmental gene expression. EMBO J *20*, 6017-6027.

Hacker, U., Albrecht, R., and Maniak, M. (1997). Fluid-phase uptake by macropinocytosis in *Dictyostelium*. J Cell Sci *110*, 105-112.

Harloff, C., Gerisch, G., and Noegel, A. A. (1989). Selective elimination of the contact sites A protein of *Dictyostelium discoideum* by gene disruption. Genes Devel *3*, 2011-2019.

Harris, J., Honigberg, L., Robinson, N., and Kenyon, C. (1996). Neuronal cell migration in C. elegans: regulation of Hox gene expression and cell position. Development *122*, 3117-3131.

Hsieh, J. C., Rattner, A., Smallwood, P. M., and Nathans, J. (1999). Biochemical characterization of Wnt-Frizzled interactions using a soluble, biologically active vertebrate Wnt protein. Proc Natl Acad Sci U S A *96*, 3546-3551.

Huang, H. C., and Klein, P. S. (2004). The Frizzled family: receptors for multiple signal transduction pathways. Genome Biol *5*, 234.

Iranfar, N., Fuller, D., and Loomis, W. F. (2003). Genome-wide expression analyses of gene regulation during early development of *Dictyostelium discoideum*. Eukaryot Cell *2*, 664-670.

Ishikawa, T., Tamai, Y., Zorn, A. M., Yoshida, H., Seldin, M. F., Nishikawa, S., and Taketo, M. M. (2001). Mouse Wnt receptor gene Fzd5 is essential for yolk sac and placental angiogenesis. Development *128*, 25-33.

Jain, R., Yuen, I. S., Taphouse, C. R., and Gomer, R. H. (1992). A density-sensing factor controls development in *Dictyostelium*. Genes Devel *6*, 390-400.

Kay, R. R., and Trevan, D. J. (1981). *Dictyostelium* amoebae can differentiate into spores without cell-to-cell contact. J Embryol Exp Morph *62*, 369-378.

Kennerdell, J. R., and Carthew, R. W. (1998). Use of dsRNA-mediated genetic interference to demonstrate that Frizzled and Frizzled 2 act in the wingless pathway. Cell *95*, 1017-1026.

Kessin, R. H. (1988). Genetics of early *Dictyostelium discoideum* development. Microbiol Rev *52*, 29-49.

Kessin, R. H. (2001). *Dictyostelium* - Evolution, cell biology, and the development of multicellularity. (Cambridge, UK, Cambridge Univ. Press).

Kim, L., Harwood, A., and Kimmel, A. R. (2002). Receptor-dependent and tyrosine phosphatase-mediated inhibition of GSK3 regulates cell fate choice. Dev Cell *3*, 523-532.

Kimmel, A. R. (1987). Different molecular mechanisms for cAMP regulation of gene expression during *Dictyostelium* development. Dev Biol *122*, 163-171.

Kimmel, A. R., and Firtel, R. A. (1991). cAMP signal transduction pathways regulating development of *Dictyostelium discoideum*. Curr Opin Genet Devel *1*, 383-390.

Klein, P., Theibert, A., Fontana, D., and Devreotes, P. N. (1985). Identification and cyclic AMP-induced modification of the cyclic AMP receptor in *Dictyostelium discoideum*. J Biol Chem *260*, 1757-1764.

Klein, P. S. (1988) The cyclic-AMP receptor of *Dictyostelium*: a eukaryotic chemoattractant receptor., PhD, The Johns Hopkins University, Baltimore, MD.

Klein, P. S., Sun, T. J., Saxe III, C. L., Kimmel, A. R., Johnson, R. L., and Devreotes, P. N. (1988). A chemoattractant receptor controls development in *Dictyostelium discoideum*. Science *241*, 1467-1472.

Klein, U., Gernold, M., and Kloetzel, P. M. (1990). Cell-specific accumulation of *Drosophila* proteasomes (MCP) during early development. J Cell Biol *111*, 2275-2282.

Konijn, T. M., van de Meene, J. G. C., Bonner, J. T., and Barkley, D. S. (1967). The acrasin activity of adenosine-3',5'-cyclic phosphate. Proc Natl Acad Sci USA *58*, 1152-1154.

Kumagai, A., Hadwiger, J. A., Pupillo, M., and Firtel, R. A. (1991). Molecular genetic analysis of two Galpha protein subunits in *Dictyostelium*. J Biol Chem *266*, 1220-1228.

LaMantia, M., Miura, T., Tachikawa, H., Kaplan, H. A., Lennarz, W. J., and Mizunaga, T. (1991). Glycosylation site binding protein and protein disulfide isomerase are identical and essential for cell viability in yeast. Proc Natl Acad Sci U S A *88*, 4453-4457.

Landfear, S. M., and Lodish, H. F. (1980). A role for cAMP in expression of developmentally regulated genes in *Dictyostelium discoideum*. Proc Natl Acad Sci USA *77*, 1044-1048.

Lee, S., Parent, C. A., Insall, R., and Firtel, R. A. (1999). A novel Ras-interacting protein required for chemotaxis and cyclic adenosine monophosphate signal relay in *Dictyostelium*. Mol Biol Cell *10*, 2829-2845.

Lehrach, H., Diamond, D., Wozney, J. M., and Boedtker, H. (1977). RNA molecular weight determinations by gel electrophoresis under denaturing conditions, a critical reexamination. Biochemistry *16*, 4743-4751.

Lindsey, D. F., Amerik, A., Deery, W. J., Bishop, J. D., Hochstrasser, M., and Gomer, R. H. (1998). A deubiquitinating enzyme that disassembles free polyubiquitin chains is required for development but not growth in *Dictyostelium*. J Biol Chem *273*, 29178-29187.

Logan, C. Y., and Nusse, R. (2004). The Wnt Signaling Pathway in Development and Disease. Annu Rev Cell Dev Biol.

Maeda, M., Lu, S., Shaulsky, G., Miyazaki, Y., Kuwayama, H., Tanaka, Y., Kuspa, A., and Loomis, W. F. (2004). Periodic signaling controlled by an oscillatory circuit that includes protein kinases ERK2 and PKA. Science *304*, 875-878.

Malchow, D., Lusche, D. F., and Schlatterer, C. (2004). A link of Ca^{2+} to cAMP oscillations in *Dictyostelium*: the calmodulin antagonist W-7 potentiates cAMP relay and transiently inhibits the acidic Ca^{2+}-store. BMC Dev Biol *4*, 7.

Malchow, D., Mutzel, R., and Schlatterer, C. (1996a). On the role of calcium during chemotactic signalling and differentiation of the cellular slime mould *Dictyostelium discoideum*. Int J Dev Biol *40*, 135-139.

Malchow, D., Nägele, B., Schwartz, H., and Gerisch, G. (1972). Membrane-bound cyclic AMP phosphodiesterase in chemotactically responding cells of *Dictyostelium discoideum*. Eur J Biochem *28*, 136-142.

Malchow, D., Schaloske, R., and Schlatterer, C. (1996b). An increase in cytosolic Ca^{2+} delays cAMP oscillations in *Dictyostelium* cells. Biochem J *319*, 323-327.

Maniak, M. (2001). Fluid-phase uptake and transit in axenic *Dictyostelium* cells. Biochim Biophys Acta *1525*, 197-204.

Mann, S. K., and Firtel, R. A. (1989). Two-phase regulatory pathway controls cAMP receptor-mediated expression of early genes in *Dictyostelium*. Proc Natl Acad Sci USA *86*, 1924-1928.

Mann, S. K., Pinko, C., and Firtel, R. A. (1988). Regulation of *Dictyostelium* early gene expression in cAMP bypass mutants. Dev Biol *130*, 406-410.

Mann, S. K. O., Devreotes, P. N., Eliott, S., Jermyn, K., Kuspa, A., Fechheimer, M., Furukawa, R., Parent, C. A., Segall, J., Shaulsky, G., *et al.* (1994). Cell biological, molecular genetic, and biochemical methods to examine *Dictyostelium*. In Cell Biology - A Laboratory Handbook., J. E. Celis, ed. (San Diego, CA, Ac. Press), pp. 412-451.

McPherson, C. E., and Singleton, C. K. (1992). V4, a gene required for the transition from growth to development in *Dictyostelium discoideum*. Dev Biol *150*, 231-242.

Mehdy, M. C., and Firtel, R. A. (1985). A secreted factor and cyclic AMP jointly regulate cell-type-specific gene expression in *Dictyostelium discoideum*. Mol Cell Biol *5*, 705-713.

Mehdy, M. C., Ratner, D., and Firtel, R. A. (1983). Induction and modulation of cell-type-specific gene expression in *Dictyostelium*. Cell *32*, 763-771.

Meima, M. E., Weening, K. E., and Schaap, P. (2003). Characterization of a cAMP-stimulated cAMP phosphodiesterase in *Dictyostelium discoideum*. J Biol Chem *278*, 14356-14362.

Michalak, M., Guo, L., Robertson, M., Lozak, M., and Opas, M. (2004). Calreticulin in the heart. Mol Cell Biochem *263*, 137-142.

Milne, J. L., Wu, L., Caterina, M. J., and Devreotes, P. N. (1995). Seven helix cAMP receptors stimulate Ca^{2+} entry in the absence of functional G proteins in *Dictyostelium*. J Biol Chem *270*, 5926-5931.

Minobe, S., Fei, K., Yan, L., Sarras Jr, M., and Werle, M. (2000). Identification and characterization of the epithelial polarity receptor "Frizzled" in Hydra vulgaris. Dev Genes Evol *210*, 258-262.

Morita, T., Saitoh, K., Takagi, T., and Maeda, Y. (2000). Involvement of the glucose-regulated protein 94 (Dd-GRP94) in starvation response of *Dictyostelium discoideum* cells. Biochem Biophys Res Commun *274*, 323-331.

Muller, H., Samanta, R., and Wieschaus, E. (1999). Wingless signaling in the *Drosophila* embryo: zygotic requirements and the role of the Frizzled genes. Development *126*, 577-586.

Nakagawa, M., Oohata, A. A., Tojo, H., and Fujii, S. (1999). A prespore-cell-inducing factor in *Dictyostelium discoideum*: its purification and characterization. Biochem J *343*, 265-271.

Newell, P. C., Malchow, D., and Gross, J. D. (1995). The role of calcium in aggregation and development of *Dictyostelium*. Experientia *51*, 1155-1165.

Noegel, A., Gerisch, G., Stadler, J., and Westphal, M. (1986a). Complete sequence and transcript regulation of a cell adhesion protein from aggregating *Dictyostelium* cells. EMBO J *5*, 1473-1476.

Noegel, A., Witke, W., and Schleicher, M. (1986b). cDNA clones coding for alpha-actinin of *Dictyostelium discoideum*. FEBS Lett *204*, 107-109.

Noegel, A. A., Blau-Wasser, R., Sultana, H., Muller, R., Israel, L., Schleicher, M., Patel, H., and Weijer, C. J. (2004). The cyclase-associated protein CAP as regulator of cell polarity and cAMP signaling in *Dictyostelium*. Mol Biol Cell *15*, 934-945.

Noegel, A. A., Rivero, F., Albrecht, R., Janssen, K. P., Kohler, J., Parent, C. A., and Schleicher, M. (1999). Assessing the role of the ASP56/CAP homologue of *Dictyostelium discoideum* and the requirements for subcellular localization. J Cell Sci *112*, 3195-3203.

Nusse, R., and Varmus, H. E. (1992). Wnt genes. Cell *69*, 1073-1087.

Pan, P., Hall, E. M., and Bonner, J. T. (1972). Folic acid as second chemotactic substance in the cellular slime moulds. Nature New Biol *237*, 181-182.

Pinter, K., and Gross, J. (1995). Calcium and cell-type-specific gene expression in *Dictyostelium*. Differentiation *59*, 201-206.

Pitt, G. S., Milona, N., Borleis, J., Lin, K. C., Reed, R. R., and Devreotes, P. N. (1992). Structurally distinct and stage-specific adenylyl cyclase genes play different roles in *Dictyostelium* development. Cell *69*, 305-315.

Plakidou-Dymock, S., Dymock, D., and Hooley, R. (1998). A higher plant seven-transmembrane receptor that influences sensitivity to cytokinins. Curr Biol *8*, 315-324.

Prassler, J., Murr, A., Stocker, S., Faix, J., Murphy, J., and Marriott, G. (1998). DdLIM is a cytoskeleton-associated protein involved in the protrusion of lamellipodia in *Dictyostelium*. Mol Biol Cell *9*, 545-559.

Rathi, A., and Clarke, M. (1992). Expression of early developmental genes in *Dictyostelium discoideum* is initiated during exponential growth by an autocrine-dependent mechanism. Mech Devel *36*, 173-182.

Rathi, A., Kayman, S. C., and Clarke, M. (1991). Induction of gene expression in *Dictyostelium* by prestarvation factor, a factor secreted by growing cells. Dev Genet *12*, 82-87.

Riley, B. B., and Barclay, S. L. (1990). Conditions that alter intracellular cAMP levels affect expression of the cAMP phosphodiesterase gene in *Dictyostelium*. Proc Natl Acad Sci USA *87*, 4746-4750.

Robitaille, J., MacDonald, M. L., Kaykas, A., Sheldahl, L. C., Zeisler, J., Dube, M. P., Zhang, L. H., Singaraja, R. R., Guernsey, D. L., Zheng, B., *et al.* (2002). Mutant Frizzled-4 disrupts retinal angiogenesis in familial exudative vitreoretinopathy. Nat Genet *32*, 326-330.

Rocheleau, C. E., Downs, W. D., Lin, R., Wittmann, C., Bei, Y., Cha, Y. H., Ali, M., Priess, J. R., and Mello, C. C. (1997). Wnt signaling and an APC-related gene specify endoderm in early C. elegans embryos. Cell *90*, 707-716.

Rosenzweig, B. A., Pine, P. S., Domon, O. E., Morris, S. M., Chen, J. J., and Sistare, F. D. (2004). Dye bias correction in dual-labeled cDNA microarray gene expression measurements. Environ Health Perspect *112*, 480-487.

Sambrook, J., Fritsch, E.F. and Maniatis,T. (1989). Molecular cloning: a laboratory manual, Cold Spring Harbour laboratory, Cold Spring Harbour, N.Y.

Sato, A., Kojima, T., Ui-Tei, K., Miyata, Y., and Saigo, K. (1999). DFrizzled-3, a new *Drosophila* Wnt receptor, acting as an attenuator of Wingless signaling in wingless hypomorphic mutants. Development *126*, 4421-4430.

Sawa, H., Lobel, L., and Horvitz, H. R. (1996). The Caenorhabditis elegans gene lin-17, which is required for certain asymmetric cell divisions, encodes a putative seven-transmembrane protein similar to the *Drosophila* Frizzled protein. Genes Dev *10*, 2189-2197.

Saxe, C. L., 3rd, Johnson, R. L., Devreotes, P. N., and Kimmel, A. R. (1991). Expression of a cAMP receptor gene of *Dictyostelium* and evidence for a multigene family. Genes Dev *5*, 1-8.

Schaap, P., and van Driel, R. (1985). Induction of post-aggregative differentiation in *Dictyostelium discoideum* by cAMP. Evidence for the involvement of the cell surface cAMP receptor. Exp Cell Res *159*, 388-398.

Shaulsky, G., Fuller, D., and Loomis, W. F. (1998). A cAMP-phosphodiesterase controls PKA-dependent differentiation. Development *125*, 691-699.

Siu, C.H., Cho, A., and Choi, H. C. (1987). The contact site a glycoprotein mediates cell-cell adhesion by homophilic binding in *Dictyostelium discoideum*. J Cell Biol *105*, 2523-2533.

Siu, C.H., Cho, A. S., and Choi, A. (1986). Mechanism of action of the contact site A glycoprotein during development of *Dictyostelium discoideum*. Paper presented at: J. Cell Biol.

Siu, C.H., Lam, T. Y., and Choi, A. H. C. (1985). Inhibition of cell-cell binding at the aggregation stage of *Dictyostelium discoideum* development by monoclonal antibodies directed against an 80,000-Dalton surface glycoprotein. J Biol Chem *260*, 16030-16036.

Siu, C.H., Lam, T. Y., and Wong, L. M. (1988). Expression of the contact site A glycoprotein in *Dictyostelium discoideum*: quantitation and developmental regulation. Biochim Biophys Acta *968*, 283-290.

Slusarski, D. C., Corces, V. G., and Moon, R. T. (1997a). Interaction of Wnt and a Frizzled homologue triggers G-protein-linked phosphatidylinositol signalling. Nature *390*, 410-413.

Slusarski, D. C., Yang-Snyder, J., Busa, W. B., and Moon, R. T. (1997b). Modulation of embryonic intracellular Ca^{2+} signaling by Wnt-5A. Dev Biol *182*, 114-120.

Soll, D. R., Wessels, D., Heid, P. J., and Zhang, H. (2002). A contextual framework for characterizing motility and chemotaxis mutants in *Dictyostelium discoideum*. J Muscle Res Cell Motil *23*, 659-672.

Soll, D. R., Wessels, D., Voss, E., and Johnson, O. (2001). Computer-assisted systems for the analysis of amoeboid cell motility. Methods Mol Biol *161*, 45-58.

Strutt, D. (2003). Frizzled signalling and cell polarisation in *Drosophila* and vertebrates. Development *130*, 4501-4513.

Sumanas, S., Strege, P., Heasman, J., and Ekker, S. C. (2000). The putative wnt receptor *Xenopus* Frizzled-7 functions upstream of beta-catenin in vertebrate dorsoventral mesoderm patterning. Development *127*, 1981-1990.

Sun, B., and Firtel, R. A. (2003). A regulator of G protein signaling-containing kinase is important for chemotaxis and multicellular development in *Dictyostelium*. Mol Biol Cell *14*, 1727-1743.

Sun, T. J., and Devreotes, P. N. (1991). Gene targeting of the aggregation stage cAMP receptor cAR1 in *Dictyostelium*. Genes Devel *5*, 572-582.

Sussman, M. (1951). The origin of cellular heterogeneity in the slime molds, Dictyosteliaceae. J Exp Zool *118*, 407-418.

Thiery, R., Robbins, S., Khosla, M., Spiegelman, G. B., and Weeks, G. (1992). The effects of expression of an activated rasG mutation on the differentiation of *Dictyostelium*. Biochem Cell Biol *70*, 1193-1199.

Traynor, D., Milne, J. L. S., Insall, R. H., and Kay, R. R. (2000). Ca^{2+} signalling is not required for chemotaxis in *Dictyostelium*. EMBO J *19*, 4846-4854.

Van Driessche, N., Shaw, C., Katoh, M., Morio, T., Sucgang, R., Ibarra, M., Kuwayama, H., Saito, T., Urushihara, H., Maeda, M., *et al.* (2002). A transcriptional profile of multicellular development in *Dictyostelium discoideum*. Development *129*, 1543-1552.

Van Haastert, P. J., Bishop, J. D., and Gomer, R. H. (1996). The cell density factor CMF regulates the chemoattractant receptor cAR1 in *Dictyostelium*. J Cell Biol *134*, 1543-1549.

van Haastert, P. J. M., and Kien, E. (1983). Binding of cAMP derivatives to *Dictyostelium discoideum* cells. Activation mechanism of the cell surface cAMP receptor. J Biol Chem *258*, 9636-9642.

van Oss, C., Panfilov, A. V., Hogeweg, P., Siegert, F., and Weijer, C. J. (1996). Spatial pattern formation during aggregation of the slime mould *Dictyostelium discoideum*. J Theor Biol *181*, 203-213.

Vauti, F., Morandini, P., Blusch, J., Sachse, A., and Nellen, W. (1990). Regulation of the Discoidin I gamma gene in *Dictyostelium discoideum*: identification of individual promoter elements mediating induction of transcription and repression by cyclic AMP. Mol Cell Biol *10*, 4080-4088.

Wallingford, J. B., Rowning, B. A., Vogeli, K. M., Rothbacher, U., Fraser, S. E., and Harland, R. M. (2000). Dishevelled controls cell polarity during *Xenopus* gastrulation. Nature *405*, 81-85.

Wang, H. Y., and Malbon, C. C. (2003). Wnt signaling, Ca^{2+}, and cyclic GMP: visualizing Frizzled functions. Science *300*, 1529-1530.

Wang, Y., Huso, D., Cahill, H., Ryugo, D., and Nathans, J. (2001). Progressive cerebellar, auditory, and esophageal dysfunction caused by targeted disruption of the Frizzled-4 gene. J Neurosci *21*, 4761-4771.

Wang, Y., Macke, J. P., Abella, B. S., Andreasson, K., Worley, P., Gilbert, D. J., Copeland, N. G., Jenkins, N. A., and Nathans, J. (1996). A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene Frizzled. J Biol Chem *271*, 4468-4476.

Wang, Y., Thekdi, N., Smallwood, P. M., Macke, J. P., and Nathans, J. (2002). Frizzled-3 is required for the development of major fiber tracts in the rostral CNS. J Neurosci *22*, 8563-8573.

Williams, H. P., and Harwood, A. J. (2003). Cell polarity and *Dictyostelium* development. Curr Opin Microbiol *6*, 621-627.

Winklbauer, R., Medina, A., Swain, R. K., and Steinbeisser, H. (2001). Frizzled-7 signalling controls tissue separation during *Xenopus* gastrulation. Nature *413*, 856-860.

Witke, W., Schleicher, M., Lottspeich, F., and Noegel, A. (1986). Studies on the transcription, translation, and structure of alpha-actinin in *Dictyostelium discoideum*. J Cell Biol *103*, 969-975.

Wong, L. L., and Adler, P. N. (1993). Tissue polarity genes of *Drosophila* regulate the subcellular location for prehair initiation in pupal wing cells. J Cell Biol *123*, 209-221.

Wu, L., Valkema, R., Van Haastert, P. J., and Devreotes, P. N. (1995). The G protein beta subunit is essential for multiple responses to chemoattractants in *Dictyostelium*. J Cell Biol *129*, 1667-1675.

Yang-Snyder, J., Miller, J. R., Brown, J. D., Lai, C. J., and Moon, R. T. (1996). A Frizzled homolog functions in a vertebrate Wnt signaling pathway. Curr Biol *6*, 1302-1306.

Yuen, I. S., Jain, R., Bishop, J. D., Lindsey, D. F., Deery, W. J., Van Haastert, P. J., and Gomer, R. H. (1995). A density-sensing factor regulates signal transduction in *Dictyostelium*. J Cell Biol *129*, 1251-1262.

Erklärung

Ich versichere, dass ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit - einschließlich Tabellen und Abbildungen -, die anderen Werke im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; dass diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; dass sie noch nicht veröffentlicht ist, sowie, dass ich eine Veröffentlichung vor Abschluss des Promotionsverfahrens nicht vornehmen werde. Die Bestimmungen dieser Promotionsordnung sind mir bekannt. Die von mir vorgelegte Dissertation ist von Frau Prof. Dr. Angelika A. Noegel betreut worden.

Köln, den
2-12-2005
Deenadayalan Bakthavatsalam

CURRICULUM VITAE

Name:	DEENADAYALAN .B
Address:	15, Krieler Str Koeln, 50935
Permanent address:	108, H street Sri Devi Vaishnavi Nagar Tamil Nadu, Chennai 600108 India. Email: bd1306@yahoo.com
Date of birth:	13/06/1973
Place of birth:	Chennai, India
Nationality:	Indian

ACADEMIC QUALIFICATIONS

High School: (1987-1988) (1989-1990)	 Vijayanta Higher Secondary School, Tamil Nadu, India. CTTE School, Tamil Nadu, India
Bachelors Degree: (1990-1993)	B.Sc. (Zoology, Botany, Chemistry) Loyola College, Madras University Tamil Nadu, India.
Masters Degree: (1993-1995)	M.Sc. (Zoology) Loyola College, Madras University Tamil Nadu, India.
Post graduation Studies: (11.2001-02.2005)	Mathematics and Natural Science Faculty University of Cologne, Germany. Doctoral work was done under the supervision of Prof. Dr. Angelika A. Noegel in the Institute for Biochemistry I, Medical Faculty, Cologne University, Germany

Ger

LEBENSLAUF

Name:	DEENADAYALAN. B
Adresse:	15, Krieler Str Koeln, 50935 Germany
Heimat adresse:	108, H street Sri Devi Vaishnavi Nagar Tamil Nadu, Chennai 600108 India. Email: bd1306@yahoo.com
Geburtsdatum:	13/06/1973
Geburtsort:	Chennai, India
Staatsangehörigkeit:	Indian

AKADEMISCHE QUALIFIKATION

Schulausbildung:	
(1987-1988)	Vijayanta Higher Secondary School, Tamil Nadu, India.
(1989-1990)	CTTE School, Tamil Nadu, India
Studium:	
Bachelors Degree: (1990-1993)	B.Sc. (Zoology, Botany, Chemistry) Loyola College, Madras University Tamil Nadu, India.
Masters Degree: (1993-1995)	M.Sc. (Zoology) Loyola College, Madras University Tamil Nadu, India.
Promotions studium: (11.2001-02.2005)	Mathematisch-Naturwissenschaftliche Fakultat Universität zu Köln Betreuerin: Prof. Dr. Angelika A. Noegel Institut für Biochemie I, Medizinische Fakultät der Universität zu Köln.