Molecular studies on a *Dictyostelium* homolog of the *tafazzin* gene, the cause of Barth Syndrome in humans

Inaugural-Dissertation zur Erlangung
des Doktorgrades der Naturwissenschaften (Dr. rer. nat.)

Vorgelegt beim Fachbereich Biologie/Chemie
der Universität Kassel

von

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November 2002

vom Fachbereich Biologie/Chemie der Universität Kassel als Dissertation angenommen.

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Tag der mündlichen Prüfung: 29. Januar 2003

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Abbreviations

aa	amino acid
Ab	antibody
ABP	Actin-binding protein
Amp	Ampicillin
APS	ammonium persulphate
AT(s)	Acyltransferase(s)
ATP	adenosine 5 ‘- triphosphate
A260(280)	absorbance at 260 nm (280nm)
b	base
BCIP	5-bromo-4-chloro-3-indolylphosphate
bp	base pair
BSA	bovine serum albumin
Bs^r	blasticidin resistance cassett
CAR	cAMP receptor
cDNA	complementary DNA
CHO	Chinese Hamster Ovary cells
Ci	Curie
CL	Cardiolipin (1,3-bis (1’, 2’-diacyl-3’-phosphoryl-sn-glycerol)-sn-glycerol)
DABCO	1, 4 – diazabicyclo 2. 2. 2. octane
DEPC	diethylpyrocarbonate
DLMC	dilysocardiolipin
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
ddNTP	Didesoxyribonucleotide: ddATP, ddCTP, ddGTP, ddTTP
dNTP	deoxyribonucleotide : dATP, dCTP, dGTP, dTTP
DTT	1.4-dithiotreitol

EDTA	ethylendiamintetraacetic acid
ESTs	Expressed sequence tags
g	gram, gravitation constant (relative centrifugal force)
G418	geneticin
GDT	growth-differentiation transition
GFP	green fluorescence protein
Gro	glycerol
GroP	Sn-glycero-3-phosphate
GTC	guanidine thiocyanate
hr	hour
HEPES	N-2-hydroxyethylpiperazine-N'-2-ethasulfonic acid
IPTG	isopropyl-β-D-thiogalactopyranoside
KA	*Klebsiella aerogenes*
kDa	kilo Dalton
μ	micro (10^{-6})
m	Milli (10^{-3})
mAb	Monoclonal antibody
Mb	Mega (10^{6}) base pairs
MCS	Multiple cloning site, polylinker
B-ME	beta-mercaptoethanol
min	minute
MLCL	monolysocardiolipin
MLCL AT	Monolysocardiolipin acyltransferase
MOPS	γ-(morpholino)-propansulfonic acid
mRNA	Messenger RNA
M_w	Molecular weight
NP 40	ethylenphenylpolyethylenglycol
nt	nucleotide
NTP	ribonucleotide, ATP, CTP, GTP, TTP
OD	optical density
OLB	Oligo labelling
ORF	open reading frame
PAGE	Polyacrylamide gel electrophoresis
PCR	Polymerase chain reaction
PDE	phosphodiesterase
PG	phosphatidylglycerol

PIPES	1,4-piperazindiethansulfonic acid
PLA	Phospholipase A
PtdCMP	Phosphatidyl-CMP
PtdGro	phosphatidylglycerol
PtdGroP	phosphatidylglycerophosphate
PtdOH	Phosphatidic acid
RACE	Rapid amplification of cDNA ends
REMI	Restriction enzyme mediated integration
RNA	ribonucleic acid
RNase	ribonuclease
RNasin	Rnase inhibitor
rpm	revolutions per minute
RT-PCR	reverse transcription-PCR
SAP	shrimp alkaline phosphatase
TAZ	tafazzin (G4.5)
TEMED	N, N, N', N',-tetramethyl-ethylendiamine
Tris	Tris-(hydroxymethyl-aminomethane
Triton X 100	octylphenylpoly-(ethylenglycolether)
Tween 20	polyxyethylen-sorbitan-monolaurate
U	Unit(s)
Vol	volume
wt	wild type
X-gal	5 - bromo- 4 – chloro – 3 – indolyl – β – D - galactopyranoside

Zusammenfassung

Der eukaryotische Mikroorganismus Dictyostelium discoideum lebt als einzellige Amöbe solange ausreichende Nahrungsressourcen zur Verfügung stehen. Sobald Nahrungsmangel eintritt, entwickeln sich die Zellen von einem einzelligen zu einem mehrzelligen Zustand, der mit einem multizellulären Fruchtkörper abschließt. Dieser Prozess wird durch eine Reihe aufeinanderfolgender Signale organisiert, die eine differentielle Genexpression regulieren. Die Gene der Discoidin I Familie gehören zu den Ersten, die im Laufe des Wachstums-Differenzierungs-Übergangs (engl. GDT) aktiviert werden. Sie eignen sich daher vorzüglich als Marker für den Beginn der Entwicklung.

Mit Hilfe einer REMI-Mutagenese und Discoidin I als molekularem Marker sind verschiedene Komponenten des Wachstums-Differenzierungs-Übergangs in unserer Arbeitsgruppe identifiziert worden (Zeng *et al.*, 2000 A und B; Riemann und Nellen, persönliche Mitteilung). Mit demselben Ansatz wurde in der vorliegenden Arbeit eine REMI-Mutante identifiziert, die eine Fehl-Expression von Discoidin zeigte und einen axenischen Wachstumsdefekt bei 15 °C aufwies. Das Gen wurde als Homolog zum humanen Tafazzin-Gen identifiziert. Dieses Gen wurde zur Rekonstruktion des Phänotyps über homologe Rekombination erneut disruptiert, was wie erwartet zu dem zuerst beschriebenen Phänotyp führte. Folgerichtig ergab eine Überexpression des Gens in den Mutanten eine Komplementation des Phänotyps. Immunfluoreszenz-Experimente zeigten eine mitochondriale Lokalisation des Dictyostelium discoideum Taffazzin Proteins. Dass ein mitochondriales Protein in Zusammenhang mit dem Wachstums-Differenzierungs-Übergang steht, ist ein unerwarteter Befund, der aber als Hinweis darauf gewertet werden kann, dass Mitochondrien einen direkten Einfluss auf die entwicklungsspezifische Signaltransduktion ausüben.

Die Taffazzin Disruptions-Mutante in Dictyostelium führte zu einem abnormalen Cardiolipin Metabolismus. Dieses Phospholipid ist ein charakteristischer Bestandteil der inneren Mitochondrienmembran und für die Funktion verschiedener Enzyme erforderlich. Unsere vorläufigen Analysen des Phospholipid-Gehalts zeigten Übereinstimmung mit Daten von Patienten mit Barth-Syndrom, einer humanen Erkrankung, bei der das Taffazzin-Gen Mutationen aufweist, und mit Hefe-Mutanten dieses Gens. Dies zeigt den Wert von Dictyostelium discoideum als einen weiteren Modelorganismus zur Untersuchung des Barth-Syndroms und zur Erprobung möglicher Therapieansätze

Summary

Dictyostelium discoideum, a eukaryotic microorganism, exists as free-living amoeba as long as a food supply is available. When starved, the cells undergo a transition from single cell amoebae to a multicellular organism that finally develops into multicellular fruiting bodies. This process is organised by various signals which regulate the differential expression of genes. The discoidin I gene family is among the first to be activated at the growth-differentiation-transition (GDT) and thus serves as an excellent marker for the onset of differentiation.

By using REMI mutagenesis and the discoidin I gene family as a molecular marker, several GDT signal components have been investigated in this laboratory (Zeng *et al.,* 2000 A and B; Riemann and Nellen, unpublished data). In this work, a *Dictyostelium discoideum* REMI mutant, which was disrupted in the human tafazzin homologue and resulted in mis-expression of discoidin and an axenic growth defect at 15°C, was found by the same approach. The *tafazzin* gene was disrupted again by homologous recombination and the initial mutant phenotypes were reconstructed in the secondary gene disruption strains. Moreover, overexpression of tafazzin complemented the mutant phenotypes. Immunofluorescence experiment indicated that the *Dictyostelium discoideum* tafazzin was mitochondria associated. Although the linkage between a putative GDT signal component and the mitochondrial protein was unexpected, our preliminary finding added at least one more example for the concept that mitochondria may be directly involved in the signal transduction.

In *Dictyostelium discoideum, tafazzin* disruption led to an abnormal metabolism of cardiolipin, which is the characteristic phospholipid of the mitochondrial inner membrane and is required for the function of several mitochondrial enzymes. Our preliminary phospholipid result is consistent with that from human Barth syndrome patients whose tafazzin gene was mutated and from yeast gene disruption strain. They strongly suggest that *Dictyostelium discoideum* could be another model organism for the study and therapy of human Barth syndrome.

1

Introduction

Part I

Dictyostelium discoideum as a model organism to study developmental and cellular biology

Mycologist Brefeld (1869) first observed *Dictyostelium mucoroides* while examining the fungal flora in the horse dung, and then grew purer cultures in rabbit dung, he named the species *Dictyostelium* (Dicty means net-like and stelium means tower) because the aggregation territories he observed looked like nets and the fruiting bodies like towers.

It was Raper (1935) who first discovered *Dictyostelium discoideum* in the woods of North Carolina (this strain is now called NC4). *Dictyostelium discoideum* is a member of the class Acrasieae which includes those species of free-living amoebae that lack a flagellated stage and aggregate to form fruiting bodies. *Dictyostelium discoideum* is found in nature as a soil amoeba in forest detritus and feeds on bacteria by phagocytosis. During this vegetative part of the life cycle, cells multiply by mitotic division. When the bacteria that the amoebae feed on are consumed, the onset of starvation forces a major revision in the life cycle and entrances into a muticellular development cycle. Starvation induces a variety of new genes whose products are necessary for chemotaxis towards cAMP (Konijin *etal.,* 1968). A pulse of cAMP is secreted first by chance within a small population and serves as a signal for the cells to stream together and form loose aggregates of approx. 10^5 cells. In the aggregates, cells undergo differentiation and morphogenesis to result in a 2mm high fruiting body that consists of thin stalk of dead, vacuolized cells supporting a ball of resistant spores. The ratio of stalk to spore cells is about 1:4. Stalk cells are no longer viable after vacuolization but the spores can remain viable. Whenever the environmental conditions are suitable, the spores are dispersed to generate small but normal amoebae which enter the vegetative life cycle again. (figure 1-1) (For details, see Kessin, 2001).

Raper showed that nearly any species of bacteria, spread as a lawn, would support the luxuriant growth of *Dictyostelium discoideum*, he employed *E.coli* or *Aerobacter* (now *Klebsiella*) *aerogenes* as *Dictyostelium discoideum* food source and solved the problem of limited material. R. Sussman and M. Sussman (1967) first isolated a laboratory strain that can grow in axenic medium, since then *Dictyostelium discoideum* can be easily and cheaply cultured either in Ax medium (Watts and Ashworth, 1970) or in suspension culture or on plates with *Klebsiella aerogenes* (KA) as a food source. Thus *Dictyostelium discoideum* became one of the model organisms to study development and cellular biology because of its unique life cycle and easy manipulation.

The main advantages are:

1. It is haploid throughout its life cycle, so loss of function mutations usually cause phenotypes without the need for further manipulation.
2. Its genome is relatively small (~34 Mb on 6 chromosomes) and believed to code for 8000 to 10000 genes (Loomis and Kuspa, 1997), compared to the 6000 genes in *S. cerevisiae* (Goffeau *et al.,* 1996) and the estimated 15000 genes in *Drosophila* and *C. elegans* (Waterston and Sulston 1995). A genomic sequence project (http://dictybase.org/dictyostelium_genomics.htm) and a cDNA project (http://www.csm.biol.tsukuba.ac.jp/cDNAproject.html) are now well under way to be finished soon.
3. It is possible to transform the cells by electopration and to knock out genes by homologous recombination and marker replacement (De Lozanne and Spudich, 1987). Because multiple selectable markers are available (Nellen *et al*., 1984; Egelhoff *et al*., 1989; Sutoh, 1993), strains can be constructed with complex genotypes. Mutagenesis by antisense RNA (Crowley *et al*., 1985) and RNAi (Martens *et al.*, 2002) are possible. The established Restriction Enzyme Mediated Integration (REMI) technique (Kuspa and Loomis, 1992) is a very elegant method for random fishing of genes by insertion mutagenesis.

Due to the above reasons, *Dictyostelium discoideum* has become a versatile model system for basic research in developmental and cell biology. The fundamental cellular processes including cytokinesis, motility, phagocytosis, chemotaxis, signal tranduction, and aspects of development such as cell sorting, pattern formation, and cell-type determination have been well investigated. (For details, see Kessin, 2001 or visit http://dicty.cmb.nwu.edu/dicty/dicty.html).

Recently, research on this model organism is applied to the study of human health and disease (Hägels *et al.*, 2000; Li *et al.*, 2000; Pukatzki *et al.*, 2002; Saxe, 1999; Solomon *et al.,* 2000).

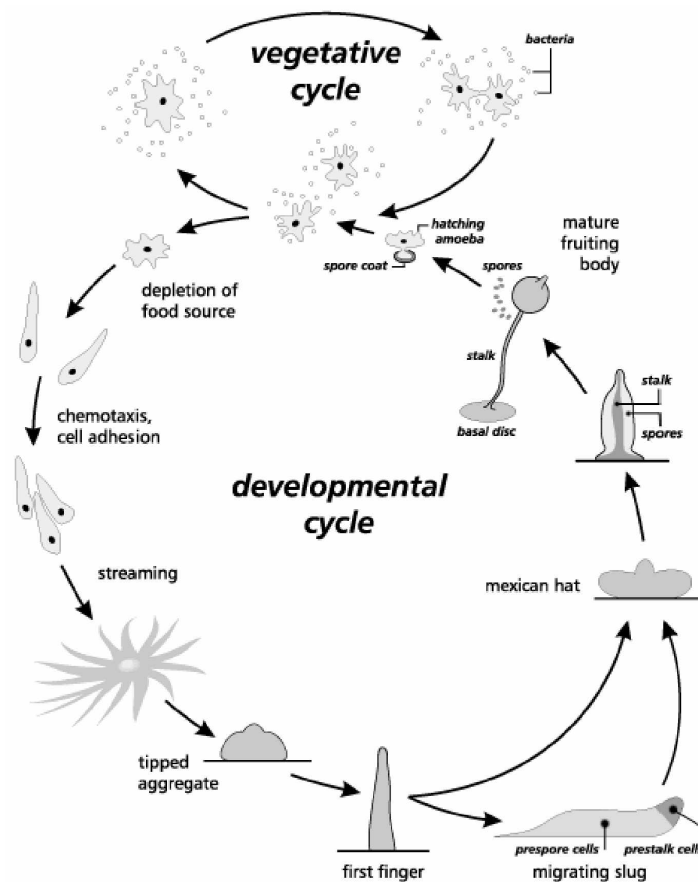


Figure 1-1. Life and development cycle of *Dictyostelium discoideum*
(Internet: http://www.uni-kassel.de/fb19/genetics)

Using the REMI strategy and discoidin gene family as a marker to study the growth–differentiation-transition (GDT) signaling in Dictyostelium discoideum

Although mutants of *Dictyostelium* can be isolated since 1953 (Sussman and Sussman), it was impossible until recently to recover the affected genes. Many of the mutated genes had been mapped to particular linkage groups by parasexual genetics (Newell, 1978; Welker and Williams, 1982), but in only a few cases had it been possible to isolate the affected genes. Even after transformation became possible, the mutations could not be complemented by transformation with libraries of genomic DNA, and therefore the affected genes could not be recovered in a manner that was available in bacteria or later became a powerful tool in the study of yeast. Transposon tagging has led to the isolation of genes in other systems, including *Myxococcus* (Kuner and Kaiser, 1981), *Drosophila* (Cooley and Spradling, 1988), and *Ceanorhabditis* (Greenwald, 1985; Moerman *et al*., 1986), but depends on the ability to mobilize transposable elements, which has not been possible in *Dictyostelium*.

REMI (**R**estriction **E**nzyme **M**ediated **I**ntegration) (Figure 1-2) is a technique developed in *Dictyostelium discoideum* by Kuspa and Loomis (1992). In *Dictyostelium discoideum* mutagenesis can be performed by electroporation of a linearized transformation vector along with high concentrations of a compatible restriction enzyme. The difference between REMI and simple transformation is that at the time of electroporation, a restriction enzyme is also included. Apparently, the enzyme enters the cell and cuts the genomic DNA. Occasionally, the compatible ends of the linearized plasmid are introduced to the restriction sites of the genomic DNA and ligated into place such that an insertion is created. REMI generates apparently random insertions into genomic DNA. Some integrations cause gene disruptions and thus result in mutations. The resulting mutant phenotypes with aberrant morphology can be directly identified by observation while the expression pattern of marker genes is detected by colony blot technique. Then the disrupted genomic DNA together with the inserted plasmid can be isolated by plasmid rescue. The resulting plasmid can be used for further molecular analysis, for example, sequence and southern analysis.

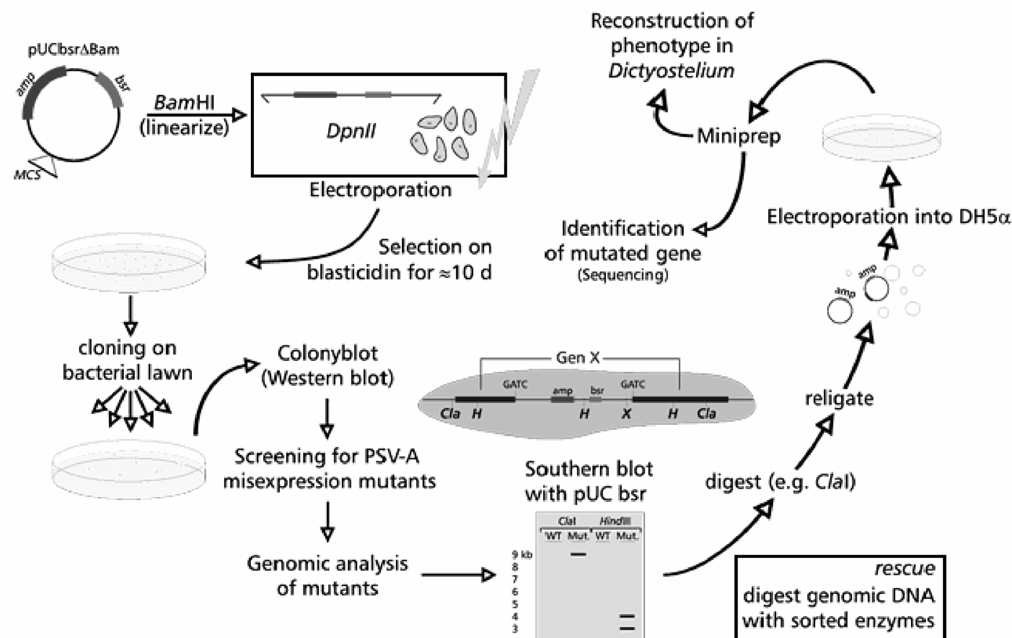


Figure 1-2. REMI (Restriction Enzyme Mediated Integration) strategy
(For details, visit ***http://www.uni-kassel.de/fb19/genetics*** or see Zeng *et al.*, 2000A)

Since development and vegetative growth are distinct phases in D*ictyostelium discoideum*, it is possible to isolate mutants defective in signal transduction pathways of development without impairing the viability of the cell. The REMI mutagenesis is used in this laboratory to isolate mutants with defects in the GDT signaling by using the discoidin I gene family as a molecular marker. This approach has been proven to be successful (Zeng *et al*., 2000A and B; Riemann and Nellen, unpublished data).

The discoidins are cytoplasmic proteins which are not essential for growth or development under laboratory conditions, but appear to be involved in cell shape changes at the onset of development. The function of the lectin, discoidin I, has been a source of interest and controversy. Discoidin was long thought to have a role in cell adhesion (Barondes *et al*., 1982), but this does not appear to be the case. Antisense and other experiments suggest however, that in strains with reduced discoidin levels, cell-substrate adhesion is altered (Barondes *et al*., 1987 and Crowley *et al*., 1985). A homology to fibronectin domains has been postulated, but never proved (Poole *et al*., 1981 and Springer *et al*., 1984). More recently, Vogel *et al*. (1997) reported there is close homology of discoidin with two mammalian receptor tyrosine kinases.

In wild type cells grown on a standard bacterial food source, discoidin is expressed several generations before the food source is exhausted, the expression level is increasing with further cell proliferation. Another boost of expression is observed where cells are completely deprived of nutrients. Discoidin can thus serve as a sensitive marker to monitor abnormalities in the growth-differentiation-transition (GDT) (Endl *et al.*, 1996; Riemann and Nellen, unpublished data; Wetterauer *et al.*, 1995 and Zeng *et al.*, 2000A and B).

After starvation, various soluble factors are secreted by the cells of *Dictyostelium discoideum* and serve as signals controlling gene expression, developmental initiation, morphogenesis and cell differentiation (Devreotes, 1989). There are two density-sensing mechanisms that function during the early stages of development. One mechanism is mediated by a molecule called prestarvation factor (PSF) and controls induction of certain very early genes (Rathi and Clarke, 1992). PSF is synthesized during growth and accumulates in the micro-environment according to the ratio of *Dictyostelium* amoebae to bacteria (Clarke *et al.*, 1992). Using partially purified PSF, a number of genes that were previously thought to be induced by complete starvation have been induced in growing cells. These genes include members of the discoidin gene family (Clarke *et al.*, 1987), cAMP receptor (*cAR1*), *pdsA* which encodes the aggregation-specific form of cyclic nucleotide phosphodiesterase, the α - mannosidase gene - *manA*, and gp24 (contact sites B). Cells growing on reduced amounts of bacteria, which do not express these genes, will express them if PSF is added (Clarke *et al.,* 1992). When the food supply has been depleted, PSF production declines, and a second density-sensing pathway, mediated by a molecule called conditioned medium factor (CMF) (Gomer *et al.*, 1991), is activated to help the cells to assess density at a slightly later period-during aggregation. The CMF signaling pathway most likely involves the G – protein α2 (Blusch *et al.*, 1995), pianissmo (Chen *et al.*, 1997), cytosolic regulater of adenylyl cyclase (CRAC) (Riemann and Nellen, unpublished data), and PKA (Endl *et al.*, 1996).

The cAMP dependent protein kinase A (PKA) plays a critical role during the early stage of development and at all later ones and is expressed early in development. The PKA of *Dictyostelium* is a dimer of one regulatory and one catalytic subunit, rather than the tetramer of higher organisms. Several of the genes involved in chemotaxis - *acaA* (adenylyl cyclase), *pdiA*, the phosphodiesterase inhibitor, and *carA,* the major cAMP receptor in early development, are not transcribed at all in the absence of the PKA catalytic subunit (Mann *et al.*, 1997; Wu *et al.*, 1995). Cells overexpressing a mutated regulatory subunit R which results in constitutive repression of PKA activity,

display undetectable levels of discoidin. In cells with a non-functional PKA, discoidin is still expressed, though at strongly reduced levels (Primpke *et al*., 2000).

yak A was recovered in a mutant screen employing REMI (Souza *et al*., 1998). *yak A* is required for the shut-off of growth stage genes and the induction of early developmental genes. The PKA catalytic subunit mRNA appears as normal in the *yak A*-null mutant, but PKA enzyme activity does not show the characteristic increase after 5 hours of starvation. *Yak A*-null cells do not turn off genes that are expressed in growing cells. Accordingly, a knock – out of *yakA* appears to reduce the levels of discoidin (Riemann, Wille and Nellen, unpublished data). *puf A* was found by a REMI suppressor screen on yak A-null cells (Souza et al., 1999). Puf A is a translational inhibitor of PKA-C mRNA and should thus serve as a negative regulator of the GDT. Puf A is downregulated by YaK A, a disruption of *puf A* can therefore partially rescue the *yak A*$^-$ phenotype.

By REMI mutagenesis and discoidin I as a molecular maker, Zeng *et al.* (2000A and B) reported a new GDT component, *gdt1*, which is a negative regulator of discoidin expression and the GDT in *Dictoystelium discoideum*. The encoded protein has four putative transmembrane regions and is localized in the cell membrane. Two PKA phosporylation consensus sequences have been detected. Disruption of the *gdt1* results in overexpression of discoidin and in a premature onset of development. However, *gdt*$^-$ cells respond normally to PSF and produce similar amounts of PSF compared to the wild type. *gdt1*$^-$*/PKA*$^-$ double mutants show no aggregation but high levels of discoidin expression, suggesting that gdt1 may be a downstream target of PKA in a branched signalling cascade initiating differentiation.

Part II

Learning from the slime mold: Dictyostelium and human disease

Yeasts and several invertebrate or vertebrate model systems are widely known for their contribution to our understanding of human diseases, but *Dictyostelium* has rarely been included in this list in the past. However, over the last few years, research on this social amoeba has revealed some common cellular characteristics shared across diverse phyla.

Dictyostelium offers numerous advantages as an experimental organism (see above). Specially, its genome is small with a low amount of noncoding sequence and, where vertebrates may express a large number of similar genes with overlapping functions, *Dictyostelium* often carries only a single orthologous gene. The organism is haploid throughout its life cycle, so loss-of-function mutations usually cause phenotypes without the need for further manipulation. For the same reason, neither recombination nor complementation is possible by the usual means (mating) in this system, but mutants may be rescued by introducing the gene of interest directly, in either wild-type or mutant form, and with variable levels of expression. This organism is uniquely suited for studies of cytokinesis, motility, phagocytosis, chemotaxis, signal transduction and aspects of development. Many of these processes, which play important roles in health and disease, are either absent or are less accessible in other model organisms. For example, leukocytes and *Dictyostelium discoideum* share certain characteristics (Devreotes and Zigmond 1988), both use G protein-mediated signaling to regulate chemotaxis. They are very similar in size, appearance and function as they both are highly motile and engulf and digest bacteria.

Complex signal transduction networks are activated when amoebae are starved and are used throughout the remainder of development to coordinate the morphogenetic and cellular differentiation events that result in the terminal structure, the fruiting body (see Parent and Devreotes, 1996; Soderbom and Loomis 1998 for reviews). Molecular genetic studies of these signaling pathways have also led to the identification of a number of other well-known signaling components, including MAP kinases, phosphatidylinositol-3 kinases, phospholipase C, protein kinase B, and STAT proteins (Drayer and Van Haastert, 1992; Zhou *et al.,* 1995; Subry *et al.,* 1997; Kawata *et al.*, 1997; Meili *et al.,* 1999). It appears that pathways from mammalian cell biology are conserved in *Dictyostelium.*

Dictyostelium cells have long been a favorite of researchers interested in the changing interactions of actin filaments and actin-associated proteins (ABPs) during cell movement and cytokinesis. For example, the *Dictyostelium* “gelation factor”, ABP-120, is related to a class of human actin-cross-linking proteins, the filamins, a targeted ablation of this gene disrupts actin filament networks, blocks pseudopodia formation, and impairs cell motility (Cox, *et al*., 1992). Interestingly, mutations in a human ABP-120 relative, *Filamin-1* (also known as “ABP-280”), lead to periventricular heterotopia in humans, a developmental abnormality in which cortical neurons fail to migrate (Fox and Walsh 1999). The possibility that this human migration defect and the motility defects seen in the *Dictyostelium* ABP-120 mutants are similar may make *Dictyostelium* a powerful system for studying the molecular basis of this disease.

Wiskott-Aldrich syndrome (WAS) is an X-linked immunodeficiency disease that results from the failure of both T and B cell function and additional defects in monocyte chemotaxis (Ochs, *et al*., 1980; for review, see Brickell et al., 1998), Clinical and laboratory evidence points out WAS being a defect in some aspect of actin organization. The protein responsible for Wiskott-Aldrich syndrome, WASp, has been shown to bind both actin and the actin-nucleating protein complex, arp2/3 (Machesky and Insall 1998). Bear *et al*., (1998) identified a *Dictyostelium* homolog , SCAR which is a suppressor of one of the cAMP receptors, cAR2. SCAR shares most of the functional domain with WASp. When *scar* was disrupted in a wild-type background, both morphogenetic and actin cytoskeletal defects were seen in *Dictyostelium* (Bear, et al., 1998), indicating that SCAR might behave in a similar manner as the closely related protein WASp. Use of *Dictyostelium* which is full of information regarding signaling and actin cytoskeletal organization may reveal many parts of Wiskott-Aldrich syndrome.

Recently, there has been interest in studying host-pathogen interactions by using simple, genetically manipulatable hosts. It is hoped that the bacterial factors and host genes involved in causing pathogenic effects in these simple organisms will be relevant to mammalian disease processes. For example, studies of the expression of antimicrobial peptides in *Drosophila melanogaster* led to the discovery of Toll receptors, critical components of innate immunity that have been recently recognized in mammals (Kopp *et al*., 1999; Lemaitre *et al.,* 1996). Labrousse *et al*., (2000) reported that *Salmonella typhimurium*, an enteropathogenic bacterium representing a major public health problem, can infect *Caenorhabditis elegans*, and that genes important for its full pathogenicity in vertebrates also play a role during infection of *Caenorhabditis elegans*.

The idea of using simple, genetically tractable host organisms to study the virulence mechanisms of pathogens date back at least to the work of Depraltère and Darmon (1978). They proposed using the predatory amoeba *Dictyostelium discoideum* as a model host, an approach that has proved to be valid at least in the case of the intracellular pathogen *Legionella* (Hägele *et al*., 2000; Solomon *et al*., 2000) and *Pseudomonas aeruginosa* (Pukatzki *et al*., 2001). *Legionella pneumophila* grows in alveolar macrophages, cells that are phagocytic and motile like amoebae to cause Legionnaires' disease (a type of pneumonia) (Chandler *et al.,* 1977). In recent years, genes and genetic loci involved in virulence of *Legionella* have been identified (Gao *et al.*, 1997; 1998; Hickey *et al.,* 1997; Segal *et al.,* 1998; Vogel *et al.,* 1998; Wintermeyer *et al*., 1995). In contrast, very little is known about specific target host cell factors or binding partners of *Legionella* virulence factors. Hägele *et al*., (2000) infected *Dictyostelium discoideum* with different *Legionella* species, and showed that *Dictyostelium discoideum* cells are able to support intracellular growth of *Legionella pneumophila* etc. highly virulent parasites in the same way as their growth in the natural host *Acanthamoeba castellanii*. Interestingly, Profillin-minus *Dictyostelium* mutant cells showed a higher rate of infection when compared with wild type. Solomon *et al*., (2000) focused on the infection of *Legionella pneumopila* and its mutants and their results are well consistent with those of Hägele *et al*., (2000). Pukatzki *et al.*, (2001) studied the interactions of *Dictyostelium discoideum* and human pathogen *Pseudomonas aeruginosa* which is an opportunistic pathogen that causes life-threatening infections in individuals with compromised immune systems and found that *Pseudomonas aeruginosa* utilizes conserved virulence pathway to infect *Dictyostelium discoideum.* All the above work clearly indicates that *Dictyostelium discoideum* is a new model host system for the investigation of pathogenicity of *Legionella*, *Pseudomonas* and maybe other pathogens. It should allow the elucidation of essential susceptibility factors of the host in the future.

Cisplatin [*cis*-diamminedichloroplatinum(II)] and its derivatives are widely used anti-cancer drugs (Chu, 1994; Eastman, 1986; Lippard, 1982). However, its therapeutic efficiency is frequently limited by the development of drug-resistant tumour cell populations (Perez, 1998). Considerable attention had been paid to the underlying mechanisms of resistance to this drug (Andrews and Howell, 1990; Chu, 1994; Perez, 1998), but all of these studies were focused on genes and mechanisms which were *a priori* suspected to modulate the cellular response to the drug. Recently, Li *et al*., (2000) reported a direct genetic approach, employing insertional mutagenesis, to specifically identify novel genetic pathways that are involved in the cellular response and resistance to cisplatin in *Dictyostelium discoideum*. They have identified 6 genes

which are involved in cisplatin resistance. Interestingly, none of these genes are directly involved in drug import or efflux, DNA repair or multi-drug resistance. Some of these genes encode proteins that are involved in signal transduction pathways which regulate cell death, cell proliferation or gene regulation. Importantly, some of the mutants exhibit abnormal developmental phenotypes, demonstrating that the genes that are involved in responding to DNA damage also function in normal development. These pathways will provide potential targets for modulating the response to this important drug. For example, one of their drug resistant genes, *regA* was identified as a central component in the pathway for spore differentiation in *Dictyostelium* (Shaulsky et al., 1996). The RegA protein is a cAMP phosphodiesterase, regulating the cAMP level in prespore cells, which in turn regulates protein kinase A (PKA). The discovery of this gene in drug resistant selection suggests that cisplatin resistance may be linked to PKA signalling pathways. In fact the work of Cvijic *et al*., (1998) has linked PKA to cisplatin resistance in CHO cells. This work clearly indicates *Dictyostelium discoideum* can be effectively used to discover genes underlying cellular responses to important pharmacological agents.

X-linked cardioskeletal myopathy and neutropenia (Barth syndrome) – MIM 302060

Barth *et al*. (1981, 1983) described a large pedigree showing X-linked inheritance of a disorder characterized by dilated cardiomyopathy, neutropenia, skeletal myopathy, diminished statural growth, and abnormal mitochondria (Figure 1-3). Neustein *et al*. (1979) reported a family that may have the same disorder. By electron microscopy, the mitochondria showed concentric, tightly packed cristae and occasional inclusion bodies. The family reported by Barth *et al*., (1981, 1983) was Dutch. Since then a number of families studied in Europe, North America, Australia and Japan present essentially the same picture with some variation (Ades *et al*., 1993; Bolhuis *et al.,* 1991; Cantlay *et al*., 1999; Hodgson *et al*., 1987; Ino *et al.,* 1988; Katsushima *et al*, 2002; Kelley *et al.,* 1991). The most common presentation is that of a young, moderately growth-retarded male infant with cardiac failure caused by dilated cardiomyopathy. Presentation may be slowly progressive or precipitous. In most patients cardiomyopathy becomes manifest in infancy. Studies on the respiratory chain in Barth syndrome patients' skeletal muscle showed impaired oxidative phosphorylation at the level of complexes III (ubihydroquinone: cytochrome oxidoreductase) and IV (Cytochrome oxidase) (Barth *et al*., 1983) and decreased activity of complex IV (Christodoulou *et al*., 1994). Barth *et al*., (1996) showed again

the decreased activities of complex III and IV from cultured fibroblasts of Barth syndrome. These findings provide evidence that respiratory-chain dysfunction is an essential component of BTHS. Results of clinical experiments pertaining to the organic acid abnormality (increased urinary excretion of 3-methylglutaconic acid, 3-methylglutaric acid and 2-ethylhydracrylic acid (Keller et al., 1991; Gibson et al., 1991) and the low serum cholesterol.

For the moment, therapeutic prospects are limited. Myocardial failure is amenable to standard treatment. In severe cases of progressive myocardial failure, cardiac transplantation has been performed (Adwani *et al.*, 1995 and 1997). Several therapies have been offered on the basis of suspected metabolic derangement, but none has been a singular success so far, probably because none has addressed the basic molecular defect of BTHS(for review, see Barth *et al.*, 1999).

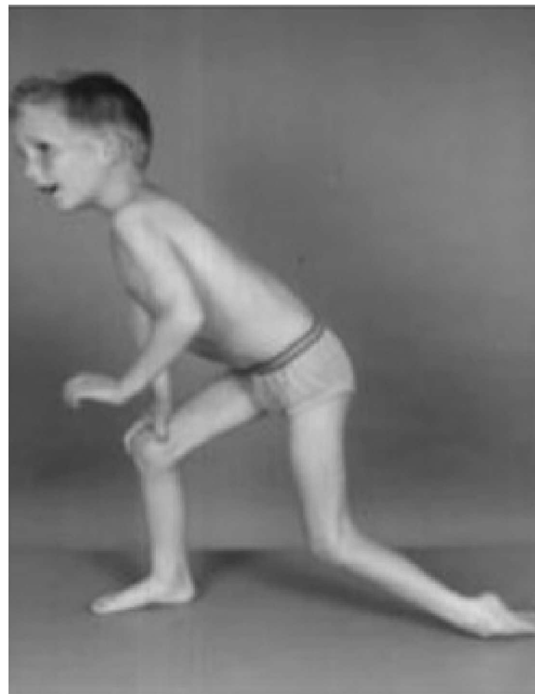


Figure 1-3. Patient with Barth syndrome at the age of 5 years is attempting to rise with typical Grower's manoeuvre, demonstrating pelvic muscle weakness (from P. G. Barth *et al.* (1999), J. Inher Metab. Dis. 22)

Obligate female carriers of Barth syndrome do not show clinical symptoms. ∅rstavik *et al.,* studied X-inactivation patterns in female carriers with BTHS and found skewed X

inactivation, likely to be the result of a selection against cells that have the mutated gene on the active chromosome. This fits well in the absence of clinical symptoms.

The last decade had seen several new developments in Barth syndrome. After its clinical and biochemical characterization (Barth *et al.*, 1983; Ino *et al.,* 1988; Kelley *et al.*, 1991), the gene was mapped to Xq28 (Bolhuis *et al.*, 1991; Adès *et al.*, 1993; Christodoulou *et al.*, 1994). And then Bione *et al.*, (1996) identified unique mutations in a gene that is located in the gene rich region Xq28 where Barth syndrome maps. The gene was termed *G4.5 (tafazzin)* by them. This made it possible to make a definite diagnosis and to offer antenatal diagnostics for future pregnancies in an affected family.

G4.5 gene and its products-tafazzins

The Barth syndrome responsible gene *G4.5* located on Xq28 is a relatively small (~4 kb) but complex gene incorporating 11 exons. Different mRNAs were produced by alternative splicing of the primary G4.5 transcript, encoding proteins that differed at their N terminus and in the central region. Two regions of the proteins may be functionally significant. There are 2 transcription initiation sites, on exon 1 and 3. Isoforms with a highly hydrophobic stretch of 30 residues at the N terminus are though to be membrane anchored. The shortest forms of tafazzins (starting from exon 3) lacking the hydrophobic stretch, may be soluble cytoplasmic proteins. The reason of 2 fundamentally different gene products, one membrance-bound and the other cytosolic, is not clear. The second variable region is the central portion between amino acids 124 and 195 (exon 5, 6 and 7). Alternative splicing of the hydrophilic central region produces 5 variants. Removal of exons 5, 6 and 7 would progressively shorten a hydrophilic domain of the protein, which may serve as an exposed loop interacting with other proteins. Two isoforms , containing all 3 exons or lacking exon 5 only are consistently abundant. Because of the two 5' ends together with various splice variants, up to 10 isoforms were found. Bione et al,. (1996) termed these proteins tafazzins (Tafazzi is a masochistic comic character from an Italian television sports show). Most isoforms are ubiquitous. Isoforms that lack the N-terminus are found in leukocytes and fibroblasts, but not in heart and skeletal muscles. Some forms appear to be restricted to cardiac and skeletal muscle or to leukocytes.
More than 30 mutations of the *G4.5* gene were published (Bione *et al.*, 1996; D'Adamo *et al.*, 1997, Ichida *et al.*, 2001, Johnston *et al.*, 1997 and Sakamoto *et al.*, 2001). The mutations involved exons 1-3 and 6-11 and some adjacent intron

sequences, resulting in a variety of gene alterations including missense and non-sense mutations, splice site mutations, and various deletions of one or more base pairs resulting in frame shifts. There is no apparent correlation between genotype and phenotype, nor is there a correlation between the location of the mutation in *G4.5* (*tafazzin*) gene and the severity of Barth syndrome, implicating that all the splicing variants seem to be equally important for cellular function or other environmental or genetic factors influencing the phenotypic severity for Barth syndrome (Johnston *et al*., 1997). Moreover, Mutations in G4.5 result in not only BTHS but also other X-linked infantile cardiomyopathies, including left ventricular noncompaction (LVNC) (Bleyl *et al*., 1997), X-linked infantile cardiomyopathy (D'Adamo *et al*., 1997 and Gedeon *et al*., 1995), and X-linked endocardial fibroelastosis (D'Adamo *et al*., 1997).

G4.5 orthologues from *S. cerevisiae*, *C. elegans*, and *D. melanogaster* have been cloned and sequenced, but no studies to investigate the function of these genes and their gene products have been published so far. It is important to note that the *C.elegans G4.5* gene (ZK809.2 gene) shares several splice sites with its human orthologue. Notably, the worm protein is missing exon 5, which appears to be removed from many of the tafazzin splice variants. Thus, the *C. elegans* mutant may serve as a useful model to explore the molecular mechanisms underlying Barth syndrome. There are no differential splicing variants in *S.cerevisiae* and *D. melanogaster*.

The function of tafazzins - Neuwald Hypothesis

The biological function of tafazzins is so far unclear. In 1997 Neuwald, using a genomic database search, reported that human tafazzins belong to a superfamily consisting of established and putative acyltransferases (PFAM 01553) involved in phospholipid biosynthesis and/or remodeling.

This superfamily includes known or putative acyltransfereas from bacteria, fungi, plants, and vertebrate and invertebrate metazoans. Characterized enzymes in this superfamily all function in phospholipid biosynthesis and have either glycerolphosphate, 1-acylglycerolphosphate, or 2-acylglycerolphosphoethanolamine acyltransferase activity. The sequence alignment contains five conserved regions that presumably reflect similar structural and functional features shared by these proteins (Figure 1-4). As all of the characterized proteins are acyltransferases involved in phospholipid biosynthesis, the uncharacterized proteins are likely to have similar catalytic activity.

The potential acyltransferase activity of tafazzins suggests a possible disease mechanism of Barth syndrome. Differential splicing of tafazzins (Bione *et al.*, 1996), at least 9 putative acyltransferases in *C.elegans* and 4 in *E.coli*, suggest a variety of substrate specific or tissue and organelle specific forms of those acyltransferases. If so, Neuwald (1996) predicted the mitochondrial structural and respiratory-chain abnormalities associated with BTHS may be due to alterations in mitochondrial membrane phospholipid composition.

protein	start	Motif A	Motif B	Motif C	Motif D	Motif E	end	description
g1263110	62	PLITVSNHQSCMDDPHLWGIL	(16)AADICFTKELHSHFFSLGKCVPVCR	(48)GDWVHIFPEGKV	(8) FKWGIGRLI	(19)VLPNSPPY	227 (65)	tafazzin (human)
g1130664	37	PLITVSNHRSNIDDPLMWCIL	(17)AHNICFTKQFHTTMFSLGRCVPCVR	(18)NKWVHIFPEGKV	(9) FKWGIGRLV	(19)VWPTQPPY	174 (74)	ZK809.2 (worm)
g1066481	70	GLMTVMNHMSMVDDPLVWATL	(16)AHNICFQNKFLANFFSLGQVLSTER	(49)PSWVHVYPEGFV	(13) FKWGITRMI	(40)EINVTIGD	262 (119)	P9659.5 (yeast)
g1458274	218	PALIIMNHRTRLDWLFFWNAL	(14)LKGMLKYVPGAGWAMQAASYIFLDR	(21)KYQLLLFPEGTD	(26) PRVTGFVHI	(12)IYDVSIGF	365 (158)	agpat (worm)
S52645	85	HALIISNHRSDIDWLIGWILA	(12)MKKSSKFLPVIGWSMWFAEYLFLER	(21)PFWLALFVEGTR	(26) PRTKGFVSA	(10)IYDTTVIV	228 (146)	agpat (maize)
g1197334	102	RAIYISNHASPIDAFFVMWLA	(7) AKKEVIWYPLLGQLYTLAHHIRIDR	(20)NLSLIMFPEGTR	(35) WRKGTFRVR	(0) PVPITVKY	238 (43)	agpat (vascular plant)
P33333	75	PYIMIANHQSTLDIFMLGRIF	(7) AKKSLKYVPFLGWEMALSGTYFLDR	(20)KRALWVFPEGTR	(10) FKKGAFHLA	(77)LPPQAIEY	263 (40)	agpat (yeast)
P26647	66	NAIYIANHQNNYDMVTASNIV	(7) GKKSLLWIPFFGQLYWLTGNLLIDR	(20)RISIWMFPEGTR	(8) FKTGAFHAA	(6) IIPVCVST	181 (64)	agpat (*E.coli*)
D64223	85	PVLVVANHKSNLDPLVLIKAF	(11)VAKIELKDTVLFKLMKLIDCVFIDR	(19)GTAIAVFAEGTR	(8) FKPGALKVA	(6) ILPVSIVG	203 (65)	agpat (*M.genitalium*)
g972978	71	GVLVAANHVSWLDIFAMSAVY	(5) AKQEIKSWPVLGKMGQNAGTVFINR	(19)GQNVSFFPEART	(10) FKAALFQSA	(6) VLAVALRY	185 (72)	agpat (N.meningitidis)
A41672	223	PLLFLPVHRSHIDYLLLTFIL	(10)ASGNNLNIPVFSTLIHKLGGFFIRR	(28)QQFLEIFLEGTR	(0) SRSGKTSCA	(21)VIPVGISY	356 (471)	gpat (mouse)
g1458332	160	PMVYLPLHRSHLDYLLITWCN	(10)ASGDNLNLSGLGWLLRATGAFFIRR	(28)DMPIEFFLEGTR	(8) PKNGLISNV	(13)LVPVSYTY	293 (425)	gpat (worm)
P30706	220	NIILMSNHQSEADPAIIALLL	(38)SKKHMLDNPELVDMKRKANTRSRKE	(8) SQIIWIAPSGGR	(9) WAPAPFDSS	(17)IYPLAILC	366 (91)	PlsB (PEA)
P00482	319	ELVYVPCHRSHMDYLLLSYVL	(10)AAGINLNFWPAGPIFRRLGAFFIRR	(22)GYSVEYFVEGGR	(8) PKTGTLSMT	(13)LIPIYIGY	446 (381)	PlsB (*E.coli*)
P31119	29	RVLITPNHVSFIDGILLGLFL	(20)LKSFIDFVPLDPTQPMAIKHLVRLV	(2) GRPVVIFPEGRI	(8) IYDGAGFVA	(6) VIPVRIEG	139 (580)	agpeat (*E.coli*)
g1841552	89	PYVVVSNHQSSLDLLGMMEVL	(7) AKRELLWAGSAGLACWLAGVIFIDR	(20)DVRVWVFPEGTR	(8) FKRGAFHLA	(6) IVPIVMSS	204 (71)	ORF (human)
g1503994	94	EAVMLVNHQATGDVCTLMMCL	(12)LMDHIFKYTNFGIVSLVHGDFFIRQ	(24)RKWIVLFPEGGF	(25) PRSGATKII	(30)IIDTTIAY	259 (111)	KIAA0205 (human)
g1256468	97	RALLLPNHLGLFDHFIFMTAA	(13)VIYNMWIYSPLGWLWSSYGNYFIDS	(25)LRWLCLYPEGSR	(25) PRLGAALSA	(24)LVDVTLGY	258 (105)	F08G5.2 (worm)
g1301695	42	PAMIVMNHRTRLDWMYMWCAL	(14)LKAQLKKLPGAGFGMAAAQFVFLER	(21)KYQILLFPEGTD	(26) PRTTGFLHL	(37)IYDITIAY	214 (225)	F55A11.5 (worm)
g1403001	91	PAVVICNHQSSLDILSMASIW	(7) MKRILAYVPFFNLGAYFSNTIFIDR	(20)NLKLWVFPEGTR	(8) FKKGAFNIA	(6) IIPVVFSD	206 (76)	T06E8.1 (worm)
g1673483	87	PYIIIANHQSALDVLGMSFAW	(7) LKSSLKYLPGFNLCAYLCDSVYINR	(20)KRKVWIYPEGTR	(8) FKKGAFILA	(6) IVPCVFSS	202 (3)	F59F4.4 (worm)
g798827	237	GGICVANHTSPIDVMVLSCDN	(20)SRSEHHIWFERGEAGDRAKVMDRMR	(7) KLPIIIFPEGTC	(8) FKKGSFEIG	(2) IYPIAVKY	348 (164)	R07E3.5 (worm)
g746580	117	KCLLLANHLGILDHFVLMQSL	(12)VIYNIWKYTPLGVMWTSHGNFFVNG	(24)YGWVIMYPEGSR	(25) PRTGAAHAV	(24)IIDATIGY	276 (115)	C01C10.3 (worm)
g1420660	149	TTLMICNHRSVNDYTLINYLF	(29)LKFLGWGKMFNFPRLDLLKNIFFKD	(18)NQAITIFPEVNI	(29) FKNFTTLMA	(79)IYDVTIIY	378 (101)	YOR298w (yeast)
P38226	105	NSVAICNHQIYTDWIFLWWLA	(12)LKKSLASIPILGFGMRNYNFIFMSR	(54)PYNLILFPEGTN	(4) TRQKSAKYA	(32)LYDITIGY	281 (116)	Ybp2p (yeast)
S54641	110	RAIIIANHQMYADWIYLWWLS	(12)LKKALQYIPLLGFGMRNFKFIFLSR	(41)AYNLIMFPEGTN	(28) PHSKGLKFA	(10)IYDVTIGY	275 (121)	YD9335.04c (yeast)
g1653690	57	PALVVSNHASYFDPPFLSCAM	(7) AKEELFNVPLLGPAIRLYGAYPVKR	(17)GWIVGVFLEGTR	(8) PKIGAAMIA	(6) IIPVSLGG	169 (56)	ORF (Synechocystis)
g1652948	64	RLLLAFRHPSINDPLCMGYLF	(7) LKKGALQPPPVSHSHFMYDRGIPLW	(37)QYPLAAAPEGAT	(9) LEPGIAQLG	(17)ILPVGIQY	208 (257)	ORF (Synechocystis)
g1652152	54	PTILAPTHRSRWDAILLSLAA	(3) VTGRDLRFMVAVTEVQGLQGWFIRH	(25)GEMLVIFPEGGI	(9) LKRGIGRIA	(13)VIPVTIAY	178 (62)	ORF (Synechocystis)
P32129	96	WYLLICNHRSWADIVVLCVLF	(10)LKQQLAWVPFLGLACWSLDMPFMKR	(30)PTTIVNFVEGSR	(18) PKAAGIAMA	(10)LLNVTLCY	238 (72)	YihG (*E.coli*)
Q11167	143	AALVVANHAGVLPFDGLMLSV	(15)AADMVFDLPVIGEAARKAGHTMACT	(9) GELTAVFPEGYK	(14) FGRGGFVSA	(7) IVPCSIIG	262 (96)	YV29 (*M. tuberculosis*)

Alignment of representative sequences in the tafazzins, or acyltransferase superfamily. A total of 53 proteins in the NCBI non-redundant database were detected by the PROBE search [9], which used default parameter settings. Conserved residues are highlighted in red (for the most conserved positions) or black. Numbers in parentheses are gap lengths. Human tafazzin is detected at the $p < 0.00001$ level of significance; this is based on a database search using an alignment lacking sequences with statistically significant pairwise similarity to tafazzins (that is, lacking sequences g1263110, g1130664, g1066481, g1841552, g1403001 and g1673483). (The database search and the p-value calculation were done as previously described [13].) Protein identifiers are highlighted according to the following color scheme: black, tafazzin and close homologs; red, 1-acylglycerol-phosphate acyltransferases (agpat); blue, glycerolphosphate acyltransferases (gpat); green, 2-acylglycerolphosphoethanolamine acyltransferase (agpeat); unhighlighted, proteins of unknown function.

Figure 1-4. Neuwald hypothesis. Human tafazzins belong to a superfamily consisting of established and putative acyltransferases (PFAM 01553) involved in phospholipid biosynthesis (from Neuwald (1997), Curr. Biol. 7, R465-466).

The work of Vreken *et al.* (2000) supported the Neuwald hypothesis (1997). They studied the biosynthesis and remodeling of the phospholipids phosphatidylglycerol (PG) and cardiolipin (CL), which is a unique phospholipid with dimeric structure, carrying 4 acyl groups, two negative charges, exclusively found in bacterial and mitochondrial membranes and is required for optimal function of many of the respiratory and ATP synthesizing enzymes (for review, see Schlame *et al.*, 2000). Their data showed that the biosynthesis rate of PG and CL is normal but that the CL pool size is 75% reduced in cultured skin fibroblasts of BTHS patients compared to control cells, indicating accelerated degradation. In particular, the incorporation of

linoleic acid which is the characteristic acyl side chain found in mammalian CL, into both PG and CL is dramatically reduced, whereas the incorporation of other fatty acids into these phospholipids is normal. These data suggest that the G4.5 gene indeed encodes an acyltransferase, or at least a co-factor required of the function of such enzymes. Their unpublished data from a *S. cerevisiae G4.5* disruption strain indicates that also in yeast, phospholipid remodeling is disturbed leading to reduced CL concentration and abnormal CL-acyl composition (Vreken, personal communication).

On 2002, Bissler *et al* and Schlame et al. respectively reported that their Barth syndrome patient tissues contained decreased unsaturated and increased saturated fatty acids.

Noticeably, Mushegian *et al*. (1997) predicted hydrolytic activity for tafazzins based on weak similarity to the *E. coli radC* gene, which may possess hydrolytic activity needed for DNA repair, thus it is possible that tafazzins perform some other hydrolytic function.

The biosynthesis and functional role of cardiolipin

Diphosphatidylglycerol Cardiolipin (1, 3-bis (1', 2'-diacyl-3'-phosphoryl-sn-glycerol)-sn-glycerol) (CL) is a unique phospholipid with dimeric structure, carrying four acyl groups and two negative charges (Figure 1-5). It is thus highly hydrophobic and acidic. It is exclusively found in bacterial and mitochondrial membranes to generate an electrochemical potential for substrate transport and synthesis (For review, see Schlame *et al*., 2000). The trivial name "cardiolipin" is derived from the fact that it was first found in animial hearts (Pangborn 1942), cardiolipin is most abundant in mammalian hearts, but it can be found in mitochondria of all animal tissues and indeed of the eukaryotic kingdom. For example, it amounts to about 10% of the phospholipids of bovine heart muscle, and 20% of the phospholipids of the mitochondrial membrane.

Figure 1-5. Structure of Cardiolipin. In the acid form of authentic cardiolipin, X and Y are hydrogens while A, B, C, and D are fatty acyl groups.

The biosynthetic pathway of cardiolipin is shown in Figure 1-5. The route is similar to other phospholipid pathways as it passes through the common intermediates, phosphatidic acid and phosphatidyl-CMP. Only the final step of cardiolipin synthesis is a unique reaction, which is utterly different in prokaryotes and eukaryotes (Schlame *et al*., 1997). Prokaryotic cardiolipin synthase catalyzes a transesterification in which the phosphatidyl moiety of one phosphatidylglycerol is transfered to the free 3'-hydroxyl group of another phosphatidylglycerol ((Hirshberg and Kennedy 1972). This reaction is mainly controlled by substrate availability. In contrast, eukaryotic CL synthase catalyzes a phosphatidyl transfer from CDP-DG to PG (Hostetler *et al*., 1972; Schlame *et al*., 1993; Tamai and Greenberg 1976). This is an irreversible reaction that involves cleavage of a high energy anhydride bond. This reaction can take place in the presence of low substrate concentration and is mainly regulated by CL synthase activity.

Notably, in animal tissues, cardiolipin contains almost exclusively 18 carbon fatty acids (Table 1-1), and 80% of this is typically linoleic acid (18:2 (n-6)). This appears to be true in higher plants also. Yeast cardiolipin can differ in having more 16:1 and 18:1 fatty acids, while the bacterial lipid contains saturated and monoenoic fatty acid with 14 to 18 carbons.

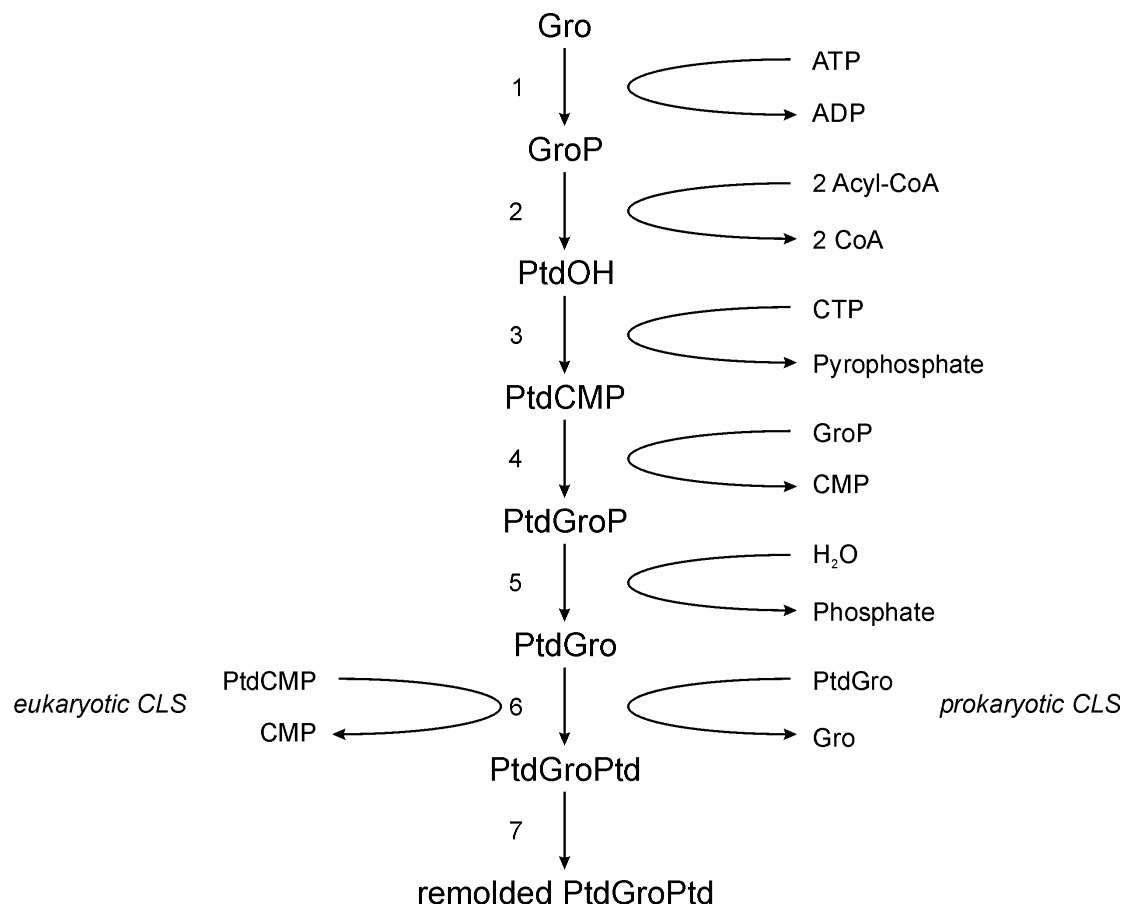


Figure 1-6. De novo biosynthesis of cardiolipin.

1, Glycerokinase

2, glycerophosphate acyltransferase and lysophosphatidate acyltransferase

3, phosphatidate cytidylyltransferase

4, phosphatidyl-CMP:glycerolphosphate phosphatidyltransferase (phosphatidylglycerophosphate snythase)

5, phosphatidylglycerophosphatase

6, cardiolipin synthase (CLS)

7. tafazzins (?)

* Abbreviations: Gro, glycerol; GroP, *sn*-glycero-3-phosphate;
PtdCMP, phosphatidyl-CMP; PtdGro, phosphatidylglycerol;
PtdGroP, phosphatidylglycerophosphate; PtdOH, phosphatidic acid;
PtdGroPtd, cardiolipin

Eukaryotic cardiolipins have their unique fatty acid pattern (C_{18} chains) with the exception of *Saccaromyces cerevisiae* (C_{16} residues). The dominant C_{18} chain in mammals is the linoleoyl group (18:2). Rüstow *et al.* 1989 suggested the generation of the characteristic acyl pattern of CL does not occur during de novo synthesis but requires additional synthetic steps, newly synthesized CL probably undergoes remodeling of its acyl groups. Neuwald hypothesis (1997) and the work of Vreken *et al.,* (2000) and Bissler *et al.,*(2002) supported the above evidence and their data suggest that the *G4.5* gene indeed encodes an acyltransferase involved in the deacylation and reacylation of CL side chain, or at least a factor required for the function of such enzymes.

Table 1-1 Major molecular species of mammalian cardiolipin[a] (from Schlame *et al.*, 2000)

Source of cardiolipin	Molecular species				Abundance (Mol%)[b]
	Residue A 3' (1-glycerol)	Residue B 3' (2-glycerol)	Residue C 1' (2-glycerol)	Residue D 1' (1-glycerol)	
Bovine heart	18:2	18:2	18:2	18:2	48
					21
	18:3	18:2	18:2	18:2	
	18:2	18:3	18:2	18:2	
	18:2	18:2	18:3	18:2	
	18:2	18:2	18:2	18:3	
	18:2	18:1	18:2	18:2	
	18:2	18:2	18:1	18:2	15
Rat liver	18:2	18:2	18:2	18:2	57
	18:2	18:1	18:2	18:2	37
	18:2	18:2	18:1	18:2	

[a] The table shows distribution of fatty acyl resides among the four ester positions in cardiolipin. Residues are as designated as shown in figure 1-4.
[b] The molecular composition was obtained from Keenan *et al.*, 1970

Defects in enzymes involved in the biosynthesis of PG and CL have so far only been documented for CHO cells, yeast and *E.coli*. Those studies showed that the presence of PG and/or CL is essential for normal respiratory-chain function and PG could substitute for CL in most essential mitochondrial functions. (Chang *et al.,* 1998 a and

b; Jiang *et al*., 1997, 1998, 1999 and 2000; Kawasaki *et al*., 1999; Koshkin and Greenberg 2000; Minskoff *et al*., 1997).
The biomembrane function of cardiolipin has been unresolved, although it is believed that its function is related to its unique ability to interact with proteins (Hoch 1992; Schlame and Greenberg 1997) because of its acidic and hydrophobic nature. Biochemical analysis suggests that CL is required for many enzymatic activities, such as cytochrome *c* oxidase (Awashti *et al.,* 1971) and the carnitine acylcarnitine translocase (Noel and Pande 1986), and is involved in cellular functions, such as mitochondrial protein import (Ardail *et al*., 1990; Ou *et al*., 1988; Schleyer and Neupert 1985) and binding of matrix Ca^{2+} (Krebs *et al*., 1979).

Part III

Aims of this thesis

In this work, *Dictyostelium discoideum* tafazzin mutant strain was initially found by chance. At the beginning, this work was focused on the signal transduction pathway of the growth differentiation transition (GDT) and started by screening REMI mutants using the discoidin gene family as a molecular marker to investigate the transition from growth to multicellular differentiation. Just during the routine REMI mutant analysis, the sequence from one of the rescue plasmid showed high similarity to human Barth Syndrome responsible gene *G4.5* (*tafazzin*).

The aim of this thesis was to characterize the *G4.5* (*tafazzin*) gene homolog and the biological function of its gene product – tafazzin in *Dictyostelium discoideum*. Hopefully, the data on this model organism can provide some useful clue to the further study and therapy of Human Barth syndrome.

2

Results

Identification of REMI mutant 17-1-J-3-1

REMI (**R**estriction **E**nzyme **M**ediated **I**ntegration) is an insertional mutagenesis technique first published for *Dictyostelium discoideum* by Kuspa and Loomis (1992). It introduces mostly single copy integration. In our laboratory, REMI mutagenesis was applied to isolate mutants and genes involved in the transition from growth to differentiation (Zeng *et al*., 2000A and B). REMI introduces into *Dictyostelium discoideum* cells a linearized plasmid DNA along with high concentration of a restriction enzyme (In our case, *Dpn*II) that will generate ends compatible with those of the linearized plasmid (In our case, *Bam*H I). This leads to random integration of the plasmid into genomic sites of the enzyme. Some integrations cause gene disruptions and thus mutations. T+he mutant phenotypes can be identified by the colony blot technique detecting the expression pattern of marker genes.

The marker gene, which is used to investigate the transition from growth to differentiation in our laboratory, is discoidin I – a developmentally regulated lectin that is expressed at relatively high levels during development and its developmental expression is among the most thoroughly studied genetic regulatory systems in Dictyostelium discoideum (Cooper and Barondes, 1984; Ma and Firtel, 1978). The expression of discoidin protein is distinct from growth to development, which is easily observed in colony blots. Since colony blots are semi-quantitative, over-expression and low-expression mutants which display stronger or weaker antibody staining than wild type can easily be detected.

The use of REMI mutagenesis and discoidin I as a molecular marker to study GDT signaling has been proven to be successful (Riemann and Nellen, unpublished data; Zeng *et al*., 2000A and B) in our laboratory. Like the others, this work started from

screening interesting REMI clones by using colony blot technique and discoidin I antibody.

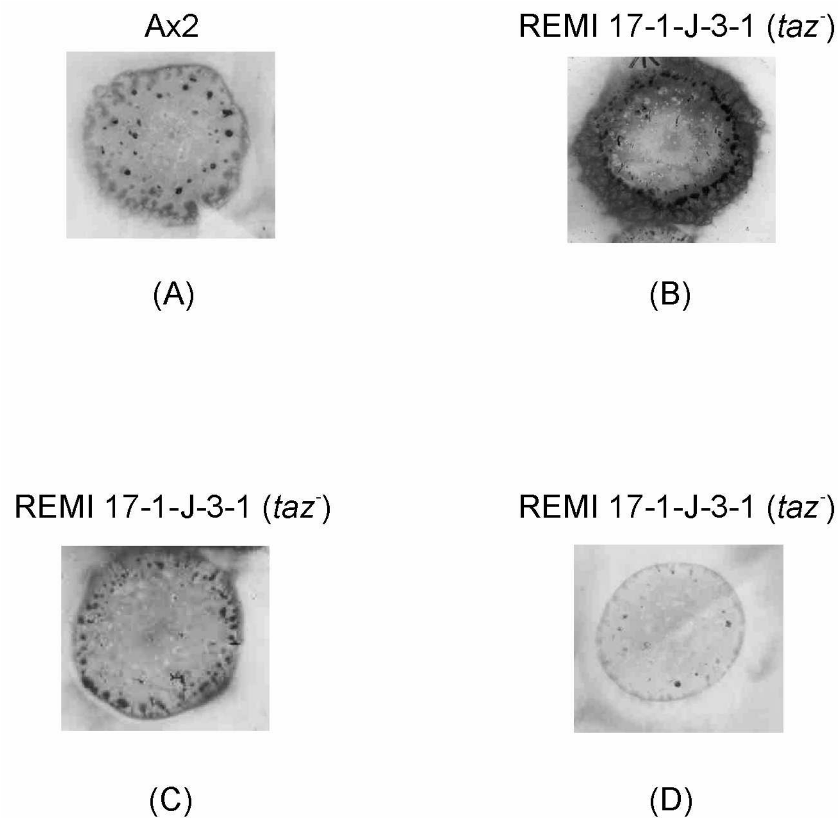


Figure 2-1. Colony blots for discoidin expression. Cells of wild type Ax2 and REMI mutant 17-1-J-3-1 (taz^-, see below) were picked on a lawn of KA, colonies were grown to a diameter of approximate 1-2 cm, blotted and incubated first with the anti-discoidin antibody (Wetterauer *et al.*, 1993), and then alkaline phosphatase coupled secondary goat-anti mouse antibody.

Just as the others, the REMI mutant 17-1-J-3-1 (taz^-, see below) was initially detected in a REMI screen as discoidin over-expression (figure 2-1). Compared to wild type Ax2 colony, in REMI mutant 17-1-J-3-1, discoidin protein was detected in vegetative growth beyond the visible border of the colony, where growing cells still have a sufficient amount of nutrients. Surprisingly, the discoidin over-expression phenotype was not always reproduced and REMI mutant 17-1-J-3-1 exhibited variable discoidin expression levels from over-expression to low-expression depending on the experiments. All efforts to control the culture conditions and to find out stable cell

lines failed and discoidin expression remained highly variable in colony blots. Meanwhile another phenotype (growth defect, see below for details) was perfectly reproducible. The colony blots shown in figure 2-1 are representative examples, where the discoidin expression is higher (figure 2-1B), normal (figure 2-1C) or lower (figure 2-1D) compared to that of wild type Ax2 (figure 2-1A).

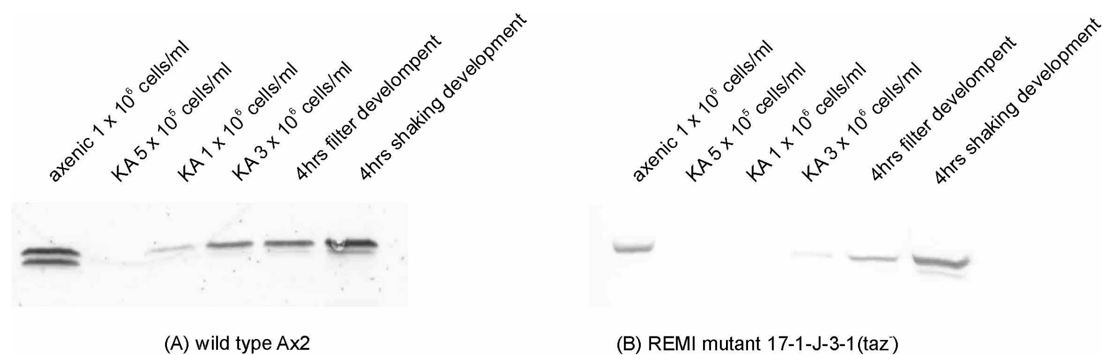


Figure 2-2. Western blots for discoidin expression. Cells of wild type Ax2 standard control and REMI mutant 17-1-J-3-1 were grown in KA suspension or axenic medium with agitation (180 rpm) and harvested at the cell densities indicated. At a density of 1 x 10^6 cells/ml, cells were harvested and set up for 4 hours development either in phosphate buffer suspension culture (2 x 10^7 cells/ml) or on filters (5 x 10^7 cells/3 cm diameter filter). Total protein was separated by SDS-PAGE, and discoidin I was detected by anti-discoidin antibody (Wetterauer *et al.*, 1993). Equal amounts of protein were loaded.

The western blot is another technique to detect discoidin I protein expression. For standard time course (see materials and methods for details), cells were grown in KA suspension and harvested at densities of 5×10^5, 1×10^6 and 3×10^6 cells/ml by differential centrifugation, the cells from 1×10^6 cells/ml were allowed to develop in shaking suspension or on filter for 4 hours. Expression of discoidin I was monitored by Western blot. As shown in figure 2-2A, when wild type Ax2 cells grow on a standard bacterial food source, discoidin I is first detected at a cell density around 1 x 10^6/ml, the amounts then gradually increase with further cell proliferation. When completely deprived of nutrients, another boost of expression is observed. The discoidin I expression of REMI mutant 17-1-J-3-1 was delayed (figure 2-2B) and could only be detected at high cell density (3 x 10^6 cells/ml), compared to wild type Ax2 where the discoidin expression can be detected from 1 x 10^6 cells/ml on. In addition, in REMI

mutant 17-1-J-3-1, the developmental expression levels of discoidin were lower than that of wild type Ax2.

Interestingly, unlike the variable colony blot results, the low discoidin expression phenotype in Western blots was perfectly reproducible, in both filter development and shaking development.

The low discoidin expression phenotype in Western blots was verified by Northern blot using an in vitro transcript of the discoidin Iγ gene as a hybridization probe (figure 2-3).

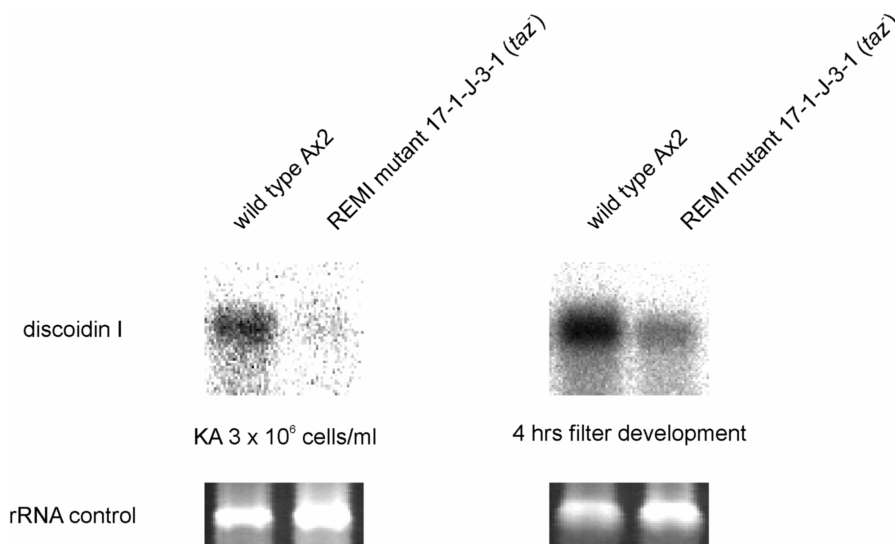
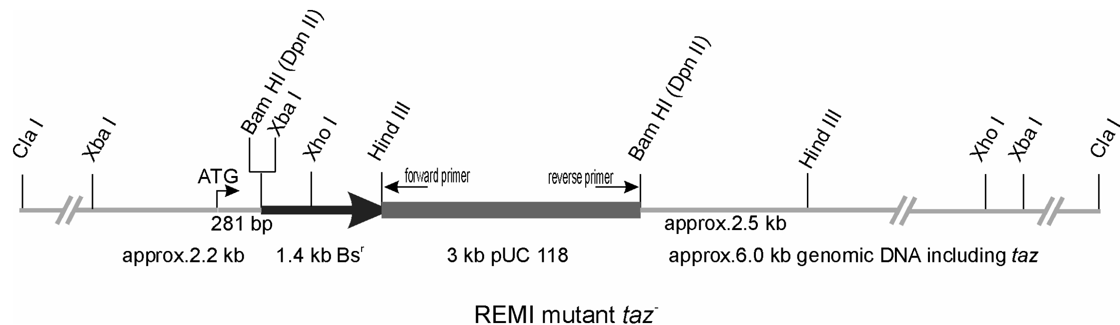


Figure 2-3. Northern blots for discoidin expression. Cells of wild type Ax2 and REMI mutant 17-1-J-3-1 were grown in KA suspension with agitation (180 rpm). RNA was isolated from the cells of wild type Ax2 and REMI mutant 17-1-J-3-1 at cell densities of 3×10^6 cells/ml and after 4 hrs filter development. 10 μg RNA were separated on a 2% agarose gel with 20 mM freshly prepared guanidine thiocyanate (GTC) and blotted onto nylon membrane. A ^{32}P labelled in vitro transcript of the discoidin Iγ gene was used as a hybridisation probe. 17s rRNA is shown as a loading control to indicate approximately equal loading.

Isolation of an approximate 10 kb genomic fragment from the integration site in REMI mutant 17-1-J-3-1



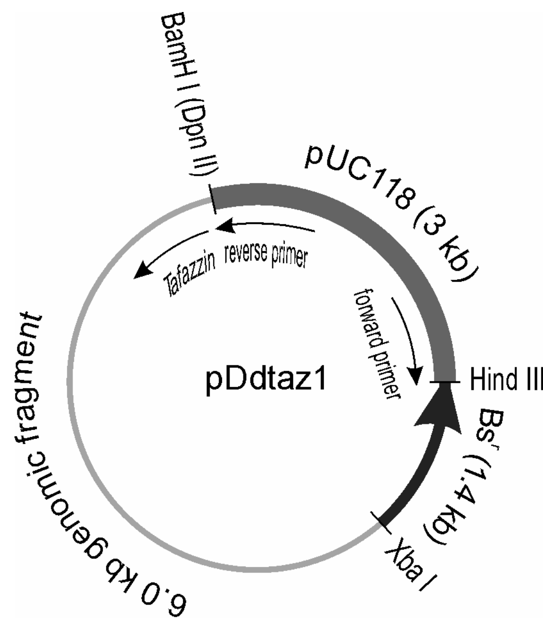


Figure 2-4. Physical map of REMI mutant 17-1-J-3-1 and rescue plasmid pDdtaz1.

(A). REMI mutant 17-1-J-3-1 was originally generated by insertion of *BamH* I linearized pUC118+Bs^R vector into a *Dpn* II site of the Ax2 genome (Zeng *et al.*, 2000A).

(B). A 10 kb *Xba* I digested genomic DNA included the entire integration plasmid (pUC118 + Bs^R) and an approximately 6 kb genomic fragment was isolated from REMI mutant 17-1-J-3-1 by plasmid rescue (See materials and methods for details), the resulting plasmid was termed pDdtaz1

An 6kb genomic fragment including approximately 800bp of the affected gene in REMI mutant 17-1-J-3-1 together with 4.4kb integrated transformation vector was isolated from the REMI mutant 17-1-J-3-1 by "plasmid rescue" (See materials and methods for details) by using *Xba*I digested genomic DNA (figure 2-3B).

The resulting plasmid termed pDdtaz1 was sequenced by reverse primer from the original integrated plasmid pUC118. The approximately 100bp sequence showed high homology to the human Barth syndrome responsible gene G4.5 (*tafazzin, taz*) by Blast search (http://www.expasy.ch/cgi-bin/blastEMBnt-CH.pl) and was used to fish the entire *Dictyostelium discoidieum tafazzin* orthologue from the Genome Sequence Center Jena, Germany (http://genome,imb-jena.de/dictyostelium). A 1041bp genomic DNA sequence with approximately 450bp non-coding region and 2 potential ATG start codons was obtained by overlapping random genomic clones: IIAFP1D41103, JC1b156g05.r1, JC1a25c03.r1, IIAFP1D84888, JC1b156g05.s1 (See appendix III, for details). For simplicity, the REMI mutant 17-1-J-3-1 will be denominated *taz*$^{-}$ in the following text.

From the multiple alignment (figure 2-9), we thus predict that the nonsense codon (TAA) at the 3 'end of the 1041bp sequence was probably the stop codon of the *Dictyostelium discoideum tafazzin* gene. 3'end RACE (Rapid Amplify cDNA End) was performed to check our prediction. cDNA was synthesized by reverse transcriptase using an oligo (dT)-adaptor primer - Smart-dT and total RNA isolated from axenically grown wild type Ax2 and *taz*$^{-}$ mutant.

A first round of PCR was performed using the *tafazzin* gene specific primer TAZ4 which is approximately 500bp downstream of gene disruption site (see appendix I for the position of the primer) and Smart-dT. This amplification resulted in only a smear of products of 100bp-400bp. These products were used as a template for a second round of PCR by using 'nested' tafazzin gene specific primer TAZ10, which is approximate 100 bp upstream of the TAA nonsense codon, and an adaptor primer – Smart. This primer pair is inside the sequence amplified in the first round PCR and a distinct 200bp product was detected (figure 2-5). Southern analysis indicated that this approximately 200bp PCR product is amplified from *tafazzin* gene (data not shown). Although the nested PCR product was not cloned and sequenced, its length and southern analysis suggested that this 3'end TAA nonsense codon could be the real stop codon. Additional, two AATAAA elements were found downstream of the TAA stop codon, probably poly A signals.

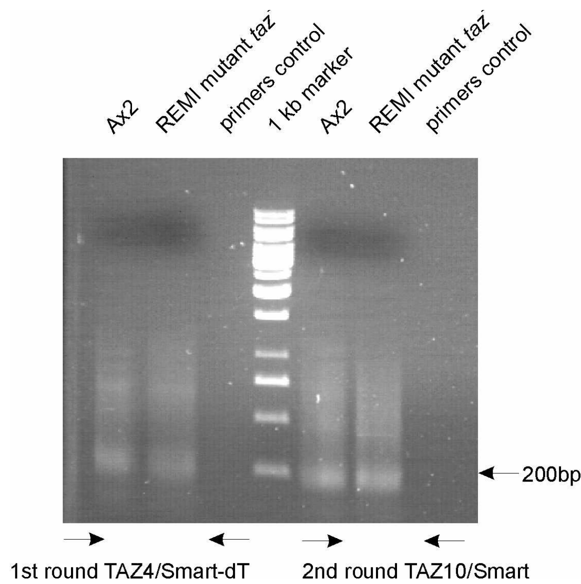


Figure 2-5.
Rapid amplification of 3' cDNA ends (3'RACE) of tafazzin gene.
3' end of *tafazzin* gene were amplified from the cDNA of wild type Ax2 and REMI mutant taz$^-$. 5 μl of the PCR products were separated on a 0.9% agarose gel by electrophoresis. The cDNA used is indicated on the top. The primers are indicated on the bottom.

There is no differential splicing in Dictyostelium discoideum tafazzin gene

The 1041bp genomic sequence obtained by overlapping randomly genomic clones was translated from different ATGs at N-terminal. Although there was no continuous open reading frame, the resulting amino acid sequence which was translated from the 4th ATG showed good similarity to the human *tafazzin* gene *(G4.5)* (figure 2-9). We thus predicted the existence of intron(s) and RT-PCR was performed to test our prediction.

The primers used for RT-PCR reaction (figure 2.6C) were TAZ7/TAZ8, TAZ7/8 primer pair nearly covered the entire coding region of the *tafazzin* gene and gave a 956bp PCR product, while the only RT-PCR product was approximately 780 bp, which was approximately 180bp smaller than the PCR product. The 780bp RT-PCR product was cloned into pGEM-T easy and sequenced. Sequence analysis indicated a 186bp intron in the middle region of *tafazzin* gene (See appendix I for details). Because this is the only intron in *Dictyostelium discoideum tafazzin* gene, we thus conclude that unlike human and *C. elegans tafazzin* genes, there is no differential splicing in the *Dictyostelium discoideum* orthologue.

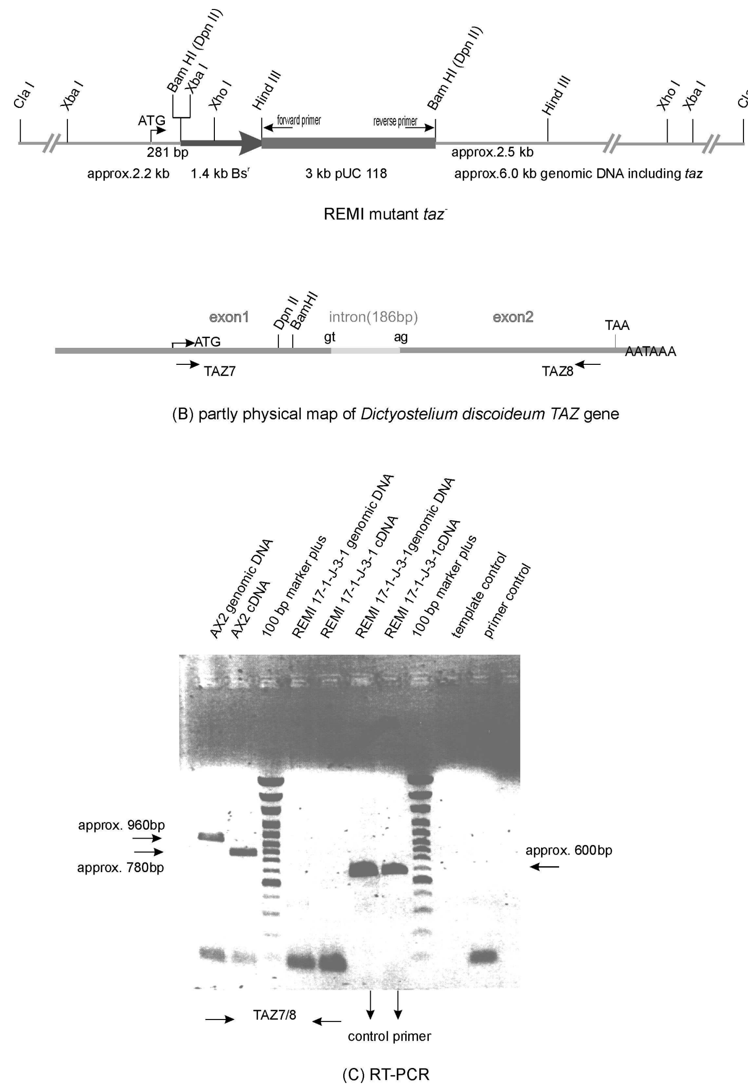


Figure 2-6. Genomic organization of the tafazzin gene

(A). REMI mutant *taz*-. (B). Schematic diagram of *Dictyostelium discoideum tafazzin* gene. The intron is from 370bp to 556bp after ATG start codon. Arrows indicate the orientation of the primers used in PCR and RT-PCR reactions. (C). PCR and RT-PCR reactions from wild type Ax2 and REMI mutant taz-. 5 µl of the PCR products were separated on a 0.9% agarose gel by electrophoresis. The DNA and cDNA used are indicated on the top, the primers are indicated on the bottom, a pair of ribosomal DNA primers is used as a positive control.

Verification of the tafazzin gene disruption

Disruption of the *tafazzin* gene was confirmed by Southern hybridization analysis (figure 2-7).

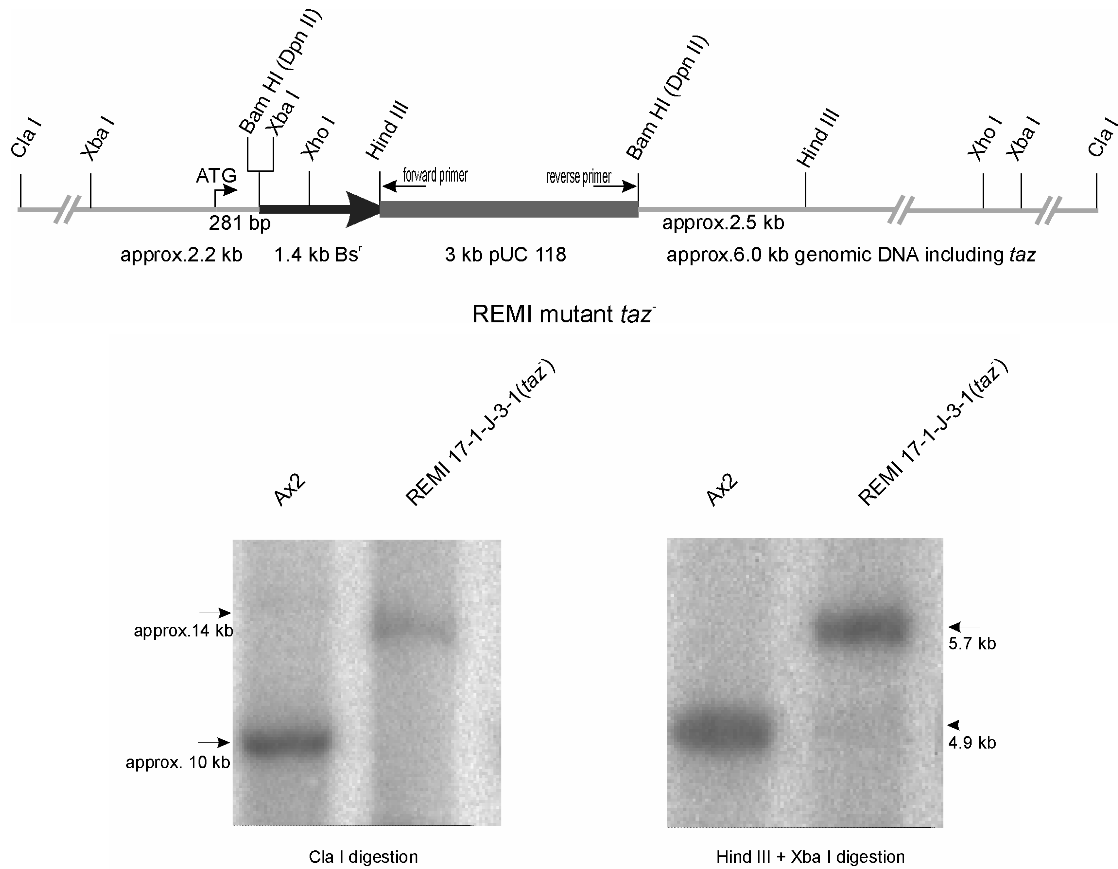


Figure 2-7. Analysis of genomic DNA from REMI mutant *taz*$^-$. Genomic DNA was prepared from wild type Ax2 and REMI mutant *taz*$^-$, digested with *Cla* I (left) or *Hind* III + *Xba*I (right), separated on 0.9% agarose gel, blotted to nylon membrane and hybridized with a ^{32}P labelled RT-PCR product of TAZ5/2 primer pair. Presence of the wild type allele is indicated by a 10 kb (*Cla* I digestion, left) or a 4.9 kb (*Hind* III + *Xba* I digestion, right) fragment, and the interrupted allele is indicated by a 14 kb (*Cla* I digestion, left) or a 5.7 kb (*Hind* III + *Xba* I digestion, right) fragment. The DNA used is indicated on the top. The restriction enzymes are indicated on the bottom. Locations of DNA are shown on the left or right

Genomic DNA from wild type Ax2 and REMI mutant *taz*$^-$ was isolated and digested with *Cla*I or *Hind* III + *Xba* I. A probe specific for the *tafazzin* gene was generated using the ^{32}P labelled RT-PCR product of the TAZ5/2 primer pair. For *Cla* I digestion,

in wild type Ax2, a 10 kb hybridizing band was seen, and in REMI mutant *taz*$^{-}$, an approximately 14 kb band consisting of predicted 10 kb from the genome and 4.4 kb from the integrated plasmid was found. *Xba* I cuts in the genome once approximately 2.2 kb upstream the tafazzin gene and once approximately 5 kb downstream the *tafazzin* gene, *Hind* III cuts once in the integrated plasmid and once in the genome approximately 2.5 kb downstream the *tafazzin* gene. For *Hind* III + *Xba* I digestion, in wild type Ax2, a predicted approximately 4.9 kb band was seen, and in REMI mutant *taz*$^{-}$, a predicted approximately 5.7 kb band was found.

The *tafazzin* gene disruption was also verified by PCR reactions (figure 2-8). A series of PCR reactions was performed by using genomic DNA from wild type Ax2, REMI mutant *taz*$^{-}$, and rescue plasmid DNA as templates and several primer pairs to confirm the *tafazzin* gene disruption. As shown in figure 2-8, when primer pair TAZ1/2 was used, the predicted approximately 580 bp PCR products should cover the *Dpn* II disruption site (239bp downstream the ATG start codon), under the conditions used, the PCR reaction cannot proceed successfully across the inserted 4.4 kb plasmid, therefore PCR products only can be obtained from wild type Ax2. When primer pair TAZ2/reverse, which is specific for the integrated plasmid pUC118, was used, PCR products only can be obtained from REMI mutant *taz*$^{-}$ and the rescued plasmids. When primer pair TAZ5/2 was used, all of the templates used should give PCR products because primer TAZ5 was designed approximately 60 bp downstream the *Dpn* II disruption site.

Taken together, the above results of southern analysis and PCR reactions confirm that the genomic structure of the *tafazzin* gene was altered in the REMI mutant *taz*$^{-}$.

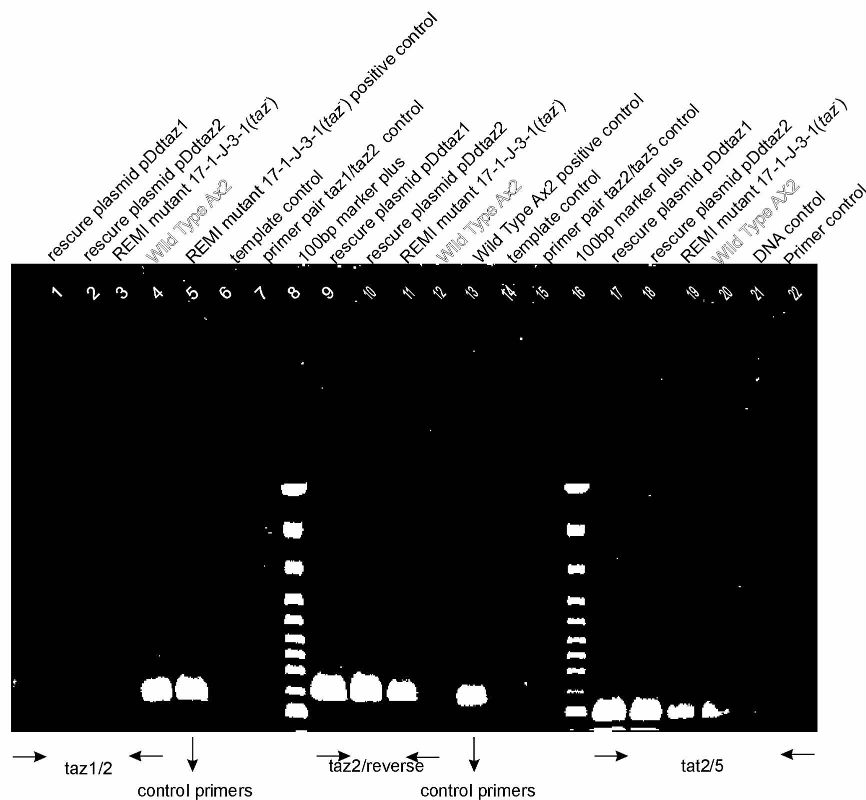


Figure 2-8. PCR reaction to verify gene disruption

Primer pair TAZ1/2 covers the *Dpn* II disruption site, under the conditions used, PCR did not proceed across the inserted plasmid (4.4 kb), therefore PCR product only from wild type Ax2 was obtained. PCR product was not obtained from wild type Ax2 using primer pair TAZ2/reverse because reverse primer is from inserted vector. Primer TAZ5 is approximate 60 bp downstream of the disruption site, therefore, products were obtained from all of them by using primer pair TAZ5/2. A pair of ribosomal DNA primer was used as a positive control.5 μl of the PCR products were separated on a 0.8% agarose gel by electrophoresis. The DNA used is indicated on the top and the primers used on the bottom.

Dictyostelium discoideum tafazzin protein shows high similarity to its orthologues from different organisms

Dictyostelium discoideum tafazzin gene defines a putative 855 bp open reading frame encoding a 285 amino acid protein with a predicted molecular weight of 30.875 kDa. *Dictyostelium discoideum* tafazzin protein showed 35% identity and 57% similarity to its human orthologue (figure 2-9 lower part). Moreover, the identity or similarity

```
                                 10        20        30        40        50        60
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  ------------------------------------------------------------  Dictyostelium
Yeast          ------------------------------------------------------------  Yeast
Human          ------------------------------------------------------------  Human
Fly            MVVCSNLRRPGHVGAASAARNINWLISEGYTPPIRAMARPYVQAPEARPVPDERYPGSQQ  Fly
Arabidopsis    ------------------------------------------------------------  Arabidopsis
Worm           ------------------------------------------------------------  Worm

                                 70        80        90       100       110       120
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  -----------------------------------------------MDSNNSNNNNKNL  Dictyostelium
Yeast          -----------------------------------------------MSFRDVLERGDEF  Yeast
Human          -------------------------------------------------------MPLHV  Human
Fly            DRKDIATQTVRSSKPKDLRPPSPPTPSQTLNSSSLPPPMSDQDADPSLDVPTGVAMPYNI  Fly
Arabidopsis    -------------------------------------------------------MGKIM  Arabidopsis
Worm           ------------------------------------------------------------  Worm

                                130       140       150       160       170       180
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  KQICDIP--KP--QFLSKGVFTLV-GVLCKFWISMNTATTSGIDKLVNEIDKTHQLKRPM  Dictyostelium
Yeast          LEAYPRR--SPLWRFLSYSTSLLTFGVSKLLLFTCYNVKLNGFEKLETALERSKRENRGL  Yeast
Human          KWPEPAV--PPLTWTLASSVWMGLVGTYSCFWTKYMNHLTVHNREVLYELIEKRGPATPL  Human
Fly            DWIFPRLRNPSKFWYVVSQFVVSAVGIFSKVVLMFLNKPRVYNRERLIQLITKRPKGIPL  Fly
Arabidopsis    EWAARS----DHLGGIPRNTVIMAVSAFAKAVANLCNKSSVHNADTLMNLVQSRPPGVPL  Arabidopsis
Worm           --------------------MSLVTSVSKLMFLGGSNKLICHNKETFVKILEN--PNQPL  Worm

                                190       200       210       220       230       240
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  ITIANHSSNLDDPLLWGVLPNRILMDPS-KQRWTLGASNILFTNWFYSKFFSLGKCVSFY  Dictyostelium
Yeast          MTVMNHMSMVDDPLVWATLPYKLFTSLD-NIRWSLGAHNICFQNKFLANFFSLGQVLSTE  Yeast
Human          ITVSNHQSCMDDPHLWGILKLRHIWNLK-LMRWTPAAADICFTKELHSHFFSLGKCVPV-  Human
Fly            VTVSNHYSCFDDPGLWGCLPLGIVCNTY-KIRWSMAAHDICFTNKLHSLFFMFGKCIPV-  Fly
Arabidopsis    ITVSNHMSTLDDPVMWGAFKGLLSLDPE-LARWVLAAEDICFRNPIFSYIFRTGKCIPI-  Arabidopsis
Worm           ITVSNHRSNIDDPLMWCILKFREFWRYKDRNRYTLAAHNICFTKQFHTTMESLGRCVPC-  Worm

                                250       260       270       280       290       300
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  INIIIKIKNFIDFYFFYFLFFIFFFEKFYNIFFFFYFFLFLFFFFYFYFKFIKIKIKIVR  Dictyostelium
Yeast          ------------------RFGVGPFQGSIDASIRLLSPDDTLDLEWTPHSEVSSSLKKAY  Yeast
Human          ----------------------------CRGAEFFQAENEGKGVLDTGRHMPGAGKRREK  Human
Fly            ----------------------------VRGI----------------------------  Fly
Arabidopsis    ----------------------------TRGG----------------------------  Arabidopsis
Worm           ----------------------------VRGE----------------------------  Worm

                                310       320       330       340       350       360
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  GDGIYQDGMNESIDRLSEGQWLHIFPEGRISQ-----QTQLLYFKWGLGRLVGECYRRTG  Dictyostelium
Yeast          SPPIIR----------SKPSWVHVYPEGFVLQLYPPFENSMRYFKWGITRMILEATKPPI  Yeast
Human          GDGVYQKGMDFILEKLNHGDWVHIFPEGKVNMS----SEFLR-FKWGIGRLIAECHLNPI  Human
Fly            --GVYQDAINLCIEKAALGHWIHVFPEGKVNMD----KEELR-LKWGVGRIIYESPKIPI  Fly
Arabidopsis    --GIYQENMNEALQRLKDGSWLHTFPEGKVFQD----DVPIRRLKWGTASLIARSPVTPI  Arabidopsis
Worm           --GVYQKGMDFCVDMLNDNKWVHIFPEGKVCTL----ESEPLRFKWGIGRLVMDAKTDPV  Worm

                                370       380       390       400       410       420
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  VVPLVVPIYHQGMEKSMP--LAKLPIPR-VGINLDNKVGDNIYCDQV----------ISK  Dictyostelium
Yeast          VVPIFATGFEKIASEAVTDSMFRQILPRNFGSEINVTIGDPLNDDLIDRYRKEWTHLVEK  Yeast
Human          ILPLWHVGRNDVLPNS-------PPYFPRFGQKITVLIGKP--FSALPVLE---RLRAEN  Human
Fly            ILPMWHEGMDDLLPNV-------EPYVIQRGKQVTLNVGQP--LDLNDFIL---DLKKRQ  Fly
Arabidopsis    VLPIIHRGFEEMMPENYNNG--RRPLVPLPNKHLKVVVGEPIEFDVPMMVE---TAVLDS  Arabidopsis
Worm           ILPVWCKEMEKVWPTQ-------PPYYPKFGNTVTVHIGEP--FFLSDLKK---TVLSKS  Worm

                                430       440       450       460       470       480
                        ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  YIDDNKISDLTDYLSQDDKKRKDFYKTITLHIEDEYQKIIPPTNRGRFSHPTIKD-----  Dictyostelium
Yeast          YYDPKNPNDLSDELKYG-KEAQDLRSRLAAELRAHVAEIRNEVRKLPREDPRFKSPSWWK  Yeast
Human          KSAVEMRKALTDFIQEEFQHLKTQAEQLHNHLQPG-R-----------------------  Human
Fly            VPEPTARKLITDKIQEAFRDLRAETEKLHRERN---------------------------  Fly
Arabidopsis    RHVTPPLQEVKWPVLTSAGQVLDETAQRHLYIALS-EKIQSSLETLRLLAKRL-------  Arabidopsis
Worm           LTTEQMRKIITDEVQTRMYQLGEKVGDLPKGSSLE-ILRKNPPIEY--------------  Worm

                                490       500       510       520
                        ....|....|....|....|....|....|....|....|
Dictyostelium  ----------------------------------------                      Dictyostelium
Yeast          RFNTTEGKSDPDVKVIGENWAIRRMQKFLPPEGKPKGKDD                      Yeast
Human          ----------------------------------------                      Human
Fly            ----------------------------------------                      Fly
Arabidopsis    ----------------------------------------                      Arabidopsis
Worm           ----------------------------------------                      Worm
```

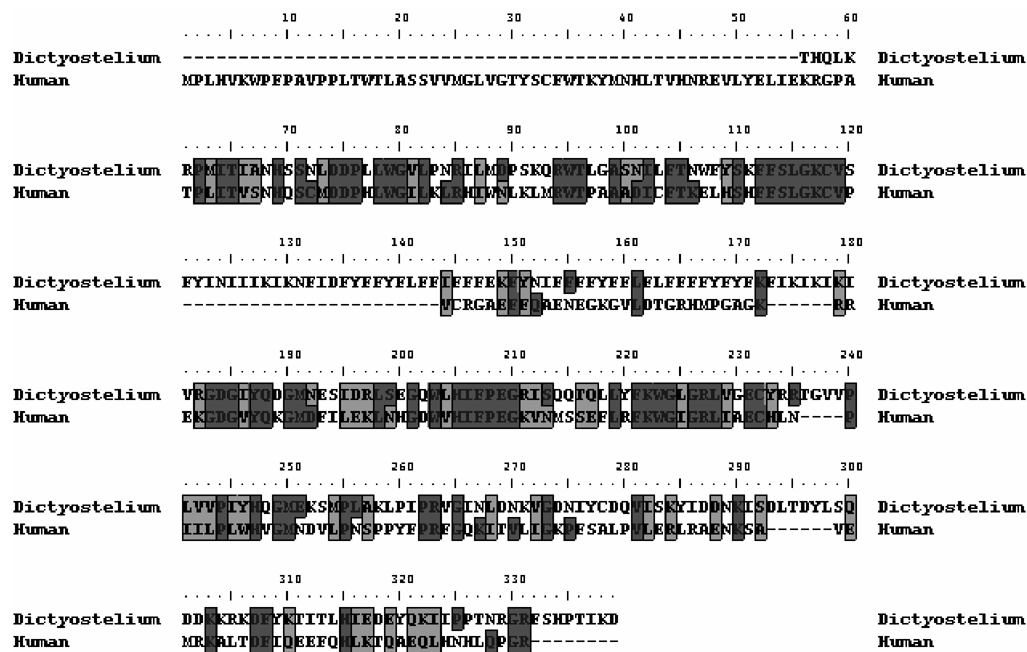


Figure 2-9. Multiple alignment of tafazzin proteins from different organisms

Upper part: Comparison of known sequences of tafazzin (or putative proteins) from *Homo sapiens* (Q16635), *Drosophila melanogaster* (AAL48681), *Caenorhabditis elegans* (Q23589), *Saccharomyces cerevisiae* (Q06510) and Arabidopsis thaliana (AAF64532).

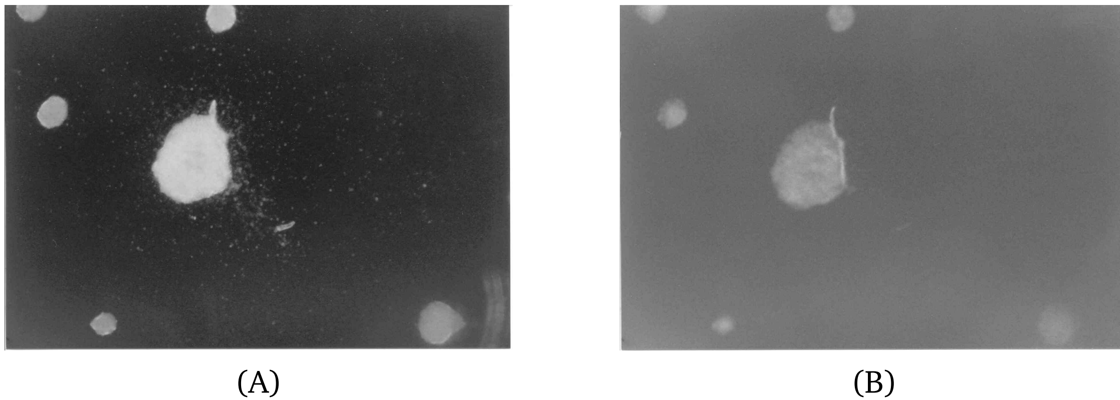
Lower part: Alignment of tafazzins from *Homo sapiens* (Q16635) and *Dictyostelium discoideum*. *Dictyostelium discoideum* tafazzin gene product is 35% identity and 57% similarity to its human orthologue.

Red: identity. Green: similarity. MultiAlign tool (Corpet *et al*., 1988) was used to perform the above alignment (http://www.toulouse.inra.fr/multalign.htm

existed through the entire amino acid sequences, the only part lacking similarity was from exon 5 of the human tafazzins, which in fact does not exist in most of the isoforms. Significant similarity to tafazzin orthologue from *Drosophila melanogaster* (Q9V6G5), *Saccharomyces cerevisiae* (Q06510) from *Ceanorhabditis elegans* (Q23598) were also found (figure 2-9 upper part).

Cells of REMI mutant taz⁻ can compete with wild type cells to enter development stage

In order to investigate if *taz*⁻ caused defect during development, a GFP transformation vector pDdA15gfp was transformed into both *taz*⁻ mutant and wild type Ax2 cells. Cell sorting experiments were performed by mixture the GFP labeled cells and non-labeled cells in the different rations and setting up for development.



(A) (B)

Figure 2-10. Cells of REMI mutant *taz*⁻ can compete with wild type cells to enter developmental cycle. Cells of REMI mutant *taz*⁻/pDdA15gfp and wild type Ax2 were grown in axenic medium with agitation (180 rpm) and harvested at the density of 1 x 10^6 cells/ml, washed twice and resuspended in phosphate to a density of 2 x 10^7 cells/ml. Cells of REMI mutant *taz*⁻/pDdA15gfp and wild type Ax2 were mixed in a ratio of 1:1 and set up for development over nigh in Costar plate. (A). The image of the cells in aggregates. (B). The image of GFP labelled cells of *taz*⁻ mutant in same aggregates. Images were captured by an OLYMPUS OM-4 camera mounted Leica DM IRB microscope equipped with appropriate filter sets to allow visualization of fluorescein and with a 40 × objective lens.

GFP labeled cells of *taz*⁻ mutant were mixed with non-labeled wild type cells in the ratio of 1:1 and set up for development in Costar plate over night (figure 2-10). Figure 2-10A shows the image of the cells in aggregates and figure 2-10B shows the image of GFP labeled cells of *taz*⁻ mutant in same aggregates, where the mutant cells are randomly dispersed in the whole aggregates. The same result was obtained by using GFP labeled wild type cell and non-labeled mutant cells (data not shown).

When the same cell sorting experiments were set up on a slide covered with a thin agarose sheet, the cells of *taz*⁻ mutant were found to scatter randomly throughout the

entire development stage, from streaming, to slug, to final fruiting body (data not shown).

These results demonstrate that the cells of *taz*⁻ mutant can compete against wild type cells and enter developmental stage in the same way as wild type cells.

REMI mutant taz⁻ exhibits aberrant cardiolipin metabolism

As reported by Vreken *et al*. (2000) and Bissler *et al*. (2002), disruption of the human *tafazzin* gene which may involve in cardiolipin remodelling leads to a defect in phospholipid metabolism. We thus investigated phospholipid metabolism of *Dictyostelium discoideum* cells. Our preliminary results showed that in the REMI *taz*⁻ mutant, the cardiolipin biosynthesis was normal (data not shown) but as shown in figure 2-11, the cardiolipin pool size of REMI mutant *taz*- was 80% reduced compared to that of wild type Ax2, while the other phospholipid classes were normal (data not shown). Our preliminary phospholipid assay indicates that *Dictyostelium discoideum* tafazzin has the same function as its human orthologue.

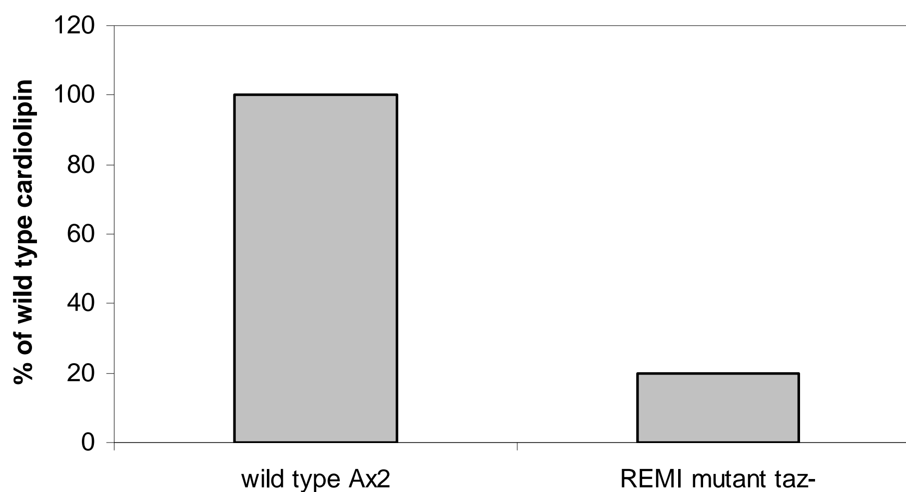


Figure 2-11. Aberrant cardiolipin metabolism. Cardiolipin pool size is 80% reduced compared to that of wild type Ax2 (data from collaboration with P. Vreken).

REMI mutant taz⁻ shows growth defect

As reported previously from Yeast *Saccharomyces cerevisiae* and Chinese hamster ovary (CHO) (Jiang *et al.*, 1999 and 2000; Kawasaki *et al.,* 1999; Ohtsuka *et al.*, 1993A and B, Ostrander *et al.*, 2001; Tuller *et al.*, 1998; Verken, personal communication), defects in enzymes involved in the biosynthesis of cardiolipin pathway lead to a loss of viability at elevated temperature. We thus examined the growth of *Dictyostelium discoideum* cells. Pre-warmed axenic medium and KA suspension were inoculated with exponentially growing cells from wild type Ax2 or REMI mutant *taz*$^{-}$, and incubated at 15°C, 22°C or 27°C with agitation (180rpm) (figure 2-12). When cells of REMI mutant *taz*$^{-}$ grew in KA suspension, they displayed no temperature sensitivity compared to wild type Ax2 cells and grew with the same doubling time as the wild type Ax2 at different temperatures (15°C, 22°C or 27°C) (figure 2-12A-C). However, when grown in axenic medium (figure 2-12 D-F), at 15°C REMI mutant *taz*$^{-}$ had significant growth defect compared to the wild type Ax2. After increasing the growth temperature to 22°C, which is the normal laboratory culture temperature for *Dictyostelium discoideum*, the mutant only had slight growth defect compared to the wild type Ax2 cells. At 27°C, mutant cells exhibited no temperature sensitivity and grew with the same doubling time as wild type Ax2 cells (table 2-1). It is worth to mention here that the growth defect phenotype of REMI mutant *taz*$^{-}$ is perfectly reproducible independent of the variability in the colony blot results

Table 2-1 Generation time of Ax2 and REMI mutant *taz*$^{-}$ at different temperature (hours)

	Growth in KA suspension	Growth in axenic medium
Ax2 (15°C)	≅ 9	≅ 20-26
REMI mutant *taz*$^{-}$ (15°C)	≅ 9	> 50
Ax2 (22°C)	≅ 4	≅ 8
REMI mutant *taz*$^{-}$ (22°C)	≅ 4	≅ 10
Ax2 (27°C)	≅ 3-4	≅ 7-8
REMI mutant *taz*$^{-}$ (27°C)	≅ 3-4	≅ 7-8

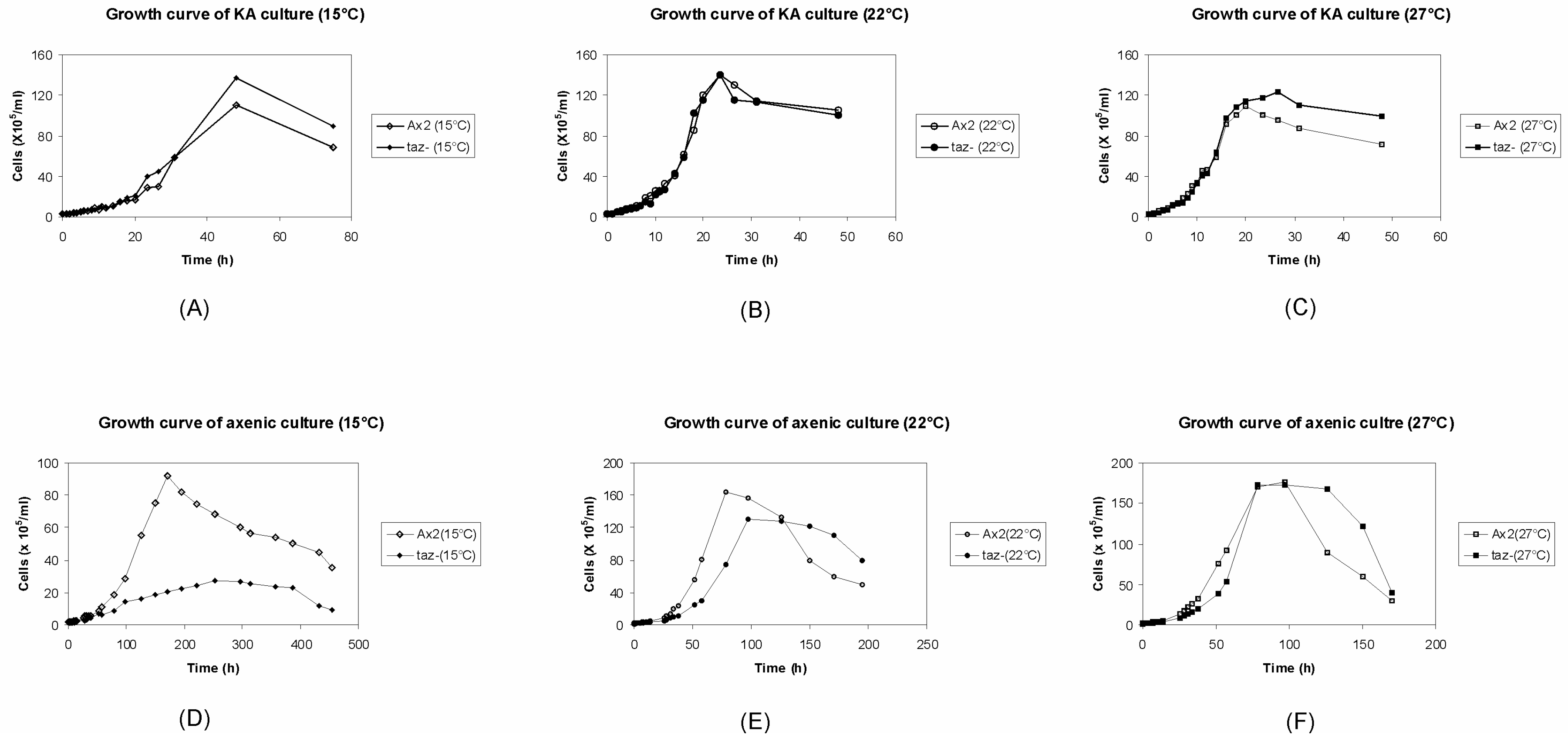


Figure 2-12. Growth curves. Prewarmed axenic medium and KA suspension were inoculated with exponential cells from wild type Ax2 and REMI mutant *taz*⁻ and incubated at 15°C, 22°C or 27°C with agitation (180rpm). At the times indicated, the cells were counted microscopically. The experiment was performed in duplicate at least three times.

Genomic disruption (knock-out, K. O.) of the tafazzin gene via homologous recombination

REMI is a random mutagenesis. By this technique, while one specific gene is disrupted, defects in different parts of the genome may be caused due to the introduced restriction enzyme, although it has been reported that *Dictyostelium discoideum* has exceptionally powerful DNA repair mechanisms which may prevent damage from restriction enzymes (Deering, 1998). To unambiguously confirm the phenotypes of the t*afazzin* mutant, the gene was knocked out by homologous recombination which is a targed, specific mutagenesis.

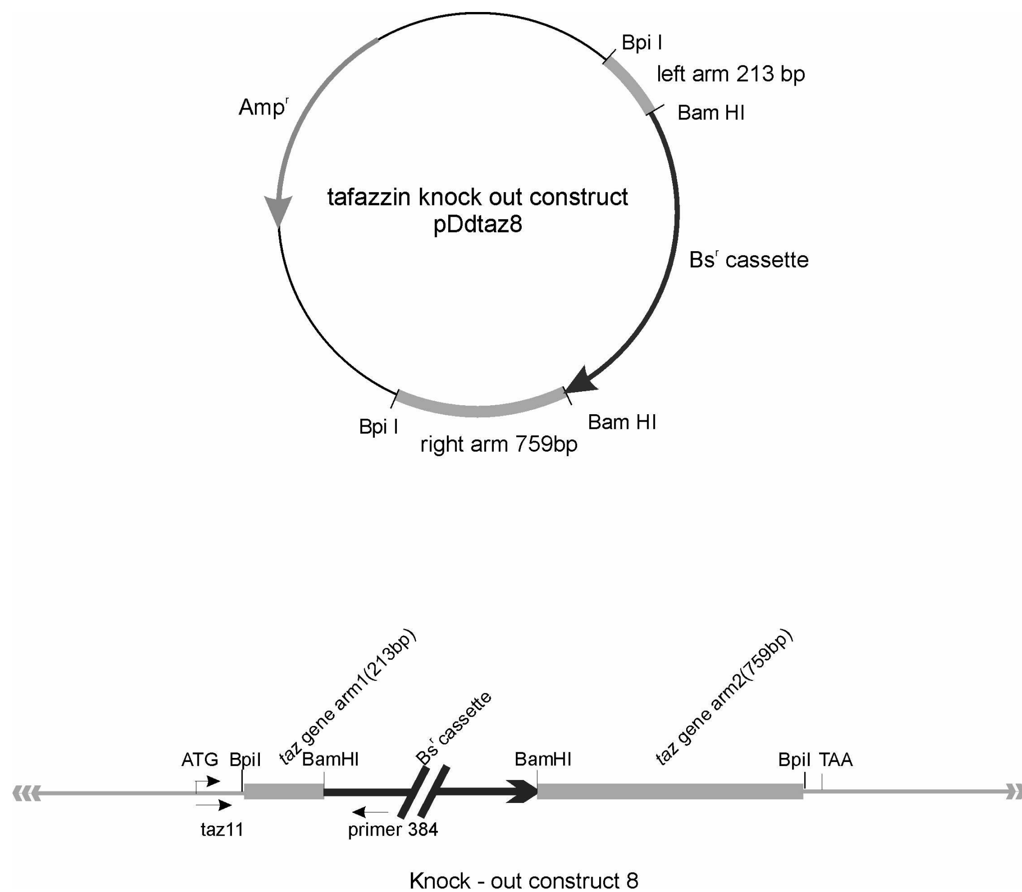


Figure 2-13. Knock-out construct

(A).The plasmid pDdtaz8 of knock-out construct.. 963bp PCR fragment of the *tafazzin* gene was cloned into the pGEM T-easy vector, the *Bam*H I site in this fragment was used to insert the Bs^R cassette. *Bpi* I sites are present in PCR primers flanking the entire fragment and allow for excision of the whole construct from the plasmid.

(B).Schematic diagram of knock out construct 8. Arrows indicate the orientation of the primers used in PCR reactions (figure 2-14)

The disruption vector was constructed as follows (figure 2-13): The *Dictyostelium discoideum tafazzin* gene was amplified by PCR using the TAZ7/TAZ8 primer pair and genomic DNA from wild type Ax2 as a template. This 972 bp PCR product was cloned into pGEM T – easy which is a specific clone vector for PCR products, then the Bs^R cassette (containing the blasticidin resistance gene under control of the actin-15 promoter (Sutoh, 1993)) was inserted into the *BamH* I site of the tafazzin gene cloned in the pGem T-easy. The resulting plasmid termed pDdtaz8 was digested with *Bpi* I that recognizes AGGAGG, but cuts 2-6 bp downstream of the recognition site. The Bs^R cassette flanked by two arms of 213 bp and 759 bp was purified from an agarose gel and electroporated into *Dictyostelium discoideum* Ax2 cells. Transformed cells were grown under blasticidin selection (10 μg/ml) on Petri dishes. After 10 days to 2 weeks, resistant cells were plated on KA plates in order to obtain single clones. 24 clones were reselected under blasticidin selection on Costar plates, some of these clones lost blasticidin resistance. DNA was prepared from the resistant single clones and a PCR strategy was used to check the gene disruption (figure 2-14).

PCR was performed by using primer TAZ11 which binds to the *tafazzin* gene, but 34 bp upstream of the left disruption arm (see appendix I, for the position of the primer used), and primer No. 384, which specifically binds to the coding region of the Bs^R cassette. Due to specific binding characteristics of the primer pair, PCR products can be obtained only when the Bs^R cassette is integrated into the correct locus. As shown in figure 2-14, 4 positive clones out of 12 blasticidin resistant clones were found. The reason that PCR product from the REMI mutant *taz*$^-$ is slightly smaller than those of knock-out clones is that the gene disruption site of original REMI mutant (Dpn II) is approximately 50 bp upstream of the secondary gene disruption site (*Bam*H I)

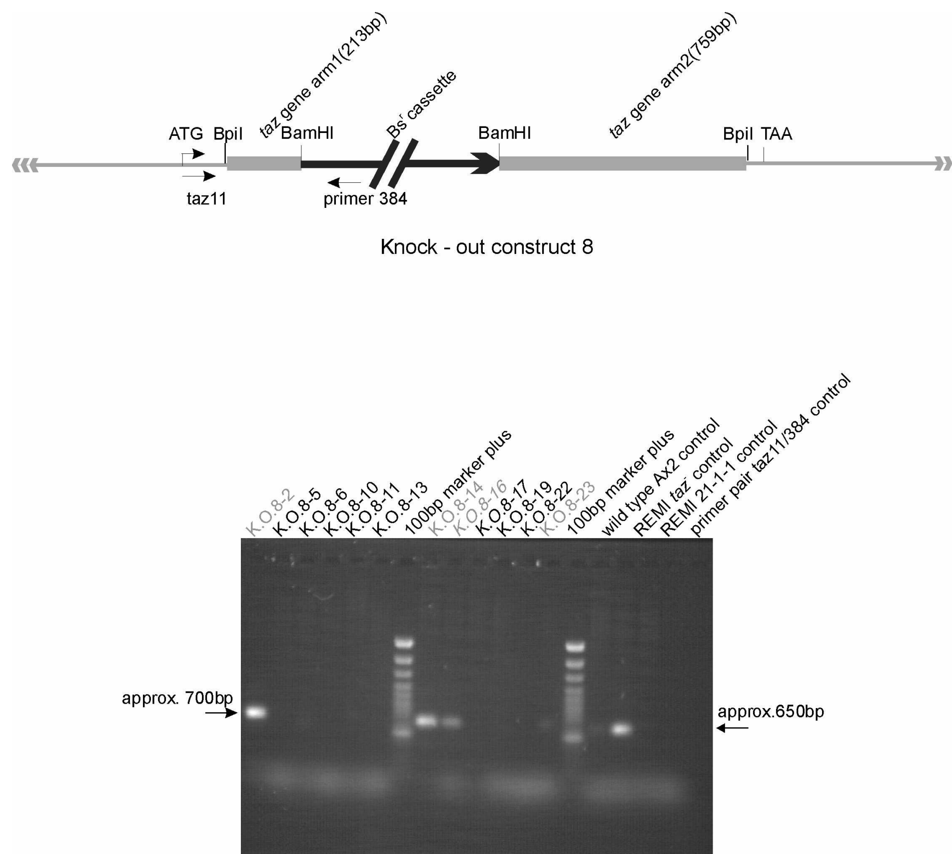


Figure 2-14. PCR analysis for the *tafazzin* gene disruption. Upper: physical map of knock out construct 8. Lower: PCR analysis for the *tafazzin* gene disruption. PCR products only from positive clones and REMI mutant *taz*$^{-}$ were obtained using the TAZ11/384 primer pair where TAZ11 binds to the tafazzin gene upstream outside the recombination arm, 384 binds specifically within the coding region of the BS^{R} cassette, PCR products from the REM mutant *taz*$^{-}$ is slightly smaller than that of knocked- out clones. 5 μl of the PCR products were separated on a 0.9% agarose gel by electrophoresis. The DNA used is indicated on the top.

Southern analysis to verify the secondary disruption

The secondary gene disruption was also confirmed by southern analysis (figure 2-15). Genomic DNA from 4 positive secondary disruption strains and REMI mutant *taz*$^{-}$ were isolated and digested with *Xho* I (see figure 2-4 and 2-14 for physical maps), A probe specific for the *tafazzin* gene was generated by using ^{32}P labelled RT-PCR product of the TAZ5/2 primer pair. *Xho* I cuts once in the Bs^{R} cassette and once in genomic DNA approximately 4-5 kb downstream of the two disruption sites. Therefore in secondary disruption strains, a single fragment of approximately 5 kb containing 0.6 kb from the C-terminal of the Bs^{R} cassette and approximately 4.4 kb from genomic DNA was seen.

In REMI mutant *taz*$^-$, a 8 kb fragment consisting of 0.6 kb Bs^R cassette, the whole integrated plasmid pUC118 (3 kb) and an approximately 4.4 kb genomic DNA fragment was observed. The hybridization bands of knock out strains differed from that of REMI mutant *taz*$^-$ 3 kb by the size of the integrated plasmid pUC118. The reason why genomic DNA from wild type Ax2 was not included here is that another *Xho* I restriction site locates upstream of the disrupted *tafazzin* gene (> 10 kb). In southern blots, *Xho* I digested genomic DNA from wild type Ax2 gave a weak, smear signal of approximately 15-20kb (data not shown).

The above PCR reactions and southern analysis indicate that the *tafazzin* gene was disrupted by homologous recombination.

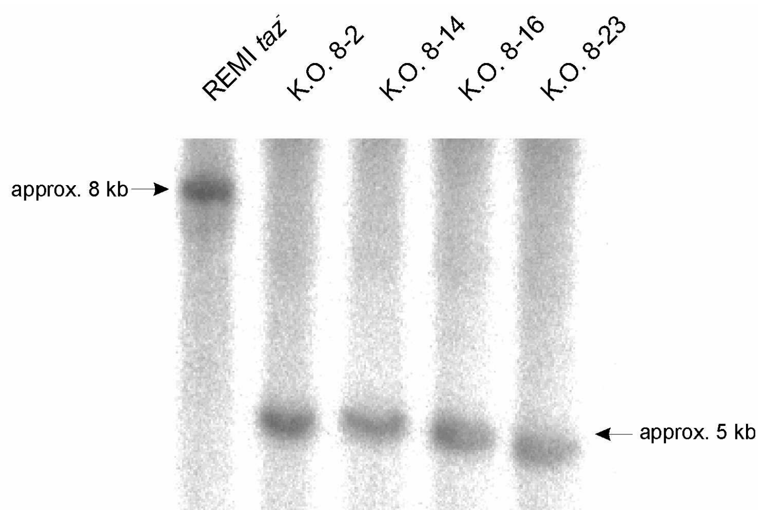


Figure 2-15. Analysis of genomic DNA from the knock out mutant.
Genomic DNA was prepared from 4 positive knock out clones and REM mutant *taz*$^-$, digested with *Xho* I, separated on 0.9% agarose gel, blotted onto nylon membrane and hybridized with a ^{32}P labeled probe (by oligo-labeling) of the TAZ2/5 RT-PCR product. A single fragment of approximate 8 kb was seen in REM mutant *taz,* and in knock out clones, a single fragment of approximate 5 kb was observed.

The secondary disruption strains display discoidin mis-expression

Because the original REMI mutant *taz*⁻ exhibits discoidin mis-expression, colony blots and Western blots were performed to investigate the discoidin expression pattern of secondary disruption strains (figure 2-16 and 2-17).

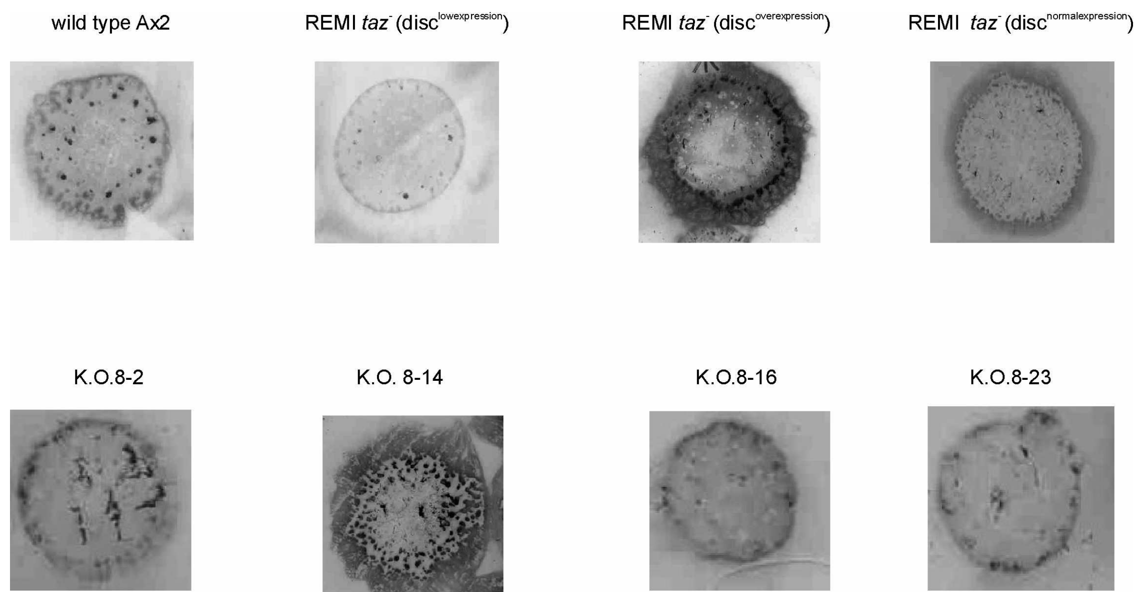


Figure 2-16. Colony blots for discoidin expression. Cells of wild type, REMI mutant *taz*⁻ and 4 knock out strains were grown on KA plates to a diameter of approximate 1-2 cm, blotted and incubated first with the anti-discoidin antibody (Wetterauer *et al.*, 1993), and then alkaline phosphatase coupled secondary goat – anti mouse antibody.

Interestingly, like the original REMI mutant *taz*⁻ that showed different levels of discoidin expression ranging from high to low compared to that of wild type Ax2 cells (figure 2-1), the 4 disruption strains also exhibited different levels of discoidin expression on colony blots. As shown in figure 2-16, K.O.8-14 showed high expression of discoidin. In comparison to a wild type Ax2 colony, in K.O.8-14, discoidin protein was found in cells beyond the visible border of the colony. K.O.8-16 showed nearly the same discoidin expression pattern as wild type Ax2, while K. O. 8-2 and K. O. 8-23 showed slightly higher or lower discoidin expression, respectively, than wild type Ax2.

Despite of the different levels of discoidin expression in colony blots, in Western blots, all the 4 disruption strains exhibited low discoidin expression compared to that of wild

type Ax2. Figure 2-17 shows the Western blots from K. O.8-2 and K.O.8-14, which represented discoidin low or high expression respectively on colony blots (figure 2-16). When the cells reached a density of 1×10^6 cells/ml, the discoidin protein expression can only be detected in wild type Ax2 cells. After 4 hours of development, discoidin expression can be detected in all strains, but both REMI mutant *taz*⁻ and two secondary disruption strains exhibited a weaker signal than that of wild type Ax2. In addition, all three disruption strains showed nearly the same discoidin expression level (figure 2-17).

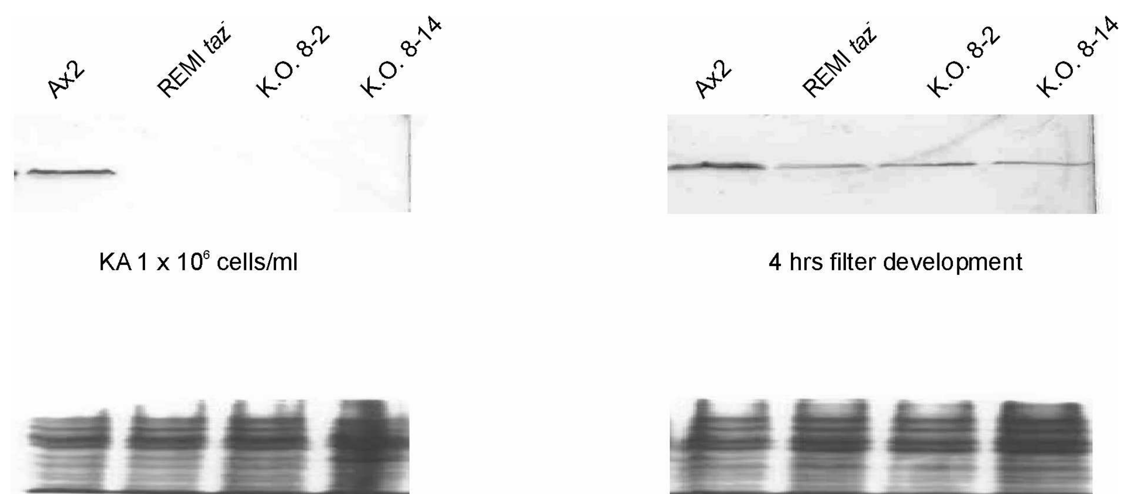


Figure 2-17. Western blots for discoidin expression. Cells from wild type Ax2, REMI mutant *taz*⁻, knock out construction K.O.8-2 and K.O.8-14 were grown in KA suspension with agitation (180 rpm) and harvested at the cell densities of 1×10^6 cells/ml, washed free of the bacteria by differential centrifugation and allowed to develop on filters for 4 hours. Total protein was separated by SDS-PAGE, and discoidin I was detected by anti-discoidin antibody (Wetterauer *et al.*, 1993). Coomassie stained SDS-PAGEs are used as a loading control

Knock out strains exhibit an axenic growth defect at 15°C

The secondary disruption strains were examined to investigate whether the axenic growth defect phenotype of the original REMI mutant *taz*⁻ could be reproduced. Prewarmed axenic medium was inoculated with exponentially growing cells from wild type Ax2, REMI mutant *taz*⁻, and two secondary disruption strains: K.O.8-2 and K.O.8-14 (figure 2-18). All the three gene disruption strains exhibited significant axenic growth defects at 15°C compared to that of wild type Ax2 cells (table 2-2).

The growth of a REMI mutant 21-1-1(blasticidin resistance) was monitored and found its axenic growth was normal at 15°C compared to that of wild type Ax2 cells (data not shown), indicating that the phenotype of axenic growth defect at 15°C is due to the *tafazzin* gene disruption, not a common phenotype of blasticidin resistant strains.

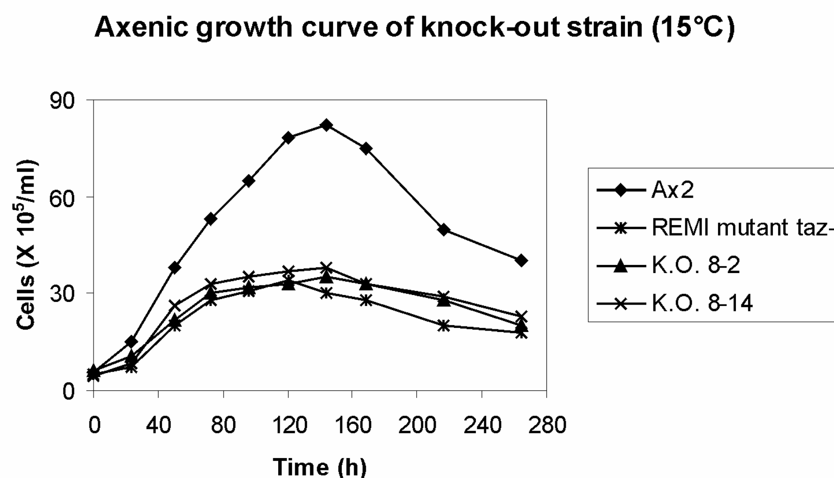


Figure 2-18. Axenic growth curves at 15°C. Prewarmed axenic medium was inoculated with exponentially growing cells from wild type Ax2, REMI mutant *taz*$^-$, K.O.8-2 and K.O.8-14, and incubated at 15°C with agitation (180 rpm). Cells were counted microscopically.

Table 2-2 Generation time of axenic growth at 15°C (hours)

	Wild type Ax2	REMI mutant *taz*$^-$	K.O.8-2	K.O.8-14
Generation time	≅ 24	> 60	>60	>60

Taken together, all the above results indicate that the axenic growth defect at 15°C and discoidin mis-expression phenotypes of original REMI mutant *taz*$^-$ and secondary gene disruption strains are due to the defect in the *Dictyostelium discoideum tafazzin* gene.

Construction of GFP tagged tafazzin

The Neuwald hypothesis (1997) and the work of Vreken *et al.* (2000) and Bissler *et al.* (2002) suggest that the human *tafazzin* gene (*G4.5*) may encode an acyltransferase involved in the deacylation and reacylation of cardiolipin side chain, or at least a co-factor required for the function of such enzymes. Cardiolipin and two most important enzymes involved in cardiolipin biosynthesis, PGP synthase and CL synthase, are exclusively found in mitochondrial inner membranes. We thus predicted that tafazzin was associated with mitochondria.

An expression vector (figure 2-19) encoding a fusion protein, in which the green fluorescent protein (GFP) of *Aequorea victoria* was attached to the carboxyl terminus of *Dictyostelium discoideum* tafazzin, was constructed by cloning the PCR product of the TAZ 17/19 primer pair (see appendix I for the positions of the primers) into the *Bgl* II restriction site of the *Dictyostelium discoideum* transformation vector pDdA15gfp (*Gerisch et al*., 1995).

The resulting plasmid termed pDdtaz15 was transformed into *Dictysotelium discoideum* cells of REMI mutant *taz*$^{-}$ and wild type Ax2 by the calcium method (Nellen *et al*., 1984). For comparison, the GFP protein alone was expressed by transforming the plasmid pDdA15GFP alone into *Dictyostelium discoideum* cells. Transformed cells were grown under G418 selection (10 μg /ml) (for the transformants in the mutant background, blasticidin and G418 double selection were used). After 10 days to 2 weeks, G418 resistant cells were plated on KA plates in order to obtain single clones. Individual clones were reselected under G418 selection on Costar plates, most of them still kept G418 resistant. Resistant cells were washed, diluted in phosphate buffer and GFP fluorescence images (figure 2-21) were taken by a conventional immunofluorescence microscope with CCD camera.

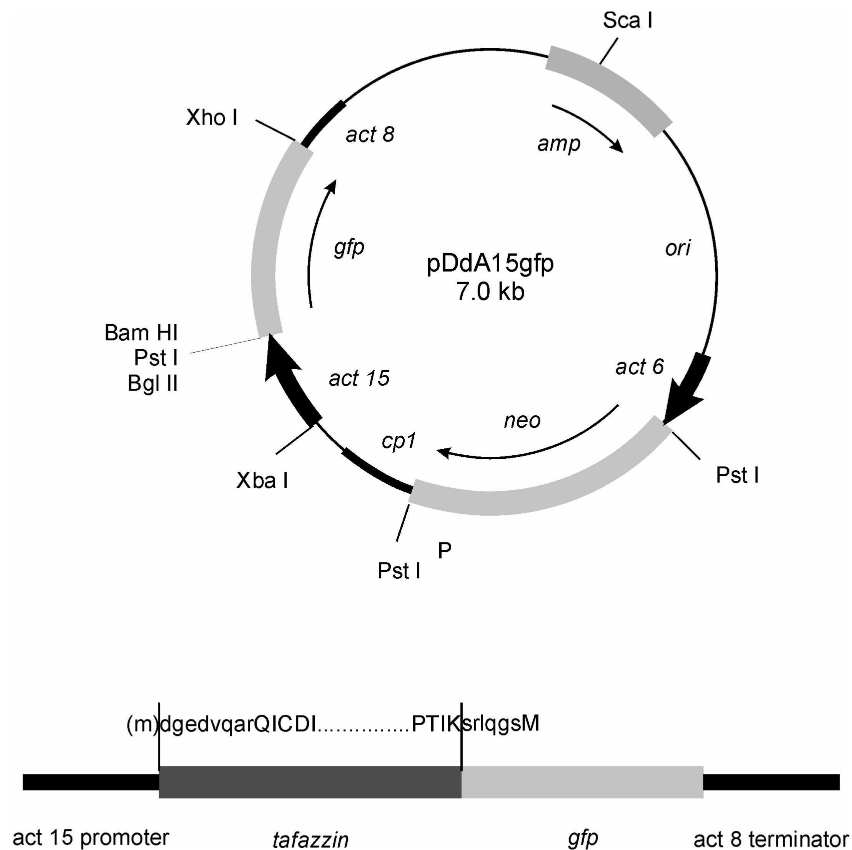


Figure 2-19. Construct used for the expression of tafazzin-GFP.

Upper part. *Dictyostelium discoideum* transformation vector pDdA15gfp (*Gerisch et al.*, 1995). In addition to the gfp cassette which was preceded by a multiple cloning site, the vector contained a neomycin-resistance cassette (*neo*) for the selection of transformants in *Dictyostelium discoideum* and an ampicillin-resistance cassette (*amp*) for selection in *E. coli*.

Lower part. A 1097 bp *tafazzin* coding region (including 186 bp intron) was amplified by PCR and cloned in front of the gfp sequence of pDdA15gfp (Bgl II). The relevant portion of the resulting plasmid, pDdA15taz-gfp (pDdtaz15) is shown here. Transcription of the gene encoding the fusion protein was controlled by *act 15* promoter, and the *act 8 terminator* from *Dictyostelium discoideum.* The tafazzin-GFP fusion protein produced by the vector pDdtaz15 contained 8 amino-terminal amino acids (behind the initial methionine) encoded by act 15 (lower case; single-letter amino-acid code), followed by the 267 amino acids of tafazzin (upper case).The tafazzin and GFP sequences were separated by 6 amino acids encoded by the polylinker (lower case).*'act 6*' refers to the *actin 6* promoter; *'cp1'* refers to the *cp1* terminator.

The clones were analyzed by immunoblotting with an anti-GFP monoclonal antibody (figure 2-20). 4 G418 resistant clones, which showed good green fluorescence images

in the microscope, and two control strains, one was REMI mutant *taz*⁻/pDdA15gfp - a GFP expression positive control, the other was REMI mutant *taz*⁻ - a GFP expression negative control, were grown in axenic medium and harvested at the cell density of 1×10^6 cells/ml. The immunoblotting experiments revealed that the relevant clones contained a GFP labeled protein corresponding to the size of the fusion protein of 58 kDa (31 kDa tafazzin + 27 kDa GFP), the GFP positive control REMI mutant *taz*⁻ /pDdA15gfp only contained a labeled protein corresponding to the size of green fluorescent protein (27 kDa), no detectable GFP signal was found in REMI mutant *taz*⁻ negative control.

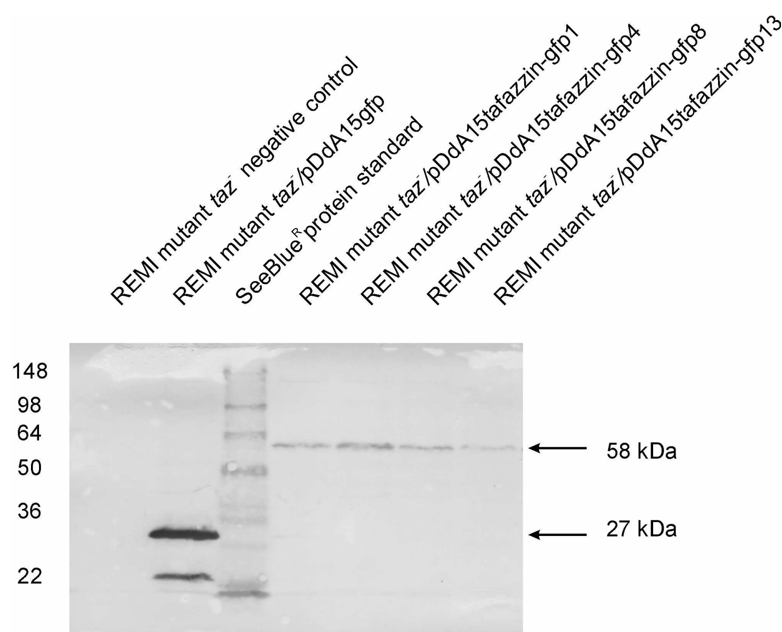


Figure 2-20. Western blot for GFP expression. Cells from several G418 resistant clones, REMI mutant *taz*⁻/pDdA15gfp and REMI mutant *taz*⁻ were grown in axenic medium with agitation (180 rpm) and harvested at the cell densities of 1×10^6 cells/ml. Total protein was separated by SDS-PAGE, and the expression of GFP was detected by anti-GFP antibody. Equal amounts of protein were loaded. The locations of see blue protein standard (kDa) are shown on the left and the locations of labeled protein are on the right.

The above results show that the fusion protein is expressed in *Dictyostelium discoideum* and may be used to localize the tafazzin protein by immunofluorescence.

All the following experiments were done by using one of the positive clones - REMI mutant *taz*⁻/pDdA15tafazzin-gfp4 which exhibited best green fluorescence in the

microscope and the strongest GFP signal in the Western blot (figure 20), compared to that of the others.

Dictyostelium discoideum tafazzin protein is mitochondria associated

The green fluorescence images produced by positive clones had a dispersed, dotted distribution (data not shown), indicating the possibility that the fusion protein was organelle associated.

From our phospholipid result, and also from Neuwald hypothesis (1997) and the work published by Vreken *et al.*(2000) and Bissler *et al,* (2002), we thus predicted the possibility that mitochondrion is the target organelle for the tafazzin-gfp fusion protein, and immunofluorescence experiments were performed to test our prediction (figure 2-21).

Exponentially growing cells of REMI mutant *taz*$^-$/pDdA15tafazzin-gfp4 were washed and fixed with picric acid/formaldehyde. Subsequently they were processed for immunolabeling according to Humbel *et al*., (1992). Porin (Zalman *et al*., 1980 and Roos *et al.*, 1982), also known as voltage-dependent anion-selective channel (VDAC) (Schein *et al*., 1976 and Colombini 1979), is a major mitochondrial outer membrane protein. An anti – mitochondria porin antibody (Troll *et al.,* 1992) was used for the immunolabeling of mitochondria. As shown in figure 2-21B and D, the porin antibody stained the periphery of mitochondria and showed sharply and dotted red images which were almost identical to those produced by tafazzin – GFP fusion protein.

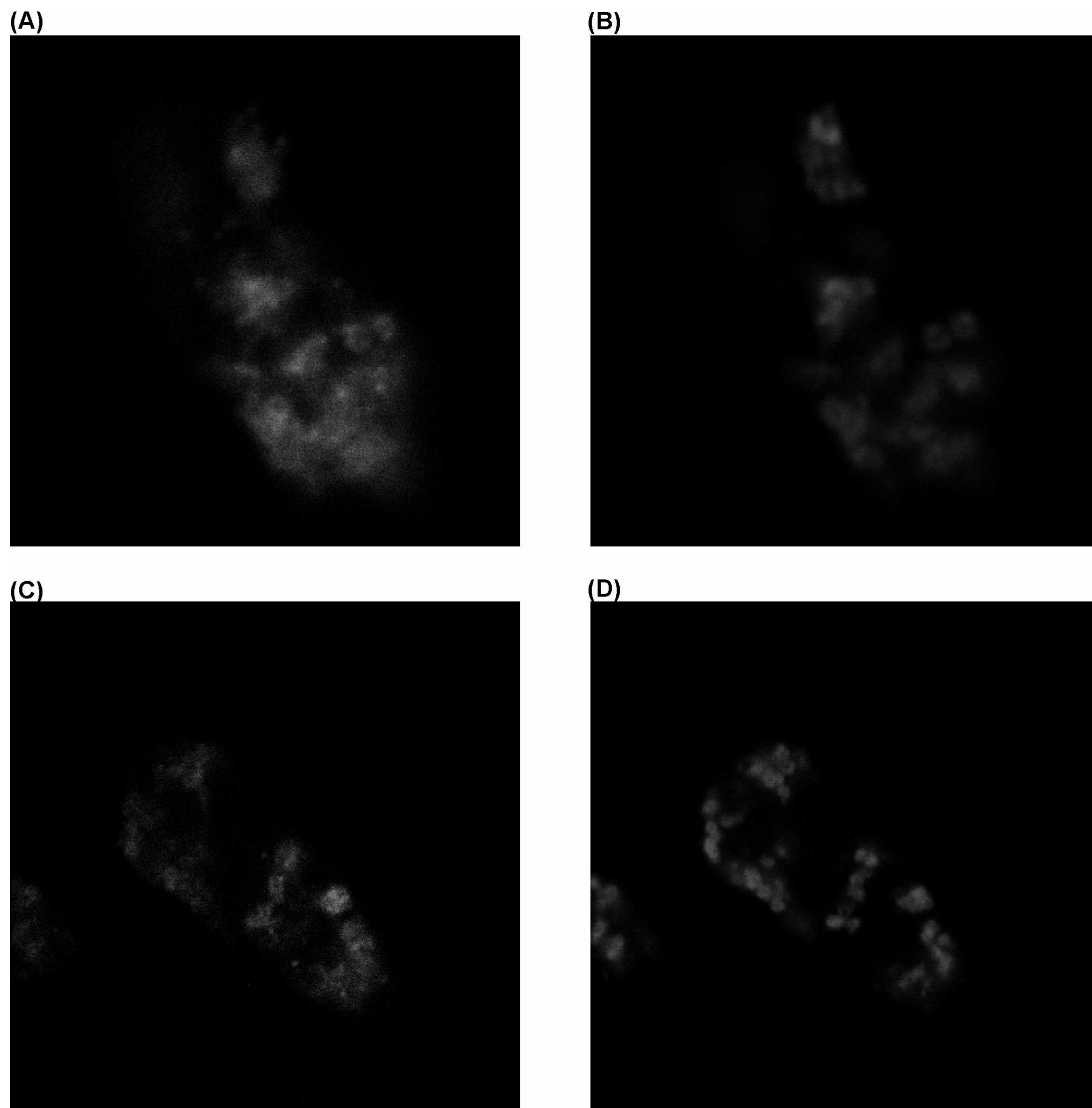


Figure 2-21. Localization of tafazzin protein in *Dictyostelium discoideum*. REMI mutant *taz*⁻/pDdA15tafazzin-gfp4 was grown in axenic medium to exponential phase, cells were allowed to settle and spread onto a clean coverslip, fixed with picric acid/formaldehyde, postfixed with 70% ethanol. Subsequently, mitochondria were detected with the *Dictyostelium discoideum* mitochondrial porin antibody (Troll *et al.*, 1992), and Cy3-conjugated goat anti-mouse IgG. (See materials and methods for details). Images were taken by a conventional immunofluorescence microscope with CCD camera. (A) and (C) fluorescence images produced by tafazzin-GFP fusion protein in a single cell. (B) and (D) Immuofluorescence images stained with a miochondrial porin antibody (red) in the same cell as (A) and (C). The red images produced by the mitochondrial porin antibody display an almost identical pattern as the GFP images

Overexpression of tafazzin rescues the mutant phenotype

Colony blots and Western blots were performed to investigate the effect of GFP tagged tafazzin on discoidin expression (figure 2-22 and 2-23). As shown in figure 2-22, discoidin I protein detected in REMI mutant *taz*$^-$/pDdA15tafazzin-gfp4 was found in cells beyond the visible border of the colony where there is still sufficient supply of nutrients.

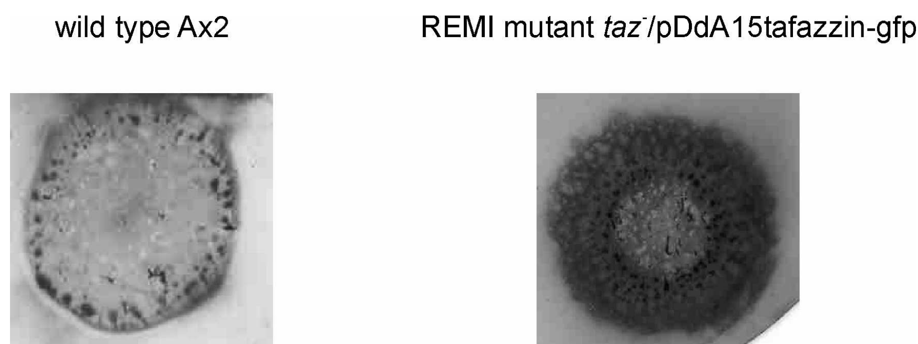


Figure 2-22. Colony blots for discoidin expression. Cells of wild type Ax2 and REMI mutant *taz*$^-$/pDdA15tafazzin-gfp4 were grown on KA plates to a diameter of approximate 1-2 cm, blotted and incubated first with the anti-discoidin antibody (Wetterauer *et al*., 1993) and then alkaline phosphatase coupled secondary goat – anti mouse antibody.

In contrast to the *taz*$^-$ mutants that showed variable expression levels of discoidin, REMI mutant *taz*$^-$/pDdA15tafazzin-gfp4 reproducibly displayed discoidin overexpression.

In Western blots, after 4 hours starvation in phosphate buffer, the discoidin I expression levels of wildtype Ax2, REMI mutant *taz*$^-$ and 4 tafazzin overexpression strains were quantified (figure 2-23), the discoidin I expression levels of the 4 tafazzin overexpression strains were obviously higher than that of their parent strain REMI mutant *taz*$^-$ and reached the expression level of wild type Ax2 cells. The faint lower molecular weight signal in western blots is from the cross reaction of the antibody with discoidin II, a related protein.

Unlike the colony blot where the tafazzin overexpression strain exhibited high discodin I expression, in Western blot, tafazzin overexpression strains just rescued the discoidin I expression level to that of wild type Ax2.

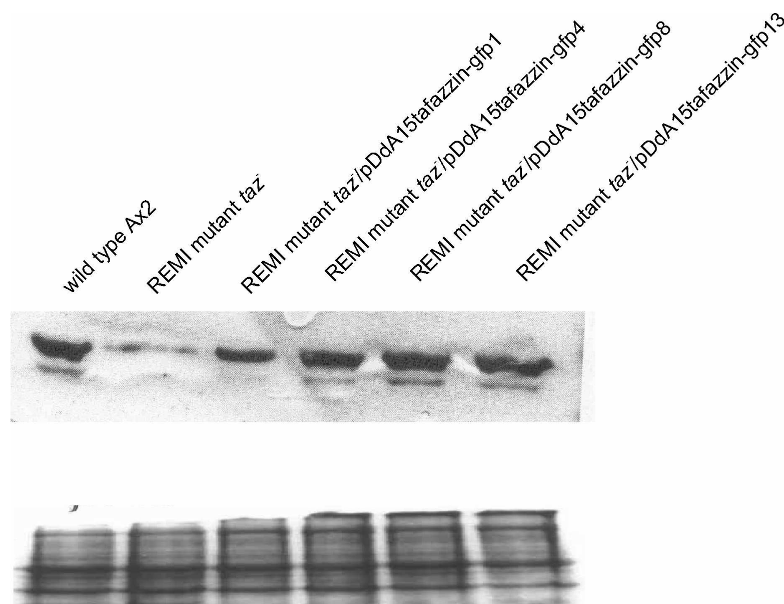


Figure 2-23. Western blot for discoidin expression. Cells from wild type Ax2, REMI mutant *taz*- and 4 overexpression strains were grown in KA suspension with agitation (180 rpm) and harvested at the cell densities of 1×10^6 cells/ml, washed the bacteria by differential centrifuge and allowed to develop in phosphate buffer for 4 hours. Total protein was separated by SDS-PAGE, and discoidin I was detected by the anti-discoidin antibody (Wetterauer *et al.*, 1993). A coomassie stained SDS-PAGE is used as a loading control.

In order to examine whether GFP tagged tafazzin can complement the growth defect phenotype of gene disruption strains, the growth of one of the strains, REMI mutant *taz*-/pDdA15tafazzin-gfp4, was compared to that of wild type Ax2 cells and its parent strain REMI mutant *taz*- (figure 2-24), the growth of the REMI mutant *taz*- /pDdA15tafazzin-gfp4 was significantly faster than that of REMI mutant *taz*-, which exhibited axenic growth defect at 15 °C (figure 2-12 and 2-18), moreover, the tafazzin overexpression strain grew with nearly the same doubling time as wild type Ax2 cells.

Taken together, the above results indicate that overexpression of GFP tagged tafazzin can rescue all of the phenotypes of the gene disruption

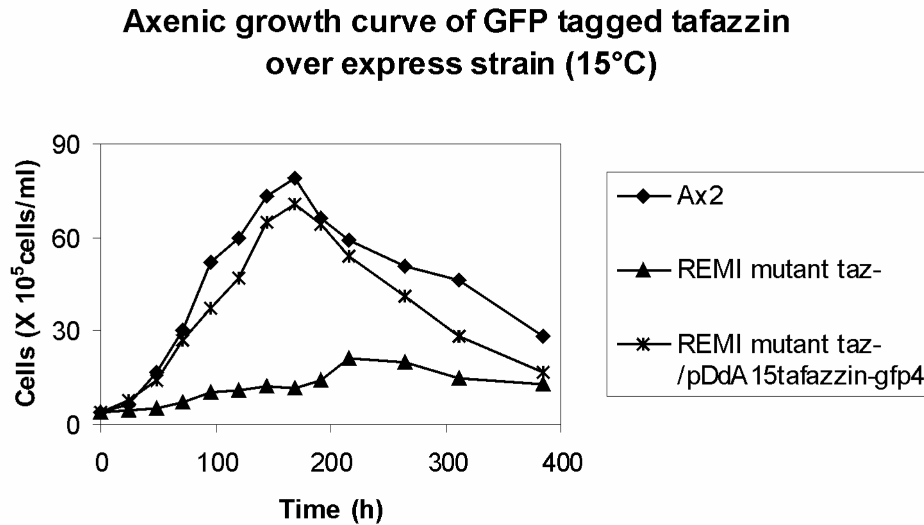


Figure 2-24. Axenic growth curve of tafazzin overexpression strain (15°) Prewarmed axenic medium were inoculated with exponential growth cells from wild type Ax2, REMI mutant *taz*⁻ and REMI mutant *taz*⁻/pDdA15tafazzin-gfp4 and incubated at 15°C with agitation (180 rpm). The total cells were counted microscopically.

Endogenous tafazzin plays a major role in Dictyostelium discoideum

In order to further investigate whether overexpression tafazzin-gfp in wild type Ax2 background can lead to even faster growth than its parent strain wild type Ax2, the growth of two tafazzin overexpression strains, one from wild type Ax2 background, the other from the original REMI mutant *taz*⁻ background were compared to that of wild type Ax2 and the original REMI mutant *taz*⁻ (figure 2-25), the growth of Ax2/pDdA15tafazzin-gfp was nearly identical to that of its parent strain wild type Ax2, and both of them grew slightly faster than the other tafazzin-gfp overexpression strain - REMI mutant *taz*⁻/pDdA15tafazzin-gfp4.

The above results demonstrate that the endogenous tafazzin protein plays a major role in *Dictyostelium discoideum* and overexpression of tafazzin protein can rescue the function of the endogenous one to a certain extent.

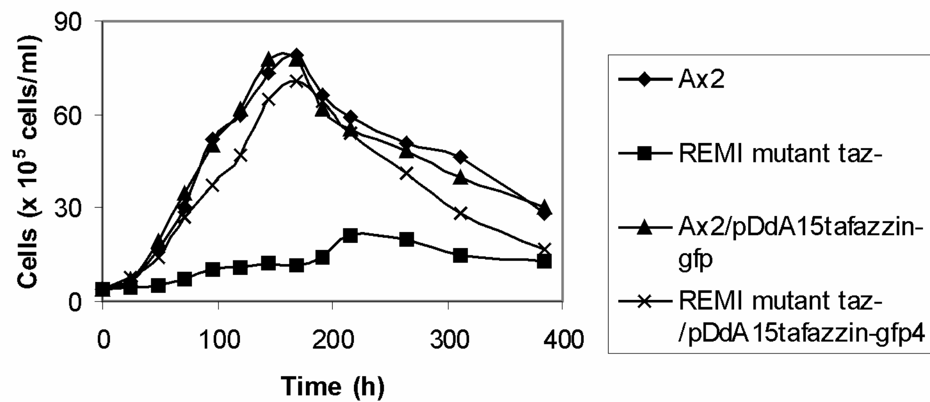


Figure 2-25. Growth curve of tafazzin overexpression strain (15°C). Prewarmed axenic medium were inoculated with exponential growth cells from wild type Ax2, REMI mutant *taz*$^-$, REMI mutant *taz*$^-$/pDdA15tafazzin-gfp4 and Ax2/pDdA15tafazzin-gfp, and then incubated at 15°C with agitation (180 rpm). The total cells were counted microscopically

3

Discussion

Using REMI mutagenesis and discoidin gene family to study growth-differentiation-transition (GDT) in Dictyostelium discoideum

Until recently it was impossible to recover the affected genes from chemically induced mutants. REMI mutagenesis had been introduced from yeast (Schiestl and Petes, 1991) and is a form of insertional mutagenesis that permits the cloning of affected genes (Kuspa and Loomis, 1992). REMI mutagenesis could be able to identify any gene that is not present in functionally redundant copies and is not essential for viability. An increasing number of REMI mutants of great interest have been recovered, and every month produces new mutants that affect fundamental processes.

As a technique, REMI has certain limitations: it creates null mutants because in general it disrupts a gene. Thus many kinds of suppressors can not be found. One would employ it preferentially to remove inhibitory genes that control a pathway and thus create second site suppression. The other problem is that REMI mutants affected during the growth cycle are not recovered by current methods, thus defects in genes that are critical to pinocytosis, phagocytosis, or the cell cycle are not available, although several non-lethal genes like coronin, vacuolin, cyclins, myosin have been investigate by this method.

In our laboratory, REMI mutagenesis was applied to isolate mutants and genes involved in the transition from growth to differentiation (Zeng *et al*., 2000A and B; Riemann and Nellen, unpublished data).

The discoidin I gene family consists of 3 major polypeptides that are co-ordinately regulated (Devine *et al.,* 1982). The developmental expression of the discoidin I gene family is among the most thoroughly studied genetic regulatory systems in *Dictyostelium*. The coordinate induction of transcription of these genes is mediated, at least in part, by the extracellular signal PSF (Clark *et al*., 1987), and this followed

later in development in down-regulation in response to cAMP (Alexander *et al.*, 1990; Vanti *et al.*, 1990). In addition, folate down-regulates discoidin mRNA accumulation in growing as well as developing cells (Alexander *et al.,* 1990; Blusch *et al.*, 1992 and Blusch and Nellen, 1994). An analysis of the discoidin gene promoter has revealed a sequence called the dIE element that is essential for the prestarvation response (Vauti *et al.*, 1990). A TTG sequence was found in the discoidin promoter which is inactive during growth on bacteria, but active when cells are grown axenically.

Discoidin I gene expression pattern was used in our laboratory as a marker for screening REMI mutants affected in the growth-differentiation-transition (GDT) and some REMI mutants, which displayed over-or under-expression of discoidin I, have been generated in our laboratory. Among others, a discoidin overexpression mutant, termed 2-9, was detected. The affected gene, denominated *gdt1*, is a negative regulator of discoidin expression and the GDT signalling in *Dictyostelium discoideum* (Zeng *et al.*, 2000A and B). Another one of these was identified as a disruption in CRAC (Riemann and Nellen, unpublished data) and confirmed the previous results that CRAC was involved in the GDT (Endl *et al.*, 1996).

The REMI technique and the use of discoidin as a marker for molecular analysis of the GDT have been proven to be successful (Zeng *et al.*, 2000A and B; Riemann and Nellen, unpublished data). Like the others, this Ph. D work was initially focused on GDT signalling by using the REMI approach and the discoidin gene family as a marker to find GDT genes. Among others, a REMI mutant 17-1-J-3-1 (*taz*$^-$) was detected. REMI mutant 17-1-J-3-1 (*taz*$^-$) exhibited variable discoidin expression levels from over-expression to low-expression on colony blots (See below, for discussion of variation), and low discoidin expression during growth and development on Western blots. In addition, the mutant cells showed an axenic growth defect at 15°C. The affected *tafazzin* gene was isolated from the REMI mutant by plasmid rescue .

REMI mutant taz$^-$ *showed variable discoidin I expression on KA plates*

In this work, initially a REMI mutant 17-1-J-3-1 (*taz*$^-$) with elevated discoidin I expression was screened on a colony blot (figure 2-1). Surprisingly, the discoidin I expression level was variable, ranging from high to low dependent on experiments. Even colonies, which were originally inoculated from the same parent colony onto the same KA plate, showed variation. Interestingly, on Western blots, the mutant cells always exhibited low discoidin expression during growth and development (figure 2-

2). When *Dictyostelium discoideum* cells grow on KA plates, the cells feed on bacteria and form plaques. Then the cells inside the plaque, where the bacteria are consumed, enter the development cycle. The KA plaque where the *Dictyostelium discoideum* cells live close to natural conditions was used for colony blots. Unlike colony blots, the samples used for Western blot in this work were isolated from KA suspension shaking culture. In this case, the cells were forced to enter development by suddenly washing the bacteria, then resuspending and shaking in phosphate buffer for a certain time, this kind of development is not the natural one, but just imitates the normal development. Due to these reasons, other samples isolated from filter development, where *Dictyostelium* cells were forced to enter the development cycle like under natural conditions, were used for Western blots (figure 2-2). Unfortunately, this discrepancy still existed because REMI mutant cells always showed low discoidin expression compared to that of wild type Ax2 cells no matter where the samples used for the Western blot were isolated. Interestingly, the 4 secondary gene disruption strains also exhibited variable discoidin expression on colony blots (figure 2-16), although PCR reactions and southern hybridization demonstrated that they were identical (figure 2-14 and 2-15). Like original gene disruption strain, the secondary gene disruption strains also showed low discoidin expression compare to that of wild type Ax2 cells on Western blots (figure 2-17).

Obviously, the original REMI mutant has a fixed genotype and the genotypes of the 4 secondary gene disruption strains are identical. One possible explanation of the phenotype variation could be the **norm of reaction**.

In general, a single genotype may produce different phenotypes, depending on the environment in which organisms develop. Such a set of environment-phenotype relationships for a give genotype is called the **norm of reaction** of the genotype. Norms of reaction to temperature for eye-size genotypes in the fruit fly *Drosophila melanogaster* is a typical example. When flies of the wild type genotype are raised at higher temperature, they develop eyes that are somewhat smaller than those of wild type flies raised at cooler temperatures. A fly that has the *ultrabar* genotype has smaller eyes than wild type files regardless of temperature during development. Temperatures have a stronger effect on development of *ultrabar* genotypes than on wild type genotypes. Any fly of the *infrabar* genotype also has smaller eyes than any wild type fly, but temperatures have the opposite effect on flies of this genotype: *infrabar* flies raised at higher temperatures tend to have larger eyes than those raised at lower temperatures. Another example is the norms of reaction to elevation for plant height genotypes in yarrow plant, *Achillea millefolium*. These norms of reaction

indicate that the relationship between genotype and phenotype is complex rather than simple.

In the *taz*$^-$ mutants (both original REMI mutant and secondary gene disruption), colony blot phenotype of discoidin expression ranged from high to low (the mutant norm of reaction) while the wild type phenotype was stable (the wild type norm of reaction), thus our findings suggest that KA plates (environment) have a stronger effect on the development of *taz*$^-$ mutants genotypes than on wild type genotype. In other words, the *taz*$^-$ mutants are more sensitive to slight changes of KA plates (environment) than wild type and show variable levels of discoidin expression. Provided that it is possible to make identical KA plates, one can imagine that the *taz*$^-$ mutants should exhibit stable phenotype on colony blot. Apparently, suspension culture and suspension development as well as filter development are better controlled conditions than KA plates, so the *taz*$^-$ mutants showed stable phenotypes on Western blots.

Mitochondria and signal transduction

As discussed above, the initial expectation of this Ph. D work was to find a signal transduction component of growth differentiation transition by using REMI technique and discoidin I as a molecular marker. Surprisingly, a *Dictyostelium discoideum* tafazzin homolog, which is a nuclear encoded mitochondrial protein and is responsible for human Barth syndrome, was found by using the above approach. Disruption of *Dictyostelium tafazzin* gene caused mis-expression of discoidin I- a molecular marker of growth differentiation transition.

The mechanism of unexpected linkage between mitochondrial protein and early development events is so far unclear. One possibility is that the simple energy problem caused by *tafazzin* gene disruption leads to development defect because the human tafazzin is believed to be involved in the remodelling of cardiolipin whose appropriate content is an important requirement for activation of enzymes involved in mitochondrial respiration. The other possibility is that the *Dictyostelium discoideum* tafazzin, a mitochondrial protein, may be directly involved in the development in addition to its putative function in energy production. In fact, several mitochondrial proteins (both nuclear and mitochondria encoded) have been published to be involved in *Dictyostelium* development in the past couple of years.

In *Dictyostelium discoideum*, several *dia* genes (**d**ifferentiation **a**ssociated **g**ene) have been reported to express specifically in response to the initial differentiation from the PS point (**p**utative **s**hift point) (Chae *et al.*, 1998; Inazu *et al.*, 1999 and Hirose *et al.*, 2000). Among them, *dia3* (Inazu *et al.*, 1999) was found to be a mtDNA encoding mitochondrial protein cluster (NADH dehydrogenase subunit 11, 5, Ribosomal protein S4 (RPS4), RPS2 and NAD4L). The dia3 expression was found to be developmentally regulated: the expression was scarcely detected at the vegetative growth phase, and the maximal expression was attained at 2 hour of starvation, followed by complete loss at the tight aggregate stage. Partial inactivation of *rps4*- a gene of the *dia3* cluster, greatly impaired the progression of cell differentiation, also the expression of *car1* which is essential for cell aggregation (Sun and Devreotes, 1991) was found to be markedly reduced. Overexpression of the *rps4* gene enhances the initial step of cell differentiation.

The finding that Tortoise, a nuclear DNA encoded mitochondrial protein is required for directional responses of *Dictyostelium* in chemotactic gradients (van Es *et al.*, 2001) is fascinating and suggests that processes like chemotaxis also require mitochondrial proteins. Murgia *et al.* (1995) reported that *Dictyostelium discoideum* ACLA, an actin-related protein that belongs to the ARP3 family of actin-related proteins, was developmentally regulated and associated with mitochondria, but its function is unknown. In *Drosophila*, the germ cell line is determined by the large subunit (mt-lrRNA) of mitochondrial rRNA (Kobayashi and Okada, 1989). More recently, mt-lrRNA of *Dictyostelium* slug cells was found to be essential for photosensory and thermosensory signal transduction (Wilcznaka *et al.*, 1997). Age related tissue dysfunction and the pathology of mitochondrial diseases might both be explained partly by defective signal transduction arising either directly from impairment of a mitochondrial role in intracellular signalling or indirectly from energy (ATP) depletion. Thus, this new aspect of mitochondria with several unexpected functions is growing up and our work added one more example to the new aspect of mitochondrial functions.

Dictyostelium discoideum is a model organism to study Barth syndrome

In the past, *S. cerevisiae* had contributed much to the study of human Barth syndrome (see introduction for details of Barth syndrome) because it is easier to be manipulated than mammalian cells. For example, in both yeast *tafazzin* gene disruption strain and human Barth syndrome patients, phospholipid remodelling is disturbed leading to

reduced cardiolipin concentration and abnormal cardiolipin-acyl composition (Vreken *et al.*, 2000).

Dictyostelium discoideum is another simple, genetically tractable organism and contributes more and more to the study of human disease and health (see introduction for examples). The *Dictyostelium* genome is about 3 times bigger than that of yeast, and *Dictyostelium* proteins are more similar to mammalian proteins than those of yeast (Loomis and Smith 1995). *Dictyostelium* genes and their products may give a better approximation to the structure and regulation of mammalian proteins than do the sequences derived from budding yeast. *Dictyostelium* contains signal transduction pathways that exist in animal cells, but not in yeast. For example, *Dictyostelium* has STAT proteins (**S**ignal **T**ransducers and **A**ctivators of **T**ranscription) which budding yeast lack (Kawata *et al.*, 1997). These protein are associated with several mammalian growth factors and cytokine-induced signal transduction pathways. Dd-STATs are made throughout growth and development and localize to the nucleus during aggregation (Araki *et al.*, 1998). Dd-STATs seem to function in the manner of mammalian STATs. Additional, yeast cells differ from both human and *Dictyostelium* cells in one important aspect-cell differentiation.

The results of this work may contribute to establish *Dictoystelium discoideum* as a new model system for the study and therapy of human Barth syndrome. In this work, the *Dictysotelium discoideum* homolog of the *tafazzin* gene was disrupted twice, initially the *tafazzin* gene was randomly disrupted by REMI mutagenesis, and then it was knocked out by homologous recombination. Both mutants exhibited identical phenotypes. Overexpression of *Dictyostelium discoideum* tafazzin protein complemented the mutant phenotypes. Immunofluorescence experiments demonstrated that *Dictyostelium discoideum* tafazzin was mitochondria associated. It is the first time that tafazzin is reported to be mitochondria associated and its location strongly suggests the molecular basis of its function. Moreover, in this work, phospholipid assays showed that cardiolipin of *Dictyostelium discoideum* REMI mutant *taz*$^-$ was 80% reduced compared to that of the wild type Ax2, while the other phospholipid classes were normal. Although the phospholipid composition from the secondary disruption strains and from the overexpression strains have not been investigated so far, from the observation that the secondary disruption strains exhibited the identical phenotypes and the overexpression strains rescued the phenotypes, we assume that cardiolipin remodelling was disturbed and rescued, respectively. Thus our preliminary results strongly demonstrate that the *Dictyostelium*

discoideum tafazzin protein has the similar function to its orthologues from human and yeast.

The results of this study clearly demonstrate that *Dictyostelium discoideum* is another model organism to the study and therapy of human Barth syndrome.

Disruption of enzymes involved in the cardiolipin biosynthesis causes growth defect

The biosynthetic pathway of cardiolipin is shown in figure 1-5. The route is similar to other phospholipid pathways and conserves through the eukaryote kingdom. For examples, the *Dictyostelium discoideum* phosphatidylglycerophosphate synthase (Q8SSY1, Gloeckner *et al.*, 2002) shows high homology to its orthologues from yeast and CHO cells (see appendix IV). Although the *Dictyostelium discoideum* cardiolipin synthase has not been published yet, a *Dictyostelium discoideum* random clone (IIBEP1D0026, 448bp) exhibits nearly 100% identity to the middle region of yeast cardiolipin synthase (Q07560) (see appendix V). One can image that the entire *Dictyostelium* cardiolipin snythase should be found to share similarity to its orthologues from another organisms after the complete *Dictyostelium* genome sequence project is finished. Due to the conservation of the cardiolopin biosynthetic pathway, the study of the pathway in model organism could answer the general questions of the cardiolipin pathway.

One approach to understanding the metabolic mechanisms and the physiological roles of phosphatidylglycerol (PG) and cardiolipin (CL) is the establishment of mutants defective in the synthesis pathway. At present, the studies of the two most important enzymes involved in cardiolipin biosynthesis pathway, phosphatidylglycerolphosphate synthase that catalyses the committed and rate-limiting step in PG/CL biosynthesis, and cardiolipin synthase that differs from prokaryotes to eukaryotes, have only been documented for Chinese hamster ovary cell (CHO) and in lower eukaryotes and prokaryotes like yeast *Saccharomyces cerevisiae* and *E.coli* (For review, see Schlame *et al*., 2000).

Studies with the CHO-K1 mutant PGS-S, deficient in PGP synthase, have revealed that the mutant was temperature-sensitive at 40°C, and displayed growth defect to some extent even when grown with glucose. While grown with glucose-deficient medium, for example, galactose, in which the majority of cellular energy is derived from

oxidative phosphorylation, the mutant had a more significant growth defect at 40°C than in glucose containing medium (Ohtsuka *et al*., 1993 A and B). In yeast, disruption of the PGP synthase gene did not lead to the loss of viability (Chang *et a*l., 1998A; Jiang *et al*., 1998 and Minskoff *et al*., 1997), although the mutant strain was dependent on a fermentable carbon source (petite lethal phenotype), specifically when the mutant cells grew at elevated temperature (37°C), its growth was significantly slower and cells reached a lower cell density in stationary phase than wild type cells.

Disruptions of the yeast cardiolipin synthase gene have been studied, the growth defect phenotypic changes brought about by this mutant are not dramatic (Chang *et al*., 1998B, Jiang *et al*., 1997 and 1999 and Koshkin and Greenberg, 2000), for example, mutants can grow on both fermentable and non-fermentable (glycol, ethanol containing medium) carbon sources at 30°C, although mutants show a slight growth defect on non-fermentable carbon source. However, when the growth temperature was increased to 37°C, cardiolipin synthase expression was essential for growth, even on a fermentable carbon source. The above studies suggest that since phosphatidylglycerol levels are elevated in these cells, phosphatidylglycerol could substitute for cardiolipin in most essential mitochondrial functions to some extent, but can not fully substitute the cardiolipin function.

Tafazzins share several conserved regions with phospholipid acyltransferase of diverse organisms (Neuwald hypothesis, 1997, figure 1-3), suggesting that Barth syndrome may be caused by a defect or defects in cardiolipin acyl transfer. Thus the growth of *Dictyostelium discoideum taz*$^{-}$ mutant was investigated (figure 2-12). When grown on axenic medium containing 1% glucose, *Dictyostelium discoideum tafazzin* mutants (both original REMI mutant and secondary gene disruption strains) exhibited a significant growth defect at 15°C. When the temperature was increased to 22°C which is the normal laboratory culture temperature for *Dictyostelium discoideum*, the growth of mutant cells was not significantly slower than that of wild type Ax2 cells. The mutant cells showed no growth defect compared to wild type Ax2 cells when the culture temperature was increased to 27°C. Because tafazzin maybe involved in cardiolipin remodelling (Bissler *et al*., 2002; Neuwald, 1997; Schlame *et al.*, 2002; Vreken *et al*., 2000 and this work), the temperature sensitive growth defect suggested that in *Dictyostelium discoideum*, phosphatidylglycerol could substitute for cardiolipin in optimal conditions (22°C and 27°C), but this substitution was only sufficient to a limited extent and under severe conditions, for example 15°C, the growth of *Dictyostelium discoideum* cells needed the normal function of cardiolipin. Our data is

consistent with the findings of Chang *et al*. (1998B), Jiang *et al*. (1999) and Tuller *et al*. (1998) from yeast *Saccharomyces cerevisiae* cardiolipin synthase mutants. However, our growth defect is low temperature sensitive (15°C), differing to their observation that yeast cardiolipin synthase mutant cells are high temperature sensitive (37°C). Because no low temperature growths of yeast and CHO have been studied and published so far, and in this work, the high temperature growth above 27°C has not been investigated, it is too earlier to draw any final conclusion before the above experiments are done.

When grown with KA suspension, the cells of *Dictyostelium discoideum* mutant *taz*$^-$ showed approximately the same doubling time as the wild type Ax2 cells no matter what the temperature was (figure 2-12A-C). *Dictyostelium discoideum* is extraordinarily efficient phagocyte, with the capability of consuming a variety of bacteria (Depratitère and Darmon, 1978; Raper, 1937; Raper and Smith, 1939). Amoebae ingest a variety of prey by coordinating surface recognition, signal transduction, and mobilization of the cytoskeleton. Most of the bacterial materials are degraded as very little remains after digestion (Braun *et al*., 1972). As a medium, bacterial material which is the natural food source of *Dictyostelium discoideum*, is a most complicated one containing all the materials for bacterial survival and bacterial secondary metabolites. The reason why the cells of *Dictyostelium discoideum* mutant *taz*$^-$ exhibited normal bacterial growth at different temperatures compared to that of wild type Ax2 cells could simply be due to the rich medium-bacterial materials, when grown with the complicated rich medium (a bacteria suspension), phosphatidylglycerol could substitute the function of cardiolipin to considerable extent compared to that of axenic growth and the cells of mutant *taz*$^-$ displayed no growth defect at different growth condition.

Additional, the prokaryotic membrane is another source of cardiolipin. The rich medium (a bacterial suspension) used in this work contains the bacterial cardiolipin. Can the exogenous cardiolipin complement the lack of endogenous one? Nevertheless, some similar evidence already existes: Dietary modification of the molecular species composition of cardiolipin was shown to alter the oxygen consumption in cardiac mitochondria ((Yamaoka-Koseki *et al*., 1990 and 1991. In addition, the activity of delipidated rat liver cytochrome c oxidase was reconstituted by the addition of cardiolipin. The fatty acid composition of the various phospholipid species in the heart was shown to be dependent upon a variety of factors including composition of diet (for review, see Van der Vusse *et al*., 1992). Shibata *et al*. (1994) reported, in vitro

incorporation of small amounts of cardiolipin can significantly stabilize the phosphatidylcholine bilayer structure.

Neuwald hypothesis: tafazzin and acyltransferase

Cardiolipin is a diphospholipid required for the structural integrity of the mitochondria and for the proper function of the electron transport chain. Cardiolipin biosynthesis occurs exclusively in mitochondria via the cytidine-5'-diphosphate-1, 2-diacyl-sn-glycerol (CDP-DG) pathway. Because the enzymes involved in cardiolipin *de novo* biosynthesis exhibit limited molecular species specificity (Rustow *et al.*, 1989), it seems very unlikely that the characteristic acyl species of cardiolipin are already present during formation of the cardiolipin precursors. Thus once cardiolipin is synthesized *de novo* by this pathway, it must be remodelled to obtain the appropriate molecular species composition found in the mitochondria inner membrane (see table 1-1, for the major molecular species of mammalian cardiolipin). This special acyl side chain of cardiolipin is very important for proper biological function. It was reported that the hydrophobic double unsaturated linolei diacylglycerol species of mammalian cells appeared to be an important structural requirement of the high protein binding affinity of cardiolipin (Schlame *et al.,* 1990).

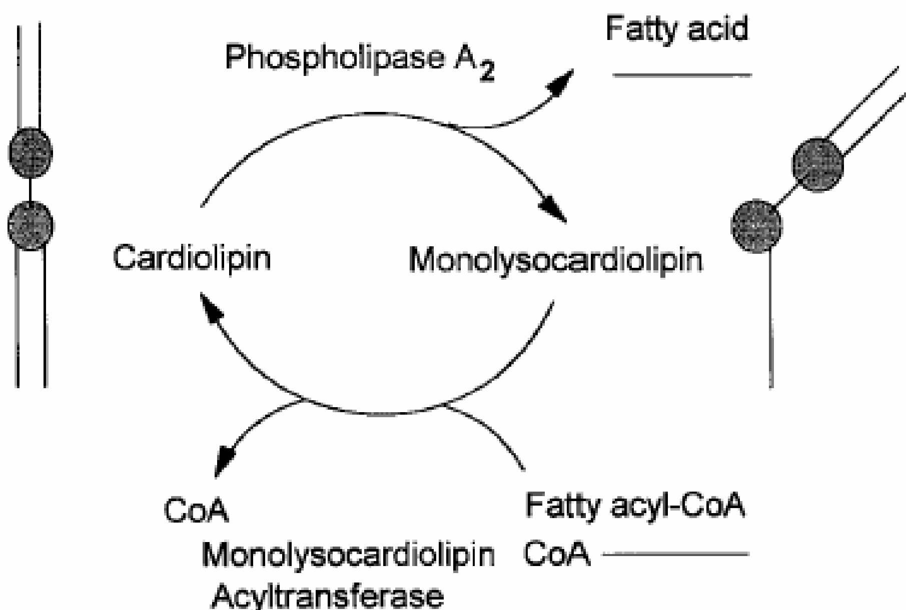


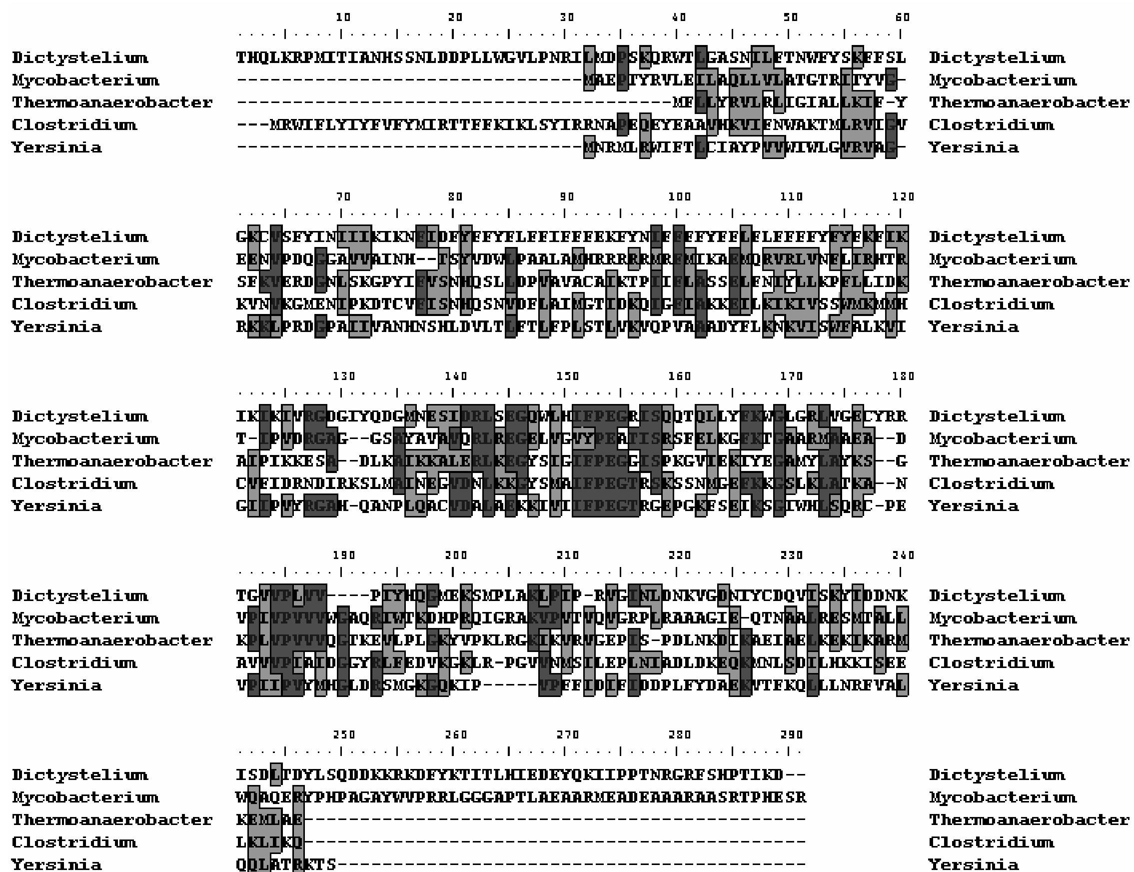
Figure 3-1. Proposed model of cardiolipin molecular remodelling in rat heart mitochondria. (From Ma *et al.* (1999), J. Lipid Res. 40, 1837-1845)

The deacylation-reacylation cycle for the molecular remodelling of glycerophospholipids was first described by Lands in 1960: Remodelling of a phospholipid requires the concerted action of phospholipase A_2 (PLA_2) followed by the reacylation of the resulting lysophospholipid. The acyltransferase (AT) activities for mammalian phosphatidylcholine remodelling have been extensively investigated (for review, see Choy *et al.*, 1997). However, limited information is available on the acyltransferases that are involved in the molecular remodelling of cardiolipin. A deacylation-reacylation cycle for the molecular remodelling of endogenous cardiolipin in rat liver mitochondria was proposed (Schlame and Rüstow, 1990). Endogenous cardiolipin was deacylated to monolysocardiolipin (MLCL) and then reacylated with linoleoyl-coenzyme A to form cardiolipin (figure 3-2). Such a deacylation followed by reacylation scheme for cardiolipin seems logical as mitochondrial phospholipase A_2 was shown to readily hydrolyse endogenous and exogenous cardiolipin (Buchland *et al.*, 1998; De Winter *et al.*, 1987; Hostetler *et al.*, 1978 and Waite and Sisson, 1971). Ma *et al.*, (1999) identified and characterized the activity of monolysocardiolipin acyltransferase (MLCL AT) that was responsible for monolysocardiolipin acylation to cardiolipin in mammalian tissues. They found that the reacylation reactions were coenzyme A-dependent and separate AT activities were present for the incorporation linoleic acid (18:2) or oleic acid (18:1) into the side chain of newly formed cardiolipin. Mutter *et al.*, (2000) postulated that MLCL AT may be a rate limiting enzyme of the molecular remodelling of cardiolipin in the heart. Additional, Taylar *et al.*, (2000) reported that the expression of monolysocardiolipin acyltransferase activity was regulated in concert with the level of cardiolipin and cardiolipin biosynthesis in the mammalian heart. It is worth to mention here that all the AT activities were detected from mammalian tissues so far, neither of the above acyltransferases were purified nor the genes were cloned.

Neuwald (1997) reported that human tafazzins shared several conserved regions with phospholipid acyltransferases of diverse organisms and suggested that Barth syndrome may be caused by a defect or defects in lipid acyl transfer. In agreement with the Neuwald hypothesis, Vreken and colleagues (2000) reported reduced content of cardiolipin and reduced incorporation of linoleic acid into polyglycerophospholipids in cultured fibroblasts from Barth syndrome patients. In yeast *tafazzin* mutant strains, reduced cardiolipin concentration and abnormal acyl composition were also found (Vreken, personal communication). More recently, Schlame *et al.* (2002) investigated cardiolipin deficiency in several tissues from Barth syndrome patients and found that L_4-cardiolipin, a cardiolipin species in which all four acyl positions are substituted by linoleic acid, was virtually undetectable in platelets, heart tissue, and skeletal muscle

from affected boys, whereas L_4-cardiolipin was specifically enriched in normal skeletal muscle and normal heart. Our preliminary phosphorlipid assay showed reduced content of cardiolipin in *Dictyostelium discoideum taz*$^-$ mutant (figure 2-11).

The findings from 3 groups by using different organisms quite agree with Neuwald's suggestion that the mutated proteins are acyltransferases, or at least co-factors. Previous studies already indicated that the in vivo molecular remodelling of phospholipid including cardiolipin was a complex process and was likely regulated at several levels (Choy *et al.*, 1997; Ma *et al.*, 1999; Mutter *et al.*, 2000, Taylor *et al.,* 2002). Since phospholipid remodelling is defined as the conversion of one molecular species to another, it has been proposed that acyltransferase may exist in multiple molecular forms, and each form has a high degree of specificity for a defined acyl group (Choy *et al.*, 1989). In fact some evidence already existed to support this (Deka *et al.*, 1986; Ma *et al.,* 1999 and Sanjanwala *et al.*, 1988). Although more and more evidences are coming recently to support Neuwald hypothesis, people still know very less about the exact role of tafazzin in the complicated cardiolipin deacylation-reacylation cycle.



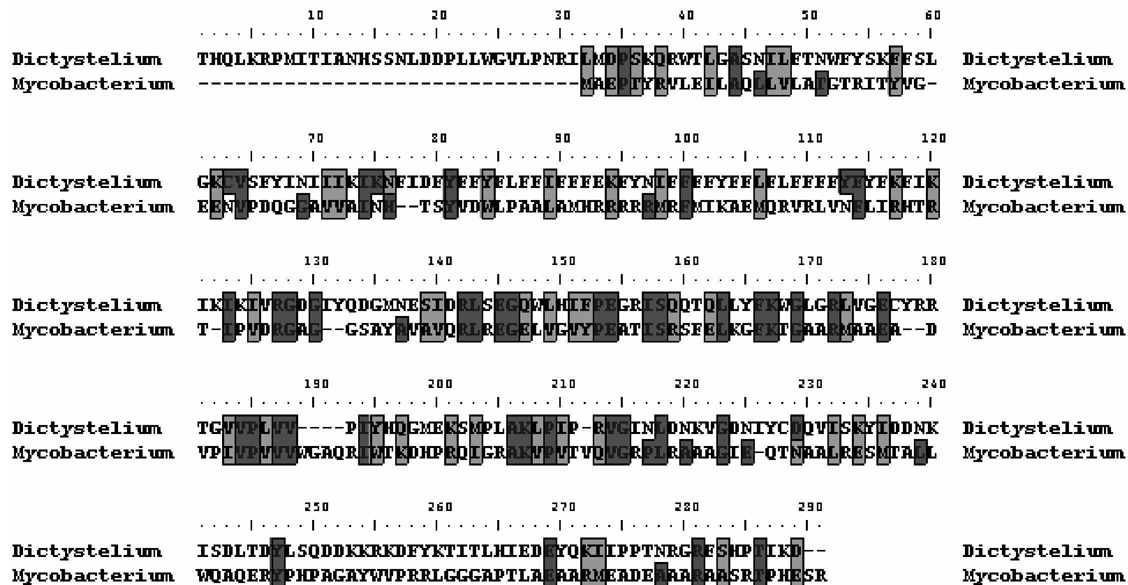


Figure 3-2. Comparison of amino acid sequence of *Dictyostelium discoideum* tafazzin with acyltransferases from other organisms.

Upper: multiple alignments between *Dictyostelium discoideum* tafazzin and acyltransferase from *Myobacterium tuberculosis* (O07808); 1-acyl-sn-glcerol-3-phosphate acyltransferase from *Thermoanaerobacter tengcongensis* (Q8R839) and from *Clostridium acetobutylicum* (Q97KF4); putative acyltransferase from *Yersinia pestis* (Q8ZCZ0).

Lower: Alignment between *Dictyostelium discoideum* tafazzin and acyltransferase from *Myobacterium tuberculosis* (O07808).

MultiAlign tool (Corpet *et al*., 1988) was used to perform the above alignment (http://www.toulouse.inra.fr/multalign.htm. Red: identity. Green: similarity.

In addition, *Dictyostelium discoideum* tafazzin shows some similarity to acyltransferase from *Mycobacterium tuberculosis* (Cole *et al*., 1998) by Blast search using *Dictyostelium discoideum* tafazzin protein. Although the identity and similarity are not high, the identical or similar amino acids are scattered through out the sequence except the N terminal of *Dictyostelium discoideum* tafazzin (figure 3-2 lower part). Moreover, *Dictyostelium discodeum* tafazzin exhibits limited similarity to other acyltransferases from *Clostridium acetobutylicum* (Noelling *et al*., 2001), *Thermoanaerobacter tengcongnesis* (Bao *et al*., 2002) and *Yersinia pestis* (Parkhill *et al*., 2001) (figure 3-2 upper part).

It is worth to mention here that all the above homologies are derived from bacterial acyltransferases, eukaryotic acyltransferases exhibit even lower similarity to *Dictyostelium discoideum* tafazzin (data not shown). Our findings are consistent with Neuwald hypothesis (1997): acyltransferases, derived by PROBE search using human tafazzins, were mainly from bacteria and lower eukaryotes, like yeast, roundworm (figure 1-3).

Since endosynbiont theory already suggested that eukaryotic mitochondria have evolved from aerobic bacteria that took to living inside the anaerobic ancestors of today's eukaryotic cells, it was not unexpected to find that tafazzin, a putative acyltransferase involved in the reacylation of mitochondria inner membrane specific cardiolipin, shares homology to bacterial acyltransferases.

The relationships between growth temperature, fatty acid composition and the physical state and fluidity of membrane lipids

Almost all organisms possess the ability to vary the fatty acid composition of their cellular lipids in a characteristic fashion in response to alterations in the environmental temperature. Changes in membrane lipid fatty acid as a function of the temperature of growth are particularly evident in prokaryotic micro-organisms, some of which have become adapted to temperature extremes ranging from -10 to nearly 100°C.

Neuwald (1997) identified 5 regions of the human tafazzin proteins with acyltransferase homology (figure 1-3). He proposed that tafazzin proteins might be involved in phospholipid biosynthesis and remodelling. Bissler *et al.* (2002) found that their Barth syndrome patient tissues had more saturated and less unsaturated fatty acids than did controls. The acyl moieties of phospholipid molecules determine many of the physical properties of the membrane. The degree of unsaturation is important in determining membrane fluidity, a measure of the ease of movement of molecules with the membrane bilayer. Membrane fluidity at a given temperature is inversely proportional to the fraction of acyl chain moieties comprised of saturated fatty acids.

Cellular membrane fluidities are maintained with a narrow range by acyltransferase-mediated remodelling of phospholipid within the membrane. As the environmental temperature is increased, the proportion of saturated fatty acids found in the membrane lipids is also markedly increased with a concomitant decrease in the

proportion of unsaturated and branched chain fatty acids. The change from one state to the other occurs via a thermally induced phase transition with the ordered (gel) state occurring at lower temperatures and the disordered (liquid-crystal) state occurring at higher temperatures. Since the lipid phase transition is dependent on the fatty acyl groups of the membrane lipids, changes in the fatty acid composition would result in shifting the temperature range of the transition. This shift is assumed to be required to minimize the effect of temperature changes on the fluidity and physical state of the membrane lipid which is compatible with cellular growth and function. *Escherichia coli*, for example, is able to maintain the fluidity of its membrane lipids nearly constant over its entire growth temperature range by appropriate alterations in the fatty acid composition of its membrane lipids-a process termed “homeoviscous adaptations” (Sinensky, 1974). Since the permeability properties of the cellular membrane and the activity of certain membrane-bound enzymes and transport systems are markedly dependent on the fluidity and physical state of the membrane lipids, it seems likely that homeoviscous adaptation represents an important mechanism for maintaining optimal levels of cell growth over a wide range of environmental temperatures. If the *tafazzin* gene product is an acyltransferase present within the mitochondrial membrane, these membranes in affected patients or *Dictyostelium discoideum taz*$^-$ mutant may have reduced fluidity, possibly reducing the normal function of integral membrane proteins.

Defects in cardiolipin biosynthesis pathway cause alterations in mitochondrial morphology

Ohtsuka *et al*. (1993) investigated the mitochondria of cultured Chinese hamster ovary (CHO) cells mutated in phosphatidylglycerophosphate synthase, which catalyses the committed step of cardiolipin biosynthesis, and found ultrastructural alterations in mitochondrial morphology (figure 3-3 left): all the mitochondria appeared greatly enlarged and swollen, cristae were lacking or disorganized, and a reduction in the electron density of the mitochondrial matrix was found. It is worthy to mention here that all the mitochondrial alterations observed by Ohtsuka *et al*. were from mutant cells cultivated at 40°C, where the cardiolipin content dropped to about 30% of that in the wild type. Mitochondria from mutant cells cultivated at 33°C were not significantly modified compared to that of the wild type cells. Furthermore, mitochondria from the mutant defect in another phospholipid biosynthesis, for example phosphatidylserine and sphingolipind, appeared similar to the wild type in shape, size, crista content, and electron density of the matrix. The results of Ohtsuka

et al. suggested that an alteration of mitochondrial ultrastructure was specifically associated with reduction in cardiolipin content.

When CHO PGS-S mutant was transfected with CHO *PGS1* cDNA (Kawasaki *et al*., 1999), the structure of mitochondria in transformant PGS-S/cPGS1 was found to be normal with respect to both size and matrix electron density (figure 3-1 right), their results indicated phosphoatidylglycerol and/or cardiolipin formation was critical for mitochondrila function and morphology.

Barth *et al.* (1983) and Neustein *et al*. (1979) independently reported mitochondrial ultrastructural abnormalities from their individual patients who are now known as suffering from Barth syndrome. They observed enlarged, spheric mitochondria and alterations in cristal structure where many mitochondria had tightly packed cristae in rectilinear or concentric array, the mitochondrial matrix in all cases was generally of low electron density except for occasional dense inclusion bodies of undetermined nature. The above observations from Barth syndrome patients were similar to that of Ohtsuka *et al*. (1993).

The consistent alteration in mitochondrial morphology observed from Barth syndrome patients whose *tafazzin* gene (G4.5) was disrupted, and from CHO phosphatidylglycerophosphate synthase (PGS) mutant cells, which had reduced cardiolipin content, could be one more evidences to support the Neuwald hypothesis that the human *tafazzin* gene (*G4.5*) is involved in cardiolipin biosynthesis. Mitochondrial ultra-structural morphology neither from *Dictyostelium discoideum* taz^- mutant (this work), nor from yeast disruption strains defect in cardiolipin biosynthesis have been investigated and published so far.

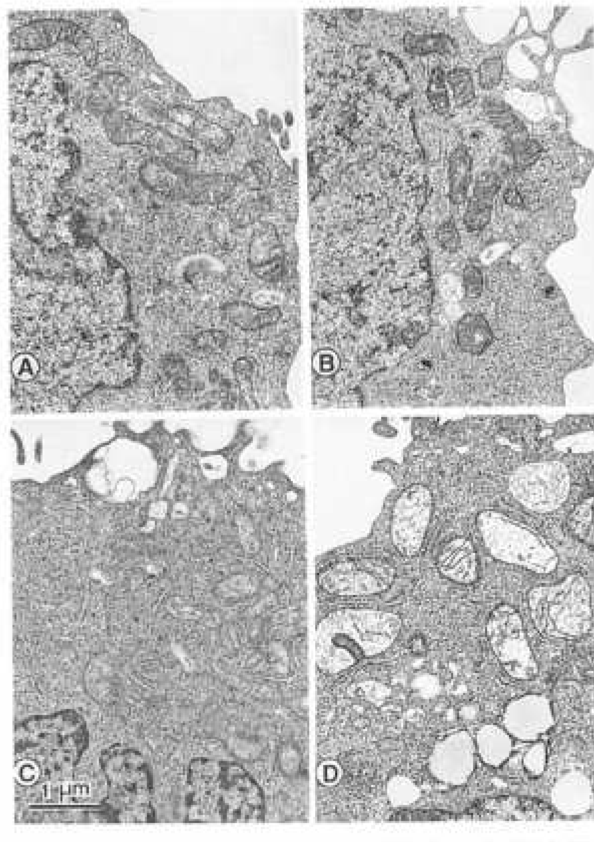


Figure 3-3(L).
Electron micrographs of PGS-S mutant and CHO-K1 wild type cells. Cells were grown at 33°C (A and C) or 40°C (B and D). A and B, CHO-K1 wild type cells; C and D, mutant PGS-S cells (from Ohtsuka *et al*. (1993). J. Biol. Chem. 268, 22914-22919)

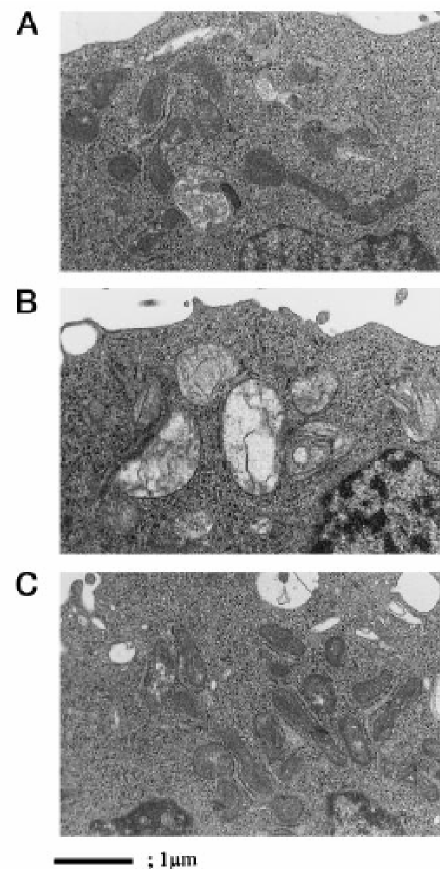


Figure 3-3(R).
Electron micrographs of transformant PGS-S/cPGS1 cells.
CHO-K1 (A), PGS-S (B), and PGS-S/cPGS1 (C) cells grown at 40°C were analysed by electron microscopy. (from Kawasaki *et al*.(1999), J. Biol. Chem. 274, 1828-1834

Dictyostelium discoideum endogenous tafazzin plays a major role and exogenous tafazzin can only complement the function to some extent

In this work, a GFP tagged tafazzin overexpression strain rescued the phenotypes of *taz*⁻ mutant, the cells of overexpression strain exhibited the normal axenic growth compared to that of wild type Ax2 cells at 15°C (figure 2-24), and the developmental discoidin expression level of tafazzin overexpression strain reached that of wild type

Ax2 (figure 2-23). These results demonstrate that the functional defects were directly due to the *tafazzin* gene disruption and the *taz*⁻ mutant transformed with GFP tagged tafazzin can complement the mutant phenotypes to the level of wild type Ax2. Noticeably, the wild type Ax2 cells transformed with GFP tagged tafazzin grew in the same double time as its parent strain Ax2 at 15°C (figure 2-26), no further increase of growth ability was observed, indicating that exogenous tafazzin only can restore the function of endogenous one to some extent.

Kawasaki and his colleagues (1999) investigated the growth of CHO PGS-S mutant transfected with the CHO *PGS1* cDNA at 40°C and their result is consistent with ours: transformant PGP-S/cPGS1 can complement the growth defect of mutant PGS-S and the growth of transformat PGP-S/cPGS1 nearly reached the levels of wild type. The above findings indicate that cardiolipin is of vital importance at severe conditions.

REMI mutant taz⁻ can compete against wild type Ax2 in development

In this work, a GFP labelled cell sorting assay was performed. GFP was transformed into both *Dicyostelium discoideum* wild type Ax2 and original REMI mutant *taz*⁻. The cells of GFP labelled *taz*⁻ and wild type Ax2 were mixed in different ratios, washed and forced to enter development stage at 22°C. The opposite assay that GFP labelled wild type Ax2 cells were mixed with non-labelled mutant *taz*⁻ cells in different ratios was also done. All of these experiments showed the cells of the *taz*⁻ mutant entered multicellular development stage in the same way as wild type Ax2 cells (figure 2-10). Our results indicate that at least at 22°C, the cells of *Dictyostelium discoideum taz*⁻ mutant can compete against wild type Ax2 cells to enter fruiting body. In order to further illuminate the function of tafazzin and cardiolipin, the cell sorting assay should be investigated under more stringent conditions, for example, low and high temperatures.

Conclusions

The REMI technique and the use of discoidin as a marker for molecular analysis of the GDT signalling have proven to be successful (Zeng *et al.*, 2000A and Riemann *et al.*, unpublished data). In this work, a *Dictyostelium discoideum* tafazzin homolog, which is responsible for Barth syndrome in human, was found by the above approach. The *Dictoyostelium discoideum* tafazzin was found to be mitochondria associated. The *taz*⁻

mutant displayed discoidin mis-expression. A linkage between GDT signalling and mitochondria was unexpected. Although the position of tafazzin, a mitochondria protein, in the GDT signalling cascade has not been investigated so far, our preliminary findings added at least one more example for the increasing evidences that mitochondria are directly involved in signal transduction network. The future work should concentrate on the interaction between tafazzin and other GDT components.

The *Dictyostelium discoideum* tafazzin showed homology to acyltransferases from different organism, especially from prokaryotes, and the cardiolipin content of the *taz*$^{-}$ mutant was 80% reduced compared to that of wild type. All the above findings are consistent with Neuwald hypothesis and with the results from human Barth syndrome patients and yeast tafazzin disruption strains. Since *Dictyostelium* is a facultative multicellular organism, the further study of *Dictyostelium* tafazzin could therefore present new aspects for the diagnosis and therapy of human Barth syndrome.

4

Materials and Methods

4.1 Materials

Antibiotics

Ampicillin	Sigma, Deisenhofen
amphotericin	Serva, Heidelberg
blasticidin	ICN, Arora
geneticin (G418)	Sigma, Deisenhofen
penicillin/streptomycin	Gibco BRL, Eggenstein
kanamycin	Sigma, Deisenhofen
chloramphenicol	Sigma, Deisenhofen
tetracyclin	Serva, Heidelberg

Antibodies

Monoclonal antibodies	
anti-*Dictyostelium discoideum* discoidin antibody	Wetterauer *et al.*, 1993
anti-GFP antibody	
anti-*Dictyostelium discoideum* mitochondrial porin antibody	Troll *et al.*, 1992
IgG, goat-anti-mouse, alkaline phosphatase-coupled	Dianova, Hamburg
IgG, goat-anti-mouse, Cy3 (Cyanine 3.18) coupled	BioTrend

Biological Materials

Bacterial strains

Escherichia coli *DH 5α*	(Hanahan, 1983)
Klebsiella aerogenes	Williams and Newell, 1976

Dictyostelium discoideum

Ax2	Watts and Ashworth, 1970

Chemicals and reagents

acetic acid 100%	Fluka, Deisenhofen
acetone	Fluka, Deisenhofen
accu Gel 40% (19:1 acrylamide: bisacrylamide)	National Diagnostics, USA
acrylamide Protogel 30%	National Diagnostics, USA
agarose SeaKem	FMC Bioproducts, USA
ammonium peroxodisulfate(APS)	Merck, Darmstadt
bacto-peptone	Difco, Augsburg
bacto-tryptone	Difco, Augsburg
ß-mercaptoethanol	Fluka, Deisenhofen
boric acid	Roth, Karlsruhe
bromophenol blue	Fluka, Deisenhofen
BSA	Roth, Karlsruhe
Calcium chloride ($CaCl_2$)	Roth,Karlsruhe
chloroform	Fluka, Deisenhofen
Coomassie Brilliant Blue R-250	Serva, Heidelberg
dATP	MBI Fermentas, St. Leon-Rot
dCTP	MBI Fermentas, St. Leon-Rot
dGTP	MBI Fermentas, St. Leon-Rot
dTTP	MBI Fermentas, St. Leon-Rot
diethylpyrocarbonate	Roth, Karlsruhe
DMSO	Serva, Heidelberg
DTT	Roth, Karlsruhe
EDTA	
ethanol 99.8%	Roth, Karlsruhe
ethanol absolut	Fluka, Deisenhofen
ethidium bromide	Fluka, Deisenhofen
fischgelatine (45%)	Sigma

formaldehyde 37%	Riedel-de-Haen, Seelze
formamide	Roth, Karlsruhe
D(+) glucose	Fluka, Deisenhofen
glycerol, 86%	Roth, Karlsruhe
glycine	Roth, Karlsruhe
guanidine thiocyanate	Roth, Karlsruhe
HEPES	Fluka, Deisenhofen
IPTG	Bts, St. Leon-Rot
isopropanol	Fluka, Deisenhofen
liquid nitrogene	Messer Griesheim, Krefeld
lithium chloride(LiCl)	Roth, Karlsruhe
magnesium chloride ($MgCl_2$)	Roth, Karlsruhe
magnesium sulphate ($MgSO_4$)	Fluka, Deisenhofen
methanol	Fluka, Deisenhofen
methylene blue	Roth, Karlsruhe
MOPS	Fluka, Deisenhofen
N-lauroylsarcosine	Roth, Karlsruhe
phenol	Roth, Karlsruhe
phenol/chloroform	Roth, Karlsruhe
picric acid	Sigma
Ponceau S	Sigma, Deisenhofen
potassium acetate(KAc)	Riedel-de-Haen, Seelze
potassium chloride(KCl)	Roth, Karlsruhe
potassium hydrogenphosphate (KH_2PO_4)	Fluka, Deisenhofen
Rotiphorese gel (acrylamide)	Roth, Karlsruhe
sucrose	Roth, Karlsruhe
SDS-sodium dodecyl(lauryl) sulfate	Riedel de Haen, seelze
sephadex(G25, G50)	Pharmacia, Freiburg
sodium acetate (NaAc)	Fluka, Deisenhofen
sodium azid(NaN_3)	Merck, darmstadt
sodium carbonate($NaCO_3$)	Roth, Karlsruhe
sodium citrate	Roth, Karlsruhe
sodium dihydrogenphosphate (NaH_2PO_4)	Fluka, Deisenhofen
sodium hydrogenphosphate (Na_2HPO_4)	Fluka, Deisenhofen
sodium hydroxide	Fluka, Deisenhofen
TEMED	Biomol, Hamburg
Tris	Riedel de Haen,Seelze
Triton-x-100	Serva, Heidelberg

Tween 20	Roth, Karlsruhe
urea	Merck, darmstadt
X-gal	Roth, Karlsruhe
xylene cyanol FF	Fluka, Deisenhofen

Chemicals of the highest purity (analytical grade) were used.

Devices

autoclave	Zirbus, Bad Grund
binocular	Olympus, Hamburg
centrifuges:	
Appligene	Appligene, France
Avanti™ 30	Beckmann, München
C0650, C1015, F2404 rotors	Beckmann, München
Centrifuge 5417 C	Eppendorf, Hamburg
Rotina 48R	Hettich, Tuttlingen
E.A.S.Y. gel documenting system	Herolab, Wiesloch
Fluorescence microscope	Leitz, Wetzlar
gel dryer	Bachofer, Reutlingen
Gene Pulser®	Bio-Rad, Canada
GeneQuant®	Pharmacia, Freiburg
Gradi Frac®	Pharmacia, Freiburg
glasspipette	Hirschmann Germany
heating block	Workshop, University of Kassel
heating plate	IKA Staufen im Breisgau
hybridization oven	Bachofer, Reutlingen
imager: Fuji X Bas 1500	Raytest, Straubenhardt
Bio Imaging Analyzer – BAS cassette 2025	Raytest, Straubenhardt
magnetic stirring plate	Bachofer, Reutlingen
microscope	Zeiss, Jena
microwave oven	Panasonic, England
Neubauer – counting chamber	Brand, Wertheim/Main
PCR-Mastercycler personal	Eppendorf, Hamburg
pH-Meter 320	Bachofer, Reutlingen
pipetboy	Integra bioscience, Fernwald
pipettes (10μl, 20μl, 200μl, 1000μl)	Eppendorf, Hamburg
photometer Uvikon 930	Kontron
power supplies:	

Power Pac 3000	Bio-Rad, Canada
EPS	Pharmacia, Freiburg
Pump	BioRad
rocking platform	Heidolf, Germany
scales	Satorius, Göttingen
semidry blotting apparatue	Von Kreuz, Reiskirchen
Speed Vac concentrator	Savant, USA
ultra-sonicator UP 200S	Dr. Hielscher GmbH, Stansdorf
Vortex Genie	Bender Hohbein AG, Germany
water bath Julabo F25	Schütt, Göttingen

DNA and protein markers

100bp DNA ladder	MBI Fermentas, St. Leon-Rot
100bp plus DNA ladder	MBI Fermentas, St. Leon-Rot
1 kb DNA ladder	MBI Fermentas, St. Leon-Rot
protein marker	Serva
SeeBlue pre-stained portein standards	Novex, USA

Enzymes and proteins

proteinase K	Boehringer Mannheim, Mannheim
restriction endonucleases	Boeheringer Mannheim, Mannheim
	Gibco BRL, Eggenstein
	MBI Fermentas, St. Leon-Rot
	New England Biolabs
reverse transcriptase (Mu-MLV)	MBI Fermentas, St. Leon-Rot
RNase A	Boehringer Mannheim, Mannheim
RNase inhibitor (RNasin)	MBI Fermentas, St. Leon-Rot
shrimp alkaline phosphatase (SAP)	USB
SP6 RNA polymerase	MBI Fermentas, St. Leon-Rot
T4 DNA ligase	MBI Fermentase, St. Leon-Rot
T7 RNA polymerase	MBI Fermentase, St. Leon-Rot
Taq DNA polymerase	Home made, Department of Genetics, University of Kassel

General buffers and solutions

6 x DNA loading buffer	40% (w/v) sucrose
	0.25% bromophenol blue and or
	0.25% xylene cyanol FF
100 x Denhardt	2% Ficoll 400
	2% polyvinylpyrollidone
	2% BSA
ethidium bormide solution	10 mg/ml
phosphate buffer, pH 6.7	56.5 ml 1M KH_2PO_4
	43.5 ml 1M K_2HPO_4
20 x SSC	3 M Nacl
	0.3 M sodium citrate
TAE buffer	40 mM Tris-acetate
	2 mM EDTA
TBE buffer	90 mM Tris-borate
	2 mM EDTA
TE buffer	10 mM Tris-Cl
	1 mM EDTA pH 7.4 or 8.0

All buffers and solutions were prepared using deionised or bidistilled water.

Kits and reagents sets

Nucleotrap elution kit	Macherey & Nagel, Düren
Nucleobond AX 100	Macherey & Nagel, Düren
pGem-T-easy cloning kit	Promega, USA
BigDye™Terminator Cycle Sequencing	ABI PRISM
fmol® DNA Cycle Sequencing System	Promega, USA

Media

Dictyostelium general media

AX medium, pH 6.7 (Watts and Ashworth, 1970)	14.3 g Bacto-Peptone 18.0 g glucose 0.616 g Na_2HPO_4 x 2 H_2O 0.486 g KH_2PO_4 7.15 g yeast extract add H_2O to 1000 ml and autoclave for 20 minutes at 121°C
G0 medium	AX medium plus 50 µg/ml ampicillin 100 U/ml penicillin 100 µg/ml streptomycin 0.25 µg/ml amphotericin
SM agar plate, pH 6.5 (Sussman, 1951)	15 g bacto-agar 10 g peptone 10 g glucose 1 g yeast-extract 1 g $MgSO_4$ x 7 H_2O 2.2 g KH_2PO_4 1 g K_2HPO_4 add H_2O to 1L, 20 ml/petridish

Bacterial media

LB medium	10 g Bacto-Tryptone 5 g yeast-extract 5 g NaCl add H_2O to 1L and autoclave for 20 minutes at 121°C
LB agar	1L LB medium plus 9g agar
LB_{amp} plate	LB agar was chilled to 55°C and Ampicillin was added to a final concentration of 50 µg/ml

Oligonucleotide primers

All oligonucleotides were synthesized by Gibco BRL oligo-service

Bs^R primer No. 384:	**GCCGCTCCCACATGATG**
pGEM primers:	
T7 promoter primer:	**TGTAATACGACTCACTATAGGG**
SP6 promoter primer	**ATTTAGGTGACACTATAGAATAC**
pUC primers:	
Universal primer	CGCCAGGGTTTTCCCAGTCACGAC
Reverse primer	GAGCGGATAACAATTTCACACAGG
RACE primers:	
Oligo(dT) – adapter	**AAGCAGTGGTAACAACGAGAGTACTTTTTTTTT**
(Smart-Clontech)	**TTTTTTTTTTTTTTTTTTTTTNN**
Smart adapter	**AAGCAGTGGTAACAACGAGAGTAC**
(Smart – Clontech)	
Ddtafazzin primers:	
TAZ1	**CATATGTTAGATGATCCATTATTATGGGGTG**
TAZ2	**CTCGAGTGGTATTGGTAATTTTGCTAATGG**
TAZ5	**CGTTGGACATTAGGTGCTTC**
TAZ6	**GCATTGATTTCTCCATACCTTG**
TAZ7	**AGGAAGACGTAAAGGAGTTTTTACATTAGTTGG**
TAZ8	**AGGAAGACCTTTAATTGTTGGATGAGAAAAACG**
TAZ9	**GAGGTCGTTTTTCTCATCCAAC**
TAZ10	**CTGATCTCACCGATTACCTTTC**
TAZ11	**CAAATATGTGATATACCAAAACCTC**
TAZ13	**TTTTAATTTTATTTTTAATCTTTAATTG**
TAZ14	**CATATGTGGTATTGGTAATTTTGCTAATGG**
TAZ15	**CCTCTAACAATTTTAATCTATTTTATTTTG**
TAZ16	**CTCGAGTTAGATGATCCATTATTATGGGGTG**
TAZ17	**AGATCTCAAATATGTAGTATACCAAAACCTC**
TAZ18	**AGATCTATGGATAGTAACAATAGTAATAATAAT**
TAZ19	**AGATCTTGTTGGATGAGAAAAACGACCTCT**

Plasmids

pDdA15gfp	Gerisch *et al.*, 1995
pGem 7z	Promega
pGem T-easy	Promega
pUC118	novagene
pUC118 Bs^r cassette	plasmid book No.967, this laboratory

Radioactive materials

[α-^{32}P] dATP (40MBq)	Hartmann Analytic, Braunschweig
[α-^{32}P] UTP (40MBq)	Hartmann Analytic, Braunschweig
[α-^{35}S] dATP (40MBq)	Hartmann Analytic, Braunschweig

Supplementary Material

3MM paper	Whatman, Göttingen
Biodyne-A Nylon membranes	Pall
charged (Hybond™) nylon membranes	Pharmacia, Freiburg
Coster-plates	Schütt, Göttingen
films	Kodak, Stuttgart
glass pipettes	Hirschmann, Germany
injection needles	B. Braun, Melsungen
injection syringes	B. Braun, Melsungen
Parafilm M	American Can ™, USA
Petri dishes	Sarstedt, Nümbrecht
PCR-tubes	New England Biolabs, Schwalbach
PVDF-transfer membranes (Immobilon P)	Pharmacia Biotech, Freiburg
sterile-filter (0.22 μm, 1.45μm pores)	Millipore, Eschborn
transfer membranes Parablot NCP	Mecherey & Nagel, Düren
scalpels	C. Bruno Bayha GmbH, Tuttlingen

4.2 Methods

Cell biological methods

Cell growth

Dictyostelium discoideum Ax2 and the derived transformants were grown either in axenic medium (Watts and Ashworth, 1970) or selection media or with *klebsiella aerogenes* (KA) (Williams and Newell, 1976) suspension as a food source. For growth in suspension, *klebsiella aerogenes* were grown on SM plates at RT for 2-3 days. Bacteria were washed off the plates with 30 ml phosphate buffer and the OD_{600} of KA suspension should be around 0.8. *Dictyostelium discoideum* cells were inoculated at 5 x 10^4 cells/ml, shaken at 180 rpm 22°C and harvested at the cell densities indicated.

Cloning of Dictyostelium discoideum on SM plates

To obtain single clones of *Dictyostelium discoideum*, around 50-200 cells were resuspended in 100 µl KA suspension and plated on SM plates. Plates were incubated at 22°C for several days until colony plaques appeared on the bacterial lawn. Single clones were picked up with tooth picks, transferred to both new KA plates and selection medium in 24 well Costar plates in order to obtain single clones without KA contamination.

Differentiation conditions

Vegetative cells were harvested at densities indicated and washed free of bacteria by differential centrifugation (1500, 1100 and 950 rpm) using phosphate buffer, the cell pellet was then resuspened in phosphate buffer to density of 2 x 10^7 cells/ml and allowed to develop in shaking (180 rpm) or on filter for the time indicated.

Dictyostelium discoideum transformation

Axenic Ax2 cells were harvested at a density of 1-2 x 10^6 cells/ml and then transformed either by the calcium method (Nellen *et al.,* 1984) or electroporation (Howard *et al.*, 1988). All gene disruption mutants were done by electroporation, and the overexpression transformants with G418 resistance were generated by calcium method.

Classic transformation (calcium method)

10 ml axenic growth cells were plated on a Petri dish and incubated at 22° for 10-20 minutes. The medium was then carefully removed without destroying the cell layer

and 10 ml MES-Hl-5 medium was added for 30 minutes, meanwhile, 38 µl 2M $CaCl_2$ was added to 10-15 µg DNA and 600 µl 1x HPS mixture and vortexed 10 seconds, then precipitated at RT 25 minutes. After this step the MES-Hl-5 medium was removed and CaCl2-DNA precipitate was dropped and spread over the cell layer, and then the DNA-cells mixture was incubated at RT for 20 minutes before 10 ml MES-Hl-5 medium was added and incubated at 22° for another 3 hrs. At the end, all medium was removed carefully and 2 ml glycerol solution was dropped for 5-8 minutes "glycerol-shock" treatment. Then the glycerol solution was completely removed, 10 ml DD20 medium was added and incubated at 22° overnight. Next day the medium was changed and the appropriate selection medium was added. Cells were kept under selection until transformants were obtained.

DD20 medium	20 g/l proteose Peptone
	7 g/l yeast extract
	8 g/l glucose
	0.35 g/l KH_2PO_4
	0.47 g/l $Na_2HPO_4*12H_2O$
	or 0.33 g $Na_2HPO_4*7H_2O$
	adjust pH to 6.5 and autoclave for 20 minutes at 121°C
2 x HBS	16 g/l NaCl
	0,72 g/l KCl
	0,2 g/l NaH_2PO_4
	10 g HEPES
	2 g/l glucose
	adjust pH to 7.05 and filter sterilize
MES-Hl-5 medium	5 g/l yeast extract
	10 g/l glucose
	10 g proteose Peptone
	1.6 g/l MOPS
	adjust pH to 7.1 and autoclave for 20 minutes at 121°C
glycerol solution	3 ml 60% glycerol
	2 ml H2O
	5 ml 2 x HBS

Electroporation

2 x 10^7 cells were collected, washed once with phosphate buffer (pH 6.0) and twice with EP buffer, then resuspended in 0.8 ml EP buffer, 10-20 µg DNA was added to the cells and incubated on ice for 10 minutes. Electroporation was performed at 1 kV, 25 µF in a 4 mm electroporation cuvette (the time constant was between 2 and 3 msec). Cells were plated on a Petri dish, mixed with two drops (8 µl) of each 0.1M $CaCl_2$ and 0.1 M $MgCl_2$ and left at RT for 15 minutes, finally 10 ml DD20 medium was added for overnight incubation. Next day the medium was changed and the appropriate selection medium was added. Cells were kept under selection until transformants were obtained.

EP buffer	10 mM Na_2HPO_4
	50 mM sucrose
	filter sterilize

Cell sorting assay

Axenically growing cells with or without GFP labelling were harvested at a density of approximate 1 x 10^6 cells/ml, washed twice with phosphate buffer, resuspended in phosphate buffer at a density of 2 x 10^7 cells/ml. GFP labelled cells and non-GFP labelled cells were mixed in the ratio of 1:9, 1:1 or 9:1 and allowed to develop on glass slide covered by a thin agarose sheet or Costar plate for a certain time. At the time indicated, images were taken with a conventional fluorescence microscope equipped with an OLYMPUS OM4 camera and Kodak film ISO 200

Standard time course

Preparation of pre-culture

A pre-culture was prepared two days before starting standard time course. Briefly, a *Dictyostelium discoideum* axenic or KA suppension growing culture was inoculated to 10 ml freshly prepared KA suspension to the density between 1 x 10^4 cells/ml to 1 x 10^5 cells/ml and incubated by shaking (180 rpm) until next day.

Preparation of over night culture for standard time course

Approximately 16 – 18 hours before starting standard time course, the pre-culture was counted, then inoculated to 3 flasks of freshly prepared KA suspension to the cell density of 2 x 10^4 cells/ml, 3 x 10^4 cells/ml, 8 x 10^4 cells/ml respectively and incubated over night by shaking (180 rpm).

Standard time course

After approximately 16 – 18 hours incubation, the cells in 3 flasks were counted and the cell density should reach approximately 5 x 10^5 cells/ml, 1 x 10^6 cells/ml, 3 x 10^6 cells/ml respectively (In case that the cell density was lower than expected, wait another couple of hours). The cells were washed free of bacteria by differential centrifugation (1500, 1100, 950 rpm) in 20 mM phosphate buffer. Part of the cells from 1 x 10^6 cells/ml were resuspended in phosphate buffer to a density of 2 x 10^7 cells/ml and set up for development in shaking suspension or filter for a certain time. The cells from the another densities, together with that from the left part of 1 x 10^6 cells/ml were collected for RNA isolation or resuspended in 6 x Laemmli buffer to a density of 5 x 10^5 cells/µl for further protein analysis. After a certain time, the developed cells were collected as mentioned above. Meanwhile, axenically growing cells were harvested at 1 x 10^6 cells/ml and washed with phosphate buffer. Cell pellets were collected as mentioned above.

Molecular biological methods

Isolation of plasmid from E.coli

Plasmid mini-prepartion-alkaline lysis (quick-dirty)

The alkaline lysis method (Birnboin and Doley, 1979) was used to extract the plasmid DNA from small culture (1ml) of *E.coli* transformants. *E.coli* cells were collected by centrifugation (4000 rpm x 5 min), and completely resuspended in 100 µl solution I by strong vortexing. After adding 200 µl solution II, the cells were lysed at RT for 5 min, then mixed with 150 µl solution III, incubated on ice for 5 min, the supernatant was collected by centrifugation (14000 rpm x 20min) and precipitated by adding 2 vol 100% ethanol, then the DNA pellet was washed with 70%, vacuum dried and resuspended in 40 µl TE buffer.

Solution I	25 mM Tris-Cl, pH 7.4
	10 mM EDTA pH7.4
	15% sucrose
Solution II	0.2 M NaOH
	1% SDS
Solution III	3 M NaAC pH 4.7

Isolation of plasmid DNA from *E. coli* using Macherey & Nagel kit (Max-prepration)

Nucleobond™ AX100 from Macherey & Nagel was used according the manual of the supplier.

Plasmid max-prepration "quick-dirty"

100-300 ml *E.coli* cultures were used to isolate larger quantities of plasmids. The mini-preparation method was up-scaled for this purpose. RNase A, phenol/chloroform and proteinase K were additionally used.

Isolation genomic DNA from Dictyostelium discoideum

Fast mini-preparation (Barth *et al.*, 1998)

Dictyostelium discoideum cells grown on Costar 24-well plates (5 x 10^6 cells) were collected, washed once with phosphate buffer and resuspended in 300 µl TES buffer, then snap frozen in liquid nitrogen. Cells were thawed slowly and 25 µg proteinase K

was added, then incubated at 60°C for 1 hr. The genomic DNA was extracted with phenol/chloroform and precipitated with ethanol. The resulted genomic DNA can be used for PCR reaction or single restriction digestion..

TES buffer	10 mM Tris/HCl
	1 mM EDTA
	0.1% SDS
	30 µg/ml RNase A

Maxi-preparation

Genomic DNA was prepared from isolated nuclei as described by Nellen *et al*., 1987. Briefly, up to 10^9 cells were collected from axenic culture, washed twice with ice-cold phosphate buffer and resuspended in NP-40 buffer. The nuclei fraction was obtained by centrifugation (2000 rpm x 10 min x 4°C). The nuclear pellet was then carefully resuspended in 5 ml SDS lysis buffer and incubated with 100 µl Proteinase K stock solution at 60°C for 3 hrs. The genomic DNA was extracted twice with phenol/chloroform, then precipitated by adding 1/10 vol. 8M LiCl and 2 vol. ethanol, the DNA pellet was washed with 70% ethanol, dried and then dissolved in a proper volume of ddH_2O

NP-40 buffer	30 mM HEPES, pH 7.5
	10 mM $Mg(OAc)_2$
	10 mM NaCl
	10% sucrose
	2% NP40
	filter sterilize
SDS lysis buffer	0.7% SDS in TE buffer
proteinase K solution	25 mg/ml in ddH_2O

Isolation of total RNA from Dictyostelium discoideum

Up to 10^7 cells were collected and dissolved in 500 µl solution D. After adding 50 µl 3M NaAc (pH 4.7) and 500 µl phenol/chloroform, the sample was strongly vortexed and incubated on ice for at least 15 min. The upper phase was collected by centrifugation (14000 rpm x 30 min) and the total RNA was precipitated by adding 1 vol. isopropanol, the RNA was then pelleted, washed with 70% ethanol, dried and dissolved in DEPC treated H_2O or formamide.

solution D	4 mM guanidine thiocyanate 25 mM sodium citrate 0.1 M β-mercaptoethanol 0.5% sarcosyl
DEPC water	Millipore water was treated with 0.1% DEPC overnight, then autoclave.

Nucleic acid electrophoresis in agarose gel

Agarose in 1 x TBE buffer was melted in microwave and ethidium bromide was added to a final concentration of 0.5 μg/ml, then poured into a horizontal gel-forming chamber. Gels were run using 1 x TBE buffer, and were documented using UV light (256nm) and the E.A.S.Y system.
1% agarose was used for DNA gels and 2% agarose with freshly prepared GTC (20 mM) was used for RNA gels.

Cloning of DNA fragments into vector

Standard techniques were used as described by Sambrook, *et al*., 1989 and Ausubel *et al*., 1994.

Restriction digestion

Restriction enzymes from Boeheringer Mannheim, Gibco BRL, MBI Fermentas or New England Biolabs were used. Restriction digestions were performed by using appropriate buffer and temperature according the manual of the supplier.

Dephosphorylation of vector

To prevent religation of linearized plasmid vector, usually the 5'- phosphate of vector was dephosphorylated by SAP (shrimp alkaline phosphatase, from USB). Briefly, 1U SAP was added to a 20 μl digestion mixture and incubated at 37°C for 30 min, SAP was then inactivated by heating 10 min at 70°C, then exacted once with phenol/chloroform.

DNA purification from agarose gel

The desired band was cut under UV light (366 nm). DNA was purified using Nucleotrap™ (Macherey & Nagel) purification kit. Purification was performed according to manual of the supplier.

Ligation

After restriction digestion, purified insert DNA fragment and the appropriate plasmid were mixed at a ration of 3:1 (cohesive ends) or 1.1 (blunt ends), ligation was performed in small volumes at 16°C over night or at RT several hours.
PCR fragments were cloned by using pGEM-T-easy vector (Promega).

Transformation of E.coli

$CaCl_2$ method (Dagert and Ehrlich, 1979)

Competent cells preparation

2 ml overnight grown *E.coli* culture was inoculated into 100 ml LB medium and grown at 37°C by shaking to an OD_{600} of 0.5. The bacteria were collected and washed once with ice-cold 0.1 M $CaCl_2$, then the cell pellets were incubated in 50 ml ice-cold 0.1 M $CaCl_2$ for 30 min. After that the cells were collected by centrifugation and carefully resuspended in 5 ml ice-cold 0.1 M $CaCl_2$. The competent cells were then aliquoted in 200 µl and frozen immediately at –80°C.

Transformation of competent *E.coli*

The competent cells were rapidly thawed, mixed with DNA, and incubated on ice for 30 min. The cells were then heat-shocked at 42°C for 2 min and 0.8 ml LB medium was added, after shaking at 37°C for 1 hour, the cells were collected by centrifugation, resuspended in the rest of the medium and spread on LB plates containing antibiotic. The transformants were then grown overnight at 37°C.

Electroporation

Competent cells preparation

2 ml overnight grown *E.coli* culture was inoculated into 100 ml LB medium and grown at 37°C by shaking to an OD_{600} of 0.5, the cells were collected by centrifugation under cold condition, and then washed twice by ice-cold H_2O. Finally the pellet was resuspened in 10ml 10% ice cold glycerol, aliquoted in 100 ul and frozen immediately at –80°C.

Transformation of *E.coli* by electroporation

The competent cells were rapidly thawed and mixed with DNA (in ddH_2O, no salt), the mixture was transferred into a chilled 2 mm BioRad electroporation cuvette and incubated on ice for 10 min. The DNA was then electroporated into *E.coli* at 3 kV, 25µF, 200 Ω. After that, 0.8 ml LB medium was immediately added and incubated at 37°C for 1 hour. Finally the cells were collected by centrifugation and spread on LB plates containing antibiotics. The transformants were then grown overnight at 37°C.

For blue-white screening (with vectors capable of α - complementation), the bacterial competent cells were spread on LB plates containing appropriate antibiotic, 0.1 mM IPTG and 40 μg/ml X – Gal and incubated overnight at 37°C. An alternative to preparing plates containing X – gal and IPTG was to spread 50 μl of X – gal stock solution and 5 μl IPTG stock solution onto previously prepared LB/antibiotic plates. Allow these components to absorb for at least 30 minutes perior to plating cells.

X-Gal stock solution (20 mg/ml)	0.2 g X – gal
	add dimethylformamide to 10 ml
IPTG stock solution (200 mg/ml)	2 g IPTG
	add H_2O to 10 ml, filter sterilize

DNA sequencing

After denaturetion, plasmid DNA was sequenced with the Sanger method (Sanger *et al.*, 1977). *fmol*® DNA Cycle Sequencing System from Promega, the T7 sequencing kit from Pharmacia and BigDye™Terminator cycle sequencing kits were used for all sequencing reactions. 6% denaturing polyacrylamide gels were used to separate the DNA products of sequencing reactions.

6% sequencing gel solution	5.7 g acrylamide
	0.3 g bisacrylamide
	42 g urea
	10 ml 10 x TBE buffer
	500 μl 10% APS*
	50 μl TEMED
	add deionised H_2O to 100 ml

* 10% ammonium persulfate should be prepared fresh weekly in deionised water and stored at 4°C.

Polymerase Chain Reaction (PCR)

Polymerase chain reaction (Saiki *et al*., 1985) was used to amply DNA in vitro. For amplification of plasmid and genomic DNA templates the following protocol was used:

reaction mix	1 ng DNA
	15 pmol each oligo-nucleotide primer
	200 µM dNTP mix
	2 µl 10 x PCR buffer
	1 µl Taq polymerase
	add H_2O to 20µl

a typical reaction protocol:	
1st step	5min 95°C
2nd step (30 cycles)	30 sec 95°C
	30 sec 42°C-52°C
	1 min 72°C
3rd step	10 min 72°C

10 x PCR reaction buffer (MBI)	100 mM Tris/Cl pH8,
	0,1% Triton X-100
	50 mM KCl
	10-25 mM$MgCl_2$

RT-PCR and 3'RACE (rapid amplification of cDNA ends) PCR

First strand synthesis

Reverse transcription was performed from total RNA isolated from axenic growth *Dictystostelium discoideum* cells. The following protocol was used for first strand cDNA synthesis:

total RNA	10 ng- 5 µg
oligo$(dT)_{18}$	0.5 µg
or sequence-specific primer	15 pmol
ddH2O	to 11 µl

The above mixture was incubate at 70°C for 5 min and chilled on ice, then added the following in the order indicated:

5 x reaction buffer	4 µl
10 mM dNTPs	2 µl
ribonuclease inhibitor	20 u
ddH_2O	to 19 µl

incubated at 37°C for 5 min, and 1 µl RevertAid™ H Minus M-MuLV reverse transcriptase (MBI Fermentas) was added, the reaction mixture were then incubated at 42°C for 1 hr, the reaction was stopped by heating at 70°C for 10 min.

PCR reaction

1st step	5min 95°C
2nd step (30 cycles)	30 sec 95°C
	30 sec 42°C-52°C
	1 min 72°C
3rd step	10 min 72°C

Northern and Southern hybridization

Northern-blot analysis (Goda and Minton, 1995)

10 µg total RNA mixed with RNA loading buffer was denatured at 95°C for 5 minutes before loaded to a 2% agarose gel with freshly prepared 20 mM GTC (GTC should be added after the agarose solution cooled below 60°C), the total RNA was separated by electrophoresis at 4°C. The RNA was photographed under UV light (the 2 ribosomal RNA bands were used as molecular weight markers), washed briefly with ddH_2O, and used for Northern transfer by capillary elution. After overnight transfer with 20 x SSC, the nylon membrane was dried, cross-linked under UV light (314 nm, 0.12 J/cm^2), then used for hybridization.

RNA loading buffer	100% formamide
	0.1% Xylene Cyanol FF
	0.1% bromophenol blue
	0.1% 10mg/ml ethidium bromide

Southern blot analysis (Southern, 1975)

DNA fragments (PCR fragment, or digested genomic and plasmid DNA) were separated on a 1% agarose gel, and photographed under UV light to document DNA migration related to a molecular marker. The DNA was first denatured for 30 min, then naturalized for another 30 min. The gel was then washed briefly in ddH_2O and used for transfer by capillary elution. After overnight transfer with 20 x SSC, the

membrane was dried first, cross-linked under UV light (314 nm, 0.12 J/cm^2), then used for hybridization.

denature buffer	0.5 M NaOH
	1.5 M NaCl
renature buffer	0.5 M Tris-HCL, pH7.0
	1.5 M NaCl

Radioactive labeling of nucleic acid

The "oligo-labelling" and in vitro transcription methods were used to label nucleic acid radioactively.

Oligo-labelling method

Approximately 1µg purified DNA template was denatured by heating at 95°C for 5 min, the following was then added:

OLB mix	10 µl
α-P^{32}-dATP	3-5 µl
Klenow fragment	1 µl
ddH_2O	adjust the volume to 50 µl

The reaction was performed at 37°C for 1 hour, the free nucleotides were separated by centrifugation through a Sephadex G50 or G25 spin column. The purified radioactive probe was then denatured by heating at 95°C for 5 min, chilled on ice and then used for hybridization.

OLB mix	200mM Tris-HCl, pH7.5
	25 mM $MgCl_2$
	10 mM β-ME
	1 M HEPES pH 6.6
	13.5 U A_{260} oligos-hexamers
	0.25 mM dCTP, dGTP, dTTP

In vitro transcription

Radioactive RNA probes were made by in vitro transcription. The pGEM vector containing the appropriate DNA fragment was linearized with a unique restriction enzyme, then extracted with phenol/chloroform and precipitated with 100% ethanol. If the PCR product was used, it was extracted with phenol/chloroform and precipitated with 100% ethanol, too. The labelling reaction was set up at 37°C and

incubated for 1 hour. The free nucleotides were separated by centrifugation through a Sephadex G50 or G25 spin column. The purified radioactive probe was then denatured by heating at 95°C for 5 min, chilled on ice and then used for hybridization.

in vitro transcription reaction	n µl linearized plasmid (1 -2 µg)
	or n µl PCR product (1-2 µg)
	5 µl transcription buffer (MBI)
	5 µl NTPs (5 mM each except 2 mM UTP)
	1 µl Rnasein
	1 µl RNA polymerise (10 U/µl)
	5 µl ^{32}P-UTP
	add H_2O to bring the volume of 50 µl

Hybridization

Southern or Northern blot were washed briefly with ddH2O and placed into a hybridization tube. The pre-hybridization was performed in hybridization solution at 37°C for 1 hour without radioactively labeled probe. Then the denatured probe was added and incubated by shaking overnight at 42°C (Southern blot) or 55°C (Northern blot). Next day the blot was washed twice with wash solution I and then once with wash solution II. The membrane was then exposed on an imaging plate for analysis in a Fuji X Bas 1500 bioimaging analyzer.

hybridization solution	50% formamide
	50 mM sodium phosphate pH 7.2
	5 x SSC
	0.1% N-lauroylsarcosine
	7% SDS
wash solution I	2 x SSC
	0.1% SDS
wash solution II	0.2 x SSC
	0.1% SDS

REMI mutagenesis (Kuspa and Loomis, 1992)

As described by Zeng *et al*, 2000. Briefly, 20 μg pUC118+Bs^r vector was linearized with *Bam*HI and electroporated into Ax2 cells together with 100 units of *Bam*HI at 2.5 kV, 3.0 μF (Howard et al., 1988). After electroporation, cells were distributed on 5 Petri dishes (9 cm diameter) and transformants were selected in Bs10 medium. When clones could be detected on the plates, cells were washed off, counted and plated in association with *Klebsiella aerogenes* on SM plates for cloning.

Plasmid rescue - isolation of the disrupted gene from REMI mutant

The strategy of this experiment is shown in figure 1-6.

An affected genomic fragment was recovered from the REMI mutant 17-1-J-3-1 by plasmid rescue as described by Kuspa and Loomis, 1992. Briefly, genomic DNA from REMI mutant 17-1-J-3-1 was digested with *Xba* I or *Xho* I, circularized by ligation in a diluted solution, and then transformed into *E.coli* DH5α.

Protein analytical methods

Preparation of total protein from Dictyostelium discoideum

Dictyostelium discoideum cells from the indicated density were washed once with phosphate buffer, and then the cell pellet was collected by centrifugation (4000 rpm x 5min). Total protein was prepared by lysing the cells in 6 x Laemmli buffer (5 x10^5 cells/µl Laemmli buffer).

6 x Leammli buffer	70 ml 4 x Tris-HCl/SDS, pH 6.8
	30 ml glycerol
	10 g SDS
	0.93 g DTT or
	6 ml ME
	12 mg bromphenol blue

Discontinuous SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

12% polyacrylamide gels were prepared according to Laemmli (1970). Protein samples in 6 x Laemmli buffer were denatured by heating at 95°C for 5 minutes, and then separated on a SDS-PAGE. Electrophoresis was carried out in 1 x protein running buffer. After that the separating gel was either used for immunoblot transfer or immersed directly in Coomassie Blue staining solution with gentle shaking, destaining was performed by shaking in several changes of the sustaining solution.

Table 4-1. Recipe for polyacrylamide separating and stacking gel*

	12% separating gel	Stacking gel
30% acrylamide/0.8% biascrylamide (ml)	4.4	0.45
lower(separating) buffer (ml)	2.67	/
upper (stacking) buffer (ml)	/	1
H_2O (ml)	4.4	2.5
EDTA (µl)	43.2	16
TEMED (µl)	2.67	2
20% APS (µl)	120	60

* The recipe is special for 2 mini gels of Hoefer Mighty Small SE 250/SE 260.

Lower buffer	181.7 g Tris (1.5 M) ***4 g SDS (14 mM)*** add 900 ml H_2O and adjust pH to 8.8 with HCl, add H_2O to final volume of 1000 ml
Upper buffer	60.6 g Tris (0.5 M) 4 g SDS (14 mM) add 800 ml H_2O and adjust pH to 6.8 with HCl, add H_2O to the final volume of 1000 ml.
5 x protein running buffer	25 mM Tris base 380 mM Glycine 0.1% SDS
20% APS*	1 g APS add H_2O to 5 ml

* 20% ammonium persulfate should be prepared fresh weekly in deionised water and stored at 4°C.

Coomassie Brilliant Blue staining solution	0.1% Coomassie bright blue G250 10% acetic acid
Coomassie Brillian Blue staining solution (improved protocol)	1.7% phosphoric acid 6% ammonium sulfate 0.1% CBB G250
Destaining solution	5% methanol 7% acetic acid

The tris-tricine system was used to separate of proteins under 15 kDa.

Table 4-2. Recipes for Tris – tricine separating and stacking gel

	Separating gel	Stacking gel
30% acrylamide/0.8% bisacrylamide (ml)	9.8	1.62
gel buffer (ml)	10	3.1
glycerol (87%) (ml)	3.17	/
H_2O (ml)	7.03	7.78
TEMED (µl)	10	5
20% APS (µl)	50	25

gel buffer

182 g Tris base
add H_2O to 300 ml and adjust pH to 8.45 with HCl, bring the volume to 500 ml with H_2O, add 1.5 g SDS and store at 4°C.

anode buffer

24.2 g Tris base (0.2 M final)
add 500 ml H_2O, adjust pH to 8.9 with HCl, add H_2O to the final volume of 1000 ml. Store at 4°C

cathode buffer

12.11 g Tris base (0.1 M final)
17.92 g tricine (0.1 M final)
1g SDS (0.1% final)
add H_2O to the final volume of 1000ml. Store at 4°C

Electroblotting of proteins (Western blotting, Semi-dry blotting)

Proteins separated by SDS-PAGE were transferred to nitrocellulose membranes by using a sem-dry blotting system (Bjerrum, 1986). The transfer was performed with the "Semi-Dry" Blot apparatus at 2 mA/cm^2 membrane (maximal 40 V) for 1 hour.

Semi-dry transfer buffer	5.8 g Tris
	2.92 g glycine
	0.38 g SDS
	200 ml methnol/ethnol
	add H_2O to 1000 ml

Immunodetection with enzyme conjugated secondary antibodies

The Western blots were immersed in blocking buffer, incubated then with the first antibody at a proper dilution over night. The blots were washed several times with 1 X NCP buffer and then exposed to alkaline phosphatase (AP) conjugated secondary antibody directed against the primary antibody. Antigens were identified by chromogenic visualization in BCIP substrate solution.

10 x NCP buffer	12.1 g Tris base
	87g Nacl
	5ml Tween
	2.0g Sodium azide
BCIP substrate buffer	0.2 mg/ml BCIP in sodium carbonate buffer (pH 10.2)
sodium carbonate buffer	100 mM Na_2CO_3, pH 10.2

Protein quantification

Protein concentration was determined by using the Amido-Black assay. 5 µl protein sample was added to 0.5 ml Amido-Black solution, mixed briefly and centrifuged at 10,000 rpm for 4 minutes. The supernatant was discarded and the pellet was carefully washed once in 0.5 ml washing solution without destroying the pellet. At the end the pellet was resuspended in 0.1 N NaOH, an optical density was measured photometrically at 615 nm. The concentration of the protein sample was derived from the standard curve by using BSA (1-50 µg) as standard.

Amido-Black solution	0.26 g amido-black 90% methanol 10% acetic acid add H_2O to 1000 ml
washing solution	90% methanol 10% acetic acid

Colony immunoblot (Wallraff and Gerisch, 1991)

Dictyostelium discoideum clones on KA plates were transferred onto nictrocellulose filter. The filter was then put on a precolded (-20°C) metal board and incubated at -80°C for 10 minutes. After defrozen at room temperature, the filter was washed several times with 1 x NCP buffer, and then boiled in 6% SDS, followed by briefly washing with 1 X NCP buffer again. The first antibody was added and incubated over night after the filter was pre-incubated in 1 X NCP plus 2% BAS. The blots were washed several times with 1 X NCP buffer and then exposed to alkaline phosphatase (AP) conjugated secondary antibody directed against the primary antibody for several hours. Antigens were identified by chromogenic visualization in BCIP substrate solution. After that, filters were then stained with ponceau S to detect all *Dictyostelium discoideum* cellular protein.

Immunofluorescence assay

Exponentially growing *Dictyostelium discoideum* cells were collected, washed twice with cold phosphate buffer (1000 rpm x 4°C) and resuspended in phosphate buffer, cells were then allowed to settle down and spread onto HCl -treated clean coverslip for 45 minutes, then fixed with picric acid/formaldehyde at RT for 30 minutes. After that washed with PBS/glycine twice x 5 min, followed by twice x 15 min wash with PBG. Subsequently the first antibody was added for 3 hours incubation, after washed 6 times x 5min with PBG, the second antibody was added for 1 hour incubation followed by several wash step: 2 times x 5 min with PBG, 3 times x 5 min with PBS, then briefly washed with H_2O. Finally DABCO (1,4 – diazabicyclo 2.2.2. octane) was dropped to the coverslip and the glass slide covered coverslip was incubated overnight at 4°C in the dark condition. Next day the images can be taken by a cooled CCD camera connected to conventional immunofluorescence microscopy.

10 x PBS	80 g/l NaCl 2 g/l KCl 11.5 g/l Na_2HPO_4 or 14.4 g $Na_2HPO_4*2H_2O$ 2.04 g/lKH_2PO_4 adjust pH to 7.4
PBS/glycine (Jungbluth *et al*.,1994)	100 ml 1 x PBS 0.75 g glycine
PBG	100 ml 1 x PBS 0.5 g BSA 0.1 g fischgelatine [Sigma G7765 (45%)]
picric acid/formaldehyde (freshly prepared)	0.2 g formaldehyde 3.5 ml H_2O 5 ml 20 mM PIPES buffer 1.5 ml picric acid adjust pH to 6.0
20 mM PIPES buffer, pH 6.0	6.05 g/l PIPES adjust pH to 6.0

5

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6

Appendix

appendix I sequence of the *Dictyostelium discoidum tafazzin* gene

The following 1584 bp sequence contains the complete coding region of the *Dictyostelium discoideum tafazzin* gene. The coding sequences for the *tafazzin* gene are shown in black and capitals and the other noncoding sequence including the intron are in grey and non-capitals. All the primers used in this work are indicated in the sequence. The start codon ATG, the stop codon taa are indicated in bold letters.

```
  1 ngccctgngc gtgcactgca ggtcgactct agnagatccc ctcaaaacga aaaaaaaaag

 61 aaaaaactaa aaaaaaaatt taaaaaaaga aaaaaaaatt aaaaaaaatt ttaaaaaaaa

121 aaaaaaaaaa aaaaaaatca aacaaaaatg atgttttaat tatttgttat tttatttatt

181 tacttttatt attattataa ttataattag aaacaattgt aattgaataa ttaattttaa

241 tttttttttt tttttttttc atatttttta attattttat tttatttttt ttttaaaatt

301 tttttttca atcaaacaaa aaaatttttt tgatatatta attttttttt aaattttttt

361 tttctttttc aaaacaaatg attaaaaata aaaataaaaa ataaaaaaat ataaaaaata

421 aaaaaaaata aaaaataaat aaATGGATAG TAACAATAGT AATAATAATA ATAAAAATCT

481 AAAACAAATA TGTGATATAC CAAAACCTCA ATTTTTAAGT AAAGGAGTTT TTACATTAGT
         CAAATA TGTGATATAC CAAAACCTC            GT AAAGGAGTTT TTACATTAGT
         → TAZ11                                 → TAZ7*
         → TAZ17§
```

```
 541 TGGAGTATTA TGTAAATTTT GGATATCAAT GAATACGGCC ACAACATCAG GTATTGATAA

 601 ATTAGTAAAT GAAATTGATA AAACTCACCA ATTAAAAAGA CCAATGATAA CAATTGCAAA

 661 TCATTCGTCA AATTTAGATG ATCCATTATT ATGGGGTGTT TTACCAAATC GTATTTTAAT
                AATTTAGATG ATCCATTATT ATGGGGTG
                → TAZ1

 721 GGATCCATCA AAACAACGTT GGACATTAGG TGCTTCAAAT ATTTTATTTA CAAATTGGTT
                     CGTT GGACATTAGG TGCTT
                     →TAZ5

 781 TTATTCTAAA TTTTTTTCAT TAGGTAAATG tgtaagtttt tacattaata ttattataaa
                                      →          intron

 841 aattaaatga aatttcattg atttttattt cttttatttt ttattttta ttttttttt

 901 tgagaaattt tactaaaata tcttttttt tttttatttt tttttatttt tattttttttt

 961 tttttatttt tattttaaat ttatcaaaat aaaatagATT AAAATTGTTA GAGGTGATGG
                                             ←

1021 AATTTATCAA GATGGTATGA ATGAATCAAT TGATAGATTA TCAGAAGGAC AATGGTTACA

1081 TATATTTCCA GAAGGTAGAA TTAGTCAACA AACTCAATTA TTATATTTTA AATGGGGTCT

1141 TGGTAGATTA GTTGGTGAAT GTTATAGAAG AACCGGTGTT GTACCATTAG TTGTACCAAT

1201 TTATCATCAA GGTATGGAGA AATCAATGCC ATTAGCAAAA TTACCAATAC CAAGAGTTGG
                                   GG TAATCGTTTT AATGGTTATG G
                                                   TAZ2 ←
             CAA GGTATGGAGA AATCAATGCC
             → TAZ4

1261 TATAAATTTA GATAATAAAG TTGGTGATAA TATTTATTGT GATCAAGTAA TTTCGAAATA

1321 TATTGATGAT AATAAAATAT CTGATCTCAC CGATTACCTT TCACAAGATG ATAAAAAACG
                           CTGATCTCAC CGATTACCTT TC
                           → TAZ10

1381 AAAAGATTTT TATAAAACAA TTACGCTTCA TATTGAAGAT GAATATCAAA AAATTATACC
```

```
1441 CCCAACCAAT AGAGGTCGTT TTTCTCATCC AACAATTAAA GATtaaaaat aaaattaaaa
                    GCAA AAAGAGTAGG TTGTTAATTT CTA
                                             TAZ8* ←
                AGAGGTCGTT TTTCTCATCC AACA
                                      TAZ19&←


1501 ttaaaattaa taatgaaaat aataaaatta aaaaaaaaaa aaaaaaaaaa gatattttat


1561 ctaggttgaa aaaaaaaaaa aaaa
```

*: Bpi I restriction digestion site was added at the 5´end of primers TAZ7 and TAZ8.

GATC: original gene disruption site

GGATCC: secondary gene disruption site

TAT17[§]: Bgl II restriction digestion site + primer TAZ11

TAZ19[&]: Bgl II restriction digestin site was added at the 5´end of the primer.

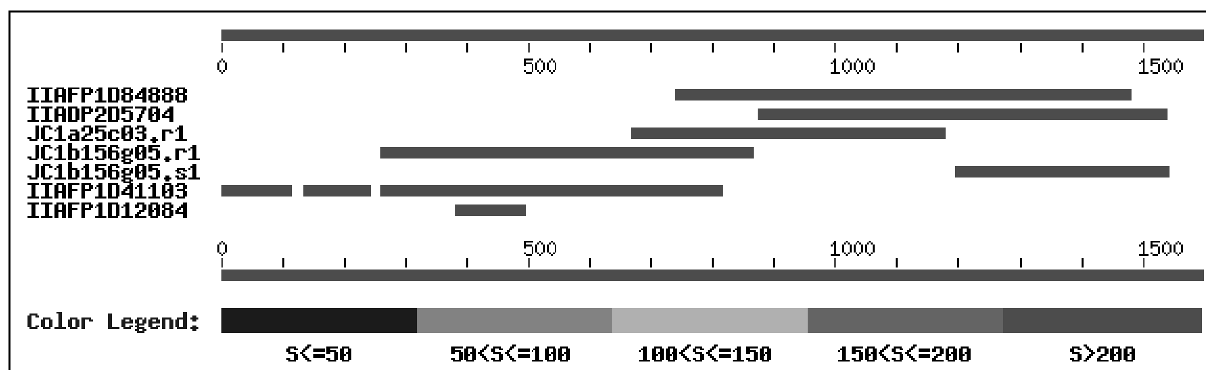
Appendix II: amino acid sequence of *Dictyostelium discoideum* tafazzin protein

```
                 10        20        30        40        50        60
....|....|....|....|....|....|....|....|....|....|....|....|
MDSNNSNNNNKNLKQICDIPKPQFLSKGVFTLVGVLCKFWISMNTATTSGIDKLVNEIDK

                 70        80        90       100       110       120
....|....|....|....|....|....|....|....|....|....|....|....|
THQLKRPMITIANHSSNLDDPLLWGVLPNRILMDPSKQRWTLGASNILFTNWFYSKFFSL

                130       140       150       160       170       180
....|....|....|....|....|....|....|....|....|....|....|....|
GKCIKIVRGDGIYQDGMNESIDRLSEGQWLHIFPEGRISQQTQLLYFKWGLGRLVGECYR

                190       200       210       220       230       240
....|....|....|....|....|....|....|....|....|....|....|....|
RTGVVPLVVPIYHQGMEKSMPLAKLPIPRVGINLDNKVGDNIYCDQVISKYIDDNKISDL

                250       260       270       280
....|....|....|....|....|....|....|....|....|
TDYLSQDDKKRKDFYKTITLHIEDEYQKIIPPTNRGRFSHPTIKD
```

Appendix III

Dictyostelium discoideum tafazzin gene was "cloned" by overlapping the random clones: IIAFP1D84888, IIADP2D5704, JC1a25c03.r1, JC1b156g05.r1, JC1b156g05.s1, IIAFP1D41103, IIAFP1D12084, from the *Dictyostelium* genome project.

BLAST Report




```
>IIAFP1D84888
   Length = 787

  Minus Strand HSPs:

 Score = 4428 (1419.6 bits), Expect = 0., P = 0.
 Identities = 738/738 (100%), Positives = 738/738 (100%), Strand = Minus / Plus

Query:  1478 GATGAGAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 1419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    50 GATGAGAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 109

Query:  1418 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 1359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   110 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 169

Query:  1358 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 1299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   170 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 229

Query:  1298 TATCACCAACTTTATTATCTAAATTTATACCAACTCTTGGTATTGGTAATTTTGCTAATG 1239
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   230 TATCACCAACTTTATTATCTAAATTTATACCAACTCTTGGTATTGGTAATTTTGCTAATG 289

Query:  1238 GCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAATGGTACAACACCGGTTC 1179
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   290 GCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAATGGTACAACACCGGTTC 349

Query:  1178 TTCTATAACATTCACCAACTAATCTACCAAGACCCCATTTAAAATATAATAATTGAGTTT 1119
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   350 TTCTATAACATTCACCAACTAATCTACCAAGACCCCATTTAAAATATAATAATTGAGTTT 409

Query:  1118 GTTGACTAATTCTACCTTCTGGAAATATATGTAACCATTGTCCTTCTGATAATCTATCAA 1059
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   410 GTTGACTAATTCTACCTTCTGGAAATATATGTAACCATTGTCCTTCTGATAATCTATCAA 469

Query:  1058 TTGATTCATTCATACCATCTTGATAAATTCCATCACCTCTAACAATTTTAATCTATTTTA 999
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   470 TTGATTCATTCATACCATCTTGATAAATTCCATCACCTCTAACAATTTTAATCTATTTTA 529

Query:   998 TTTTGATAAATTTAAAATAAAAATAAAAAAAAAAAAATAAAAATAAAAAAAAATAAAAAA 939
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   530 TTTTGATAAATTTAAAATAAAAATAAAAAAAAAAAAATAAAAATAAAAAAAAATAAAAAA 589
```

```
Query:   938 AAAAAAAGATATTTTAGTAAAATTTCTCAAAAAAAAAAATAAAAAATAAAAAATAAAAGA 879
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   590 AAAAAAAGATATTTTAGTAAAATTTCTCAAAAAAAAAAATAAAAAATAAAAAATAAAAGA 649

Query:   878 AATAAAAATCAATGAAATTTCATTTAATTTTTATAATAATATTAATGTAAAAACTTACAC 819
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   650 AATAAAAATCAATGAAATTTCATTTAATTTTTATAATAATATTAATGTAAAAACTTACAC 709

Query:   818 ATTTACCTAATGAAAAAAATTTAGAATAAAACCAATTTGTAAATAAAATATTTGAAGCAC 759
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   710 ATTTACCTAATGAAAAAAATTTAGAATAAAACCAATTTGTAAATAAAATATTTGAAGCAC 769

Query:   758 CTAATGTCCAACGTTGTT 741
             ||||||||||||||||||
Sbjct:   770 CTAATGTCCAACGTTGTT 787


>IIADP2D5704
   Length = 715

  Minus Strand HSPs:

 Score = 3752 (1203.0 bits), Expect = 0., P = 0.
 Identities = 651/664 (98%), Positives = 651/664 (98%), Strand = Minus / Plus

Query:  1538 AATTTTATTATTTTCATTATTAATTTTAATTTTAATTTTATTTTTAATCTTTAATTGTTG 1479
             |||||||| ||  ||||| |||||| || |||||||||| ||||||||||||||||||||
Sbjct:    49 AATTTTATAATAGTCATTCTTAATTCTATTTTTAATTTTGTTTTTAATCTTTAATTGTTG 108

Query:  1478 GATGAGAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 1419
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   109 GATGAGAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 168

Query:  1418 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 1359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   169 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 228

Query:  1358 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 1299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   229 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 288

Query:  1298 TATCACCAACTTTATTATCTAAATTTATACCAACTCTTGG-TATTGGTAATTTTGCTAAT 1240
             |||||||||||||| ||||||||||||||||||||||||| ||||||||||| |||||||
Sbjct:   289 TATCACCAACTTTAATATCTAAATTTATACCAACTCTTGGGTATTGGTAATTATGCTAAT 348

Query:  1239 GGCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAATGGTACAACACCGGTT 1180
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   349 GGCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAATGGTACAACACCGGTT 408

Query:  1179 CTTCTATAACATTCACCAACTAATCTACCAAGACCCCATTTAAAATATAATAATTGAGTT 1120
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   409 CTTCTATAACATTCACCAACTAATCTACCAAGACCCCATTTAAAATATAATAATTGAGTT 468

Query:  1119 TGTTGACTAATTCTACCTTCTGGAAATATATGTAACCATTGTCCTTCTGATAATCTATCA 1060
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   469 TGTTGACTAATTCTACCTTCTGGAAATATATGTAACCATTGTCCTTCTGATAATCTATCA 528

Query:  1059 ATTGATTCATTCATACCATCTTGATAAATTCCATCACCTCTAACAATTTTAATCTATTTT 1000
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   529 ATTGATTCATTCATACCATCTTGATAAATTCCATCACCTCTAACAATTTTAATCTATTTT 588

Query:   999 ATTTTGATAAATTTAAAATAAAAATAAAAAAAAAAAAATAAAAATAAAAAAAAATAAAAA 940
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   589 ATTTTGATAAATTTAAAATAAAAATAAAAAAAAAAAAATAAAAATAAAAAAAAATAAAAA 648

Query:   939 AAAAAAAAGATATTTTAGTAAAATTTCTCAAAAAAAAAAATAAAAAATAAAAAATAAAAG 880
             ||||||| ||||||||||||||||||||||||||||||||||||||||||||||| || |
Sbjct:   649 AAAAAAACGATATTTTAGTAAAATTTCTCAAAAAAAAAAATAAAAAATAAAAAATTAACG 708

Query:   879 AAAT 876
             ||||
Sbjct:   709 AAAT 712
```

```
>IIAFP1D41103
   Length = 808

  Plus Strand HSPs:

 Score = 1556 (499.3 bits), Expect = 0., Sum P(4) = 0.
 Identities = 322/356 (90%), Positives = 322/356 (90%), Strand = Plus / Plus

Query:   466 TAATAATAAAAATCTAAAA-CAAATATGTCAAATATGTGATATACCAAAACCTCAATTTT 524
             ||||||||| |||  |||| | || |        ||||||||||||||||||||||||||
Sbjct:   460 TAATAATAATAATA-AAAATCTAA-AC--CAAATATGTGATATACCAAAACCTCAATTTT 515

Query:   525 TAAGTAAAGGAGTTTTTACATTAGTTGGAGTATTATGTAAATTTTGGATATCAATGAATA 584
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||| 
Sbjct:   516 TAAGTAAAGGAGTTTTTACATTAGTTGGAGTATTATGTAAATTTTGGATATCAATGAATC 575

Query:   585 CGGCCACAACATCAGGTATTGATAAATTAGTAAATGAAATTGATAAAACTCACCAATTAA 644
             ||| ||||||||||||| ||||||||||||||||||||||||||||||||||||| ||||
Sbjct:   576 CGGTCACAACATCAGGTTTTGATAAATTAGTAAATGAAATTGATAAAACTCACCATTTAA 635

Query:   645 AAAGACCAATGATAACAATTGCAAATCATTCGTCAAATTTAGATGA-TCCATTATTATGG 703
             |||||||| ||||| ||||||||||||||||| ||||||||||||| ||| |||||||||
Sbjct:   636 AAAGACCATTGATACCAATTGCAAATCATTCGCCAAATTTAGATGACTCC-TTATTATGG 694

Query:   704 GG-TGTTTTA-CCAAATCGTATTTTAATGGATCCATCAAAACAAC-GTTGGACATTA-GG 759
             || ||||||| ||||||||||||||| |||| ||||||||||| | |||| || ||| ||
Sbjct:   695 GGGTGTTTTAACCAAATCGTATTTTATTGGACCCATCAAAACACCCGTTG-ACCTTAAGG 753

Query:   760 TGCTT-CAAATATTTTATTTACAAATTGGTTTTA-TTCTAAATTTTTT-TCATTAG 812
              |||| ||||||||||||||||||||||| |||| ||||||| ||||| | |||||
Sbjct:   754 -GCTTTCAAATATTTTATTTACAAATTGGGTTTAATTCTAAAATTTTTCTAATTAG 808

 Score = 1044 (335.2 bits), Expect = 0., Sum P(4) = 0.
 Identities = 214/234 (91%), Positives = 214/234 (91%), Strand = Plus / Plus

Query:   260 CATATTTTTTAATTATTTTATTTTATTTTTTTTTTAAAATTTTTTTTTCAATCAAACAA 319
             ||||||||| ||| |||||||||||||||||||||||||||||||||||||||| ||| |
Sbjct:   260 CATATTTTTAAATAATTTTATTTTATTTTTTTTTTAAAATTTTTTTTTCAATCGAACGA 319

Query:   320 AAAAATTTTTTGATATATTAATTTTTTTTTAAATTTTTTTTTTCTTTTTCAAAACAAAT 379
             |||||||||| ||||||||||||||||||||||||||||||||||||||||||| ||| |
Sbjct:   320 AAAAATTTTTGGATATATTAATTTTTTTTTAAATTTTTTTTTTCTTTTTCAAACCAATT 379

Query:   380 GATTAAAAATAAAAATAAAAAATAAAAAAATATAAAAAATAAAAAAAAATAAAAAATAAA 439
             |||||||| ||||| |||||| ||||||| | |||||| |||||||||||||||| ||||
Sbjct:   380 GATTAAAATTAAAATTAAAAATTAAAAAATTCTAAAAATTAAAAAAAAATAAAAATTAAA 439

Query:   440 TAAATGGATAGTAACAATAGTAATAATAATAATAAAAATCTAAAACAAATATGT 493
             ||| ||||| ||| || | ||||||||||||||||||||||||| |||||||||
Sbjct:   440 TAATTGGATGGTACCATTGGTAATAATAATAATAAAAATCTAAACCAAATATGT 493

 Score = 638 (205.1 bits), Expect = 0., Sum P(4) = 0.
 Identities = 109/111 (98%), Positives = 109/111 (98%), Strand = Plus / Plus

Query:     2 GCCCTGNGCGTGCACTGCAGGTCGACTCTAGNAGATCCCCTCAAAACGAAAAAAAAAAGA 61
             |||||| |||||||||||||||||||||||| ||||||||||||||||||||||||||||
Sbjct:     2 GCCCTGTGCGTGCACTGCAGGTCGACTCTAGAAGATCCCCTCAAAACGAAAAAAAAAAGA 61

Query:    62 AAAAACTAAAAAAAAAATTTAAAAAAAGAAAAAAAAATTAAAAAAAATTTT 112
             |||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    62 AAAAACTAAAAAAAAAATTTAAAAAAAGAAAAAAAAATTAAAAAAAATTTT 112

 Score = 618 (198.7 bits), Expect = 0., Sum P(4) = 0.
 Identities = 103/103 (100%), Positives = 103/103 (100%), Strand = Plus / Plus

Query:   138 TCAAACAAAATGATGTTTTAATTATTTGTTATTTTATTTATTTACTTTTATTATTATTA 197
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   138 TCAAACAAAATGATGTTTTAATTATTTGTTATTTTATTTATTTACTTTTATTATTATTA 197

Query:   198 TAATTATAATTAGAAACAATTGTAATTGAATAATTAATTTTAA 240
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct:   198 TAATTATAATTAGAAACAATTGTAATTGAATAATTAATTTTAA 240
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>JC1a25c03.r1 Clone JC1a25c03, reverse read, bases 41 through 547, from
  1999-11-25
   Length = 505

  Plus Strand HSPs:

 Score = 2804 (899.2 bits), Expect = 5.3e-262, P = 5.3e-262
 Identities = 494/508 (97%), Positives = 494/508 (97%), Strand = Plus / Plus

Query:   671 CATTCGTCAAATTTAGATGATCCATTATTATGGGGTGTTTTACCAAATCGTATTTTAATG 730
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:     1 CATTCGTCAAATTTAGATGATCCATTATTATGGGGTGTTTTACCAAATCGTATTTTAATG 60

Query:   731 GATCCATCAAAACAACGTTGGACATTAGGTGCTTCAAATATTTTATTTACAAATTGGTTT 790
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    61 GATCCATCAAAACAACGTTGGACATTAGGTGCTTCAAATATTTTATTTACAAATTGGTTT 120

Query:   791 TATTCTAAATTTTTTTCATTAGGTAAATGTGTAAGTTTTTACATTAATATTATTATAAAA 850
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   121 TATTCTAAATTTTTTTCATTAGGTAAATGTGTAAGTTTTTACATTAATATTATTATAAAA 180

Query:   851 ATTAAATGAAATTTCATTGATTTTTATTTCTTTTATTTTTTATTTTTTATTTTTTTTTTT 910
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   181 ATTAAATGAAATTTCATTGATTTTTATTTCTTTTATTTTTTATTTTTTATTTTTTTTTTT 240

Query:   911 GAGAAATTTTACTAAAATATCTTTTTTTTTTTTTATTTTTTTTTTATTTTTATTTTTTTTT 970
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   241 GAGAAATTTTACTAAAATATCTTTTTTTTTTTTTATTTTTTTTTTATTTTTATTTTTTTTT 300

Query:   971 TTTTATTTTTATTTTAAATTTATCAAAATAAAATAGATTAAAATTGTTAGAGGTGATGGA 1030
             ||||||| | ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   301 TTTTATTCTAATTTTAAATTTATCAAAATAAAATAGATTAAAATTGTTAGAGGTGATGGA 360

Query:  1031 ATTTATCAAGATGGTATGAATGAATCAATTGATAGATTATCAGAAGGACAATGGTTACAT 1090
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   361 ATTTATCAAGATGGTATGAATGAATCAATTGATAGATTATCAGAAGGACAATGGTTACAT 420

Query:  1091 ATATTTCCAGAAGGTAGAATTAGTCAACAAACTCAATTATTATATTTTAAATGGGGTCTT 1150
             ||||||||||||||  ||||||| || ||||||||||||||||||||||||||||| |||
Sbjct:   421 ATATTTCCAGAAGGG-GAATTAGACAGCAAACTCAATTATTATATTTTAAATGGGGGCTT 479

Query:  1151 GGTAGATTAGT-TGGTGAATGTTATAGA 1177
             || |||||| | ||| ||| | || |||
Sbjct:   480 GGCAGATTA-TATGGGGAAAGGTA-AGA 505


>JC1b156g05.r1 Clone JC1b156g05, reverse read, bases 44 through 661, from
  2000-09-06
   Length = 616

  Plus Strand HSPs:

 Score = 2568 (823.6 bits), Expect = 2.8e-237, P = 2.8e-237
 Identities = 555/627 (88%), Positives = 555/627 (88%), Strand = Plus / Plus

Query:   264 TTTTTT-A-ATTATTT--T-ATTTTATTTT-TTTTTTA---AAATTTTTTTTTTCAATCA 314
             |||||| | ||| |||  | |||||||||| ||||||    ||| |||||||||   |||
Sbjct:    14 TTTTTTCATATTTTTTAATTATTTATTTATTTTTTTTTTTAAAATTTTTTTTTT---TCA 70

Query:   315 AACAAA--AAAAT-TTTTTTGAT-ATAT-TAATTTTTTTTTAAATTTTTTTTTTCT-TTT 368
             | ||||  ||||  ||||||  | |||| | | |||||||| || ||||||||| | |||
Sbjct:    71 ATCAAACAAAAAAATTTTTT--TGATATATTAATTTTTTTTTTAAATTTTTTTTT-TCTTT 127

Query:   369 TCAAAACA-AATGATTAAAAATAAAAATAAAAAATAAAAAAATATAAAAA-ATAAAAAA- 425
             |  ||| | ||  || |    |||||||||||| ||||||| || ||||| ||||||||
Sbjct:   128 TTCAAA-ACAA--ATGAT---TAAAAATAAAAA-TAAAAAA-TAAAAAAATATAAAAAAT 179

Query:   426 AAATAAAAAATAAATAAATGGATAGTAACA---ATAGTAATAATAATAATAAAAATC-TA 481
             ||| |||||||||| ||||  | | ||| |   ||||||| |||| |||||| |||  ||
Sbjct:   180 AAA-AAAAAATAAA-AAATA-A-A-TAA-ATGGATAGTAACAATAGTAATAATAATAATA 233

Query:   482 AAA--C-AAATATGTCAAATATGTGATATACCAAAACCTCAATTTTTAAGTAAAGGAGTT 538
             |||  | ||| |   |||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   234 AAAATCTAAA-A---CAAATATGTGATATACCAAAACCTCAATTTTTAAGTAAAGGAGTT 289
```

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Query:   539 TTTACATTAGTTGGAGTATTATGTAAATTTTGGATATCAATGAATACGGCCACAACATCA 598
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   290 TTTACATTAGTTGGAGTATTATGTAAATTTTGGATATCAATGAATACGGCCACAACATCA 349

Query:   599 GGTATTGATAAATTAGTAAATGAAATTGATAAAACTCACCAATTAAAAAGACCAATGATA 658
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   350 GGTATTGATAAATTAGTAAATGAAATTGATAAAACTCACCAATTAAAAAGACCAATGATA 409

Query:   659 ACAATTGCAAATCATTCGTCAAATTTAGATGATCCATTATTA-TGGGGTGTTTTACCAAA 717
             ||||||||||||||||||||||||||| |||||||||||||| |||||||||||||||||
Sbjct:   410 ACAATTGCAAATCATTCGTCAAATTTAAATGATCCATTATTAGTGGGGTGTTTTACCAAA 469

Query:   718 TCGTATTTTAATGGATCCATCAAAACAACGTTGGACATTAGGTGCTTCAAATATTTTATT 777
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   470 TCGTATTTTAATGGATCCATCAAAACAACGTTGGACATTAGGTGCTTCAAATATTTTATT 529

Query:   778 TACAAATTGGTTTTATTCTAAATTTTTTTCATTAGGTAAATG-TGTAAGTTTTTACATTA 836
             ||||||| |||||||||||||||||||||||||||||||||| |||||||||||||||||
Sbjct:   530 TACAAATGGGTTTTATTCTAAATTTTTTTCATTAGGTAAATGGTGTAAGTTTTTACATTA 589

Query:   837 ATATTATTATAAAAATTAAATGAAATT 863
             |||||||||||||||||||||||||||
Sbjct:   590 ATATTATTATAAAAATTAAATGAAATT 616

 Score = 1404 (450.6 bits), Expect = 2.1e-124, P = 2.1e-124
 Identities = 234/234 (100%), Positives = 234/234 (100%), Strand = Plus / Plus

Query:   260 CATATTTTTTAATTATTTTATTTTATTTTTTTTTTAAAATTTTTTTTTTCAATCAAACAA 319
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    20 CATATTTTTTAATTATTTTATTTTATTTTTTTTTTAAAATTTTTTTTTTCAATCAAACAA 79

Query:   320 AAAAATTTTTTGATATATTAATTTTTTTTTAAATTTTTTTTTTCTTTTTCAAAACAAAT 379
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    80 AAAAATTTTTTGATATATTAATTTTTTTTTAAATTTTTTTTTTCTTTTTCAAAACAAAT 139

Query:   380 GATTAAAAATAAAAATAAAAAATAAAAAAATATAAAAAATAAAAAAAAATAAAAAATAAA 439
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   140 GATTAAAAATAAAAATAAAAAATAAAAAAATATAAAAAATAAAAAAAAATAAAAAATAAA 199

Query:   440 TAAATGGATAGTAACAATAGTAATAATAATAATAAAAATCTAAAACAAATATGT 493
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   200 TAAATGGATAGTAACAATAGTAATAATAATAATAAAAATCTAAAACAAATATGT 253


>JC1b156g05.s1 Clone JC1b156g05, standard read, bases 42 through 441, from
  2000-09-05
   Length = 398

  Minus Strand HSPs:

 Score = 2022 (648.6 bits), Expect = 2.1e-186, P = 2.1e-186
 Identities = 341/343 (99%), Positives = 341/343 (99%), Strand = Minus / Plus

Query:  1538 AATTTTATTATTTTCATTATTAATTTTAATTTTAATTTTATTTTTAATCTTTAATTGTTG 1479
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    56 AATTTTATTATTTTCATTATTAATTTTAATTTTAATTTTATTTTTAATCTTTAATTGTTG 115

Query:  1478 GATGAGAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 1419
             ||||| ||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   116 GATGAAAAAAACGACCTCTATTGGTTGGGGGTATAATTTTTTGATATTCATCTTCAATAT 175

Query:  1418 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 1359
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   176 GAAGCGTAATTGTTTTATAAAAATCTTTTCGTTTTTTATCATCTTGTGAAAGGTAATCGG 235


Query:  1358 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 1299
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   236 TGAGATCAGATATTTTATTATCATCAATATATTTCGAAATTACTTGATCACAATAAATAT 295

Query:  1298 TATCACCAACTTTATTATCTAAATTTATACCAACTCTTGGTATTGGTAATTTTGCTAATG 1239
             |||||||||||||| |||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   296 TATCACCAACTTTAATATCTAAATTTATACCAACTCTTGGTATTGGTAATTTTGCTAATG 355
```

```
Query:  1238 GCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAA 1196
             |||||||||||||||||||||||||||||||||||||||||||
Sbjct:   356 GCATTGATTTCTCCATACCTTGATGATAAATTGGTACAACTAA 398
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Appendix IV: Multiple alignment of phosphatidylglycerophosphate synthase from different organism.

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                        10        20        30        40        50        60
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  MIKRALAPIVQPQRLFAALMVIGGGGRSATTTTTTTTKACGNGSSQSPPSTPLLSSKSST
yeast          ------------------------------------------------------------
CHO            ------------------------------------------------------------

                        70        80        90       100       110       120
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  ITSNKKSAIPSSHLYIPKKSTLDSRQIGNYSREYSTSSSSSSKKSIENDTYLNDLFWQLS
yeast          ------MTTRLLQLTRPHYRLLSLPLQKPENIKRQMSAANPSPFGNYLNTITKSLQQNLQ
CHO            ------------------------------------------------------------

                       130       140       150       160       170       180
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  SQGPAFEVNPNNIDEIQEPIDFYNHLIDGVKRSKKRITMASLYLGTSKQEIELVKEMKLA
yeast          T---CEHFQAKEIDIIESPSQEYDLLKTKILNSQNRIFIASLYLG--KSETELVDCISQA
CHO            -------------------------MKGQIRVAKRRVVMASLYLGTGPLEQELVDCLEST

                       190       200       210       220       230       240
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  MERN------KELKIHILLDGLRGTRIGLDKESSATILGELLSLY-SDRVTISMYHTPDL
yeast          LTKN------PKLKVSFLLDGLRGTR-ELPSACSATLLSSLVAKYGSERVDCRLYKTPAY
CHO            LEKSLQAKEPSNLKVSILLDFTRGSR---GRKNSRIMLLPLLRRE-PEQVRVSLEHTPHL

                       250       260       270       280       290       300
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  NGILKKVLPPRINETIGVQHIKTYIFDDDLLLSGANLSKDYETNRQDRYVLIRSTSTVSN
yeast          HGWKKVLVPKRENEGLGLQHMKIYGFDNEVILSGANLSNDYETNRQDRYYLFKSRN-FSN
CHO            RGLLRLLIPERENETIGLQHIKVYLFDNSVILSGANLSDSYETNRQDRYVFLQDCAEIAD

                       310       320       330       340       350       360
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  YENEIVEIIGSLSLHVDKDN----RN-QLLLSSGSIDPVTQSNEEKNLAYTKLSTLLKSH
yeast          YYEKLHQLISSESYQIIKPM----VDGSINLIWPDSNPTVEPTKNKRLFLREASQLLDGE
CHO            FETELVDAVGDVSLQLQGDDTVQVVDGMVHPYKGDRAEYCKAANKRVMDVINSARTRQQM

                       370       380       390       400       410       420
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  SYYPS-NSNSNSSVDSPEDCNNINNGETTWIEPTIQMGPENIRQDEVVTSHIFESVPNDS
yeast          LKSSK-QSLPITAVGQ-ESTLVYPISQETPLEPKYND---KSTEKRTILSLLSTITSNAI
CHO            LHAQTEHSNSLLTQEDAAAAGDRRPAPDTWIYPLIQMKPEEIQIDEIVTETLLTEAERGA

                       430       440       450       460       470       480
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  KEEITSPYENLTENY-LNLILTGKPKLDIITCSPQANGFYGSKGLSSAVPDCYAIIEKRE
yeast          SWTFTAGYENILPDIKAKLLATPVAEANVITASPEANGFYQSKGVSSNLPGAYLYLSKKE
CHO            KVYLTTGYENLTQAY-MDLVLGTRAEYQILLASPEVNGFFGAKGVAGAIPAAYVHIERQE

                       490       500       510       520       530       540
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  LQRVQDTDNGDRISVQEYIR------DKWTYHAKGLWIQVK-----NQQHPSITLIGSPN
yeast          LQDVCRYRQDHAITLREWQRGVVNKPNGWSYHAKGIWLSARDKNDANNWKPEITVIGSSN
CHO            FSEVCSLGQQERVQLQEYWR------RGWTEHAKGLWLYLA-----GSSLPCLTLIGSPN

                       550       560       570       580       590       600
               ....|....|....|....|....|....|....|....|....|....|....|....|
Dictyostelium  FGSRSVEKDLEAQIILITQNKQLQQKMENEKNYLWTDTQNANLELFE---KRKVSLMVRE
yeast          YTRRAYSLDLESNALIITRDEELRKKMKAELDNLLQYTKPVTLEDEQSDPERHVGTGVKI
CHO            FGYRSVHRDLEAQIAIVTENQALQQQLHQEQEQLYLRSGVVSSATFEQ-PSRQVKLWVKM

               ....|....
Dictyostelium  LVYIEGNYL
yeast          ATSILGKKL
CHO            VTPLIKNEF
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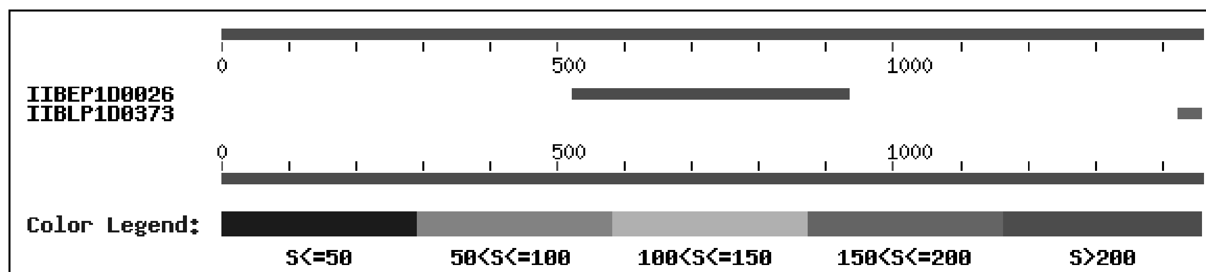
Appendix V:

A *Dictyostelium* BLAST search by using yeast cardiolipin synthase (Q07560) as a query shows that a *Dictyostelium* random clone IIBEP1D8026 possesses 100% identity to the middle region of yeast cardiolipin synthase.

BLAST Report

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>IIBEP1D0026
   Length = 448

  Minus Strand HSPs:

 Score = 2460 (789.0 bits), Expect = 9.2e-229, P = 9.2e-229
 Identities = 410/410 (100%), Positives = 410/410 (100%), Strand = Minus / Plus

Query:   934 CCGAAAAGGCCATTCTATCATGTGTTATCAGGGTTAACTGTACGCTTTAAGGTAAACCCG 875
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    39 CCGAAAAGGCCATTCTATCATGTGTTATCAGGGTTAACTGTACGCTTTAAGGTAAACCCG 98

Query:   874 CAATTAAATTACAATCTTTTCAGAGATCTCACTAGGAGGGAATATGCTACCAATCCGAGT 815
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:    99 CAATTAAATTACAATCTTTTCAGAGATCTCACTAGGAGGGAATATGCTACCAATCCGAGT 158

Query:   814 AAAACTCCTCATATAAAGAGCAAGTTGCTCAATATTCCCAACATTTTGACTTTATCACGA 755
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   159 AAAACTCCTCATATAAAGAGCAAGTTGCTCAATATTCCCAACATTTTGACTTTATCACGA 218

Query:   754 ATAGGATGTACACCCTTTATCGGACTCTTCATTATAACGAATAATTTGACCCCAGCATTA 695
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   219 ATAGGATGTACACCCTTTATCGGACTCTTCATTATAACGAATAATTTGACCCCAGCATTA 278

Query:   694 GGTTTGTTTGCATTTTCCAGCATCACTGATTTTATGGATGGGTATATAGCAAGAAAATAC 635
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   279 GGTTTGTTTGCATTTTCCAGCATCACTGATTTTATGGATGGGTATATAGCAAGAAAATAC 338

Query:   634 GGCCTGAAAACCATTGCAGGAACCATATTAGATCCACTTGCAGATAAACTACTCATGATC 575
             ||||||||||||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   339 GGCCTGAAAACCATTGCAGGAACCATATTAGATCCACTTGCAGATAAACTACTCATGATC 398

Query:   574 ACAACAACTTTGGCATTATCTGTACCATCCGGCCCTCAGATTATACCGGT 525
             ||||||||||||||||||||||||||||||||||||||||||||||||||
Sbjct:   399 ACAACAACTTTGGCATTATCTGTACCATCCGGCCCTCAGATTATACCGGT 448
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Acknowledgments

I would like to thank my supervisor Prof. Dr. Wolfgang Nellen for his continual support during this work, for his patience, guidance and encouragement throughtout this work.

Particularly, I express here my gratitude to Mrs. Petra Zahnwetzer and Mr. Karsten Riemann, who set me up in the laboratory.

I sincerely appreciate our co-operators, Dr. Peter Vreken and his co-worker from University of Amsterdam, The Nederland, for useful discussion, for providing the data of phospholipid assay.

I am deeply indebted to Dr. Jürgen Oberstrass for his inexhaustible supply of experience and and many laboratory tricks, as well as for useful discussion.

I acknowledge Prof. Dr. Marcus Maniak, who was always patient to answer any question concerning cell biology and kindly provided anti-mitochondrial porin antibody.

I thank all co-workers in the laboratory Nellen, who have accompanied me all these years and shared all my exciting sucesses and depressing failures.

I own thanks to all co-workers in the laboratory of Prof. Maniak, who help me a lot on microscopy and provided photoes of immunofluorescence expriment.

I thank *Dictyostelium* data (http://dictybase.org/) and genome sequencing project (http://dictybase.org/dictyostelium_genomics.htm). Without them, the tafazzin gene would not been cloned so simply.

I also sincerely appreciate the critical reading of my thesis by Dr. Jürgen oberstrass, and Mr. Dayananda Rao.

I am particularly thankful to my dear husband, Yi Zhao. Without his support, this PhD thesis would not have been finished.

This work was supported by a grant of the Deutsche Forschungsgemeinschaft to Prof. Dr. Wolfgang Nellen.

Erklärung

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