Characterlisation and identification of transcription factors required for
*D. discoideum* pst-cell differentiation

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Characterisation and identification of transcription factors required for \textit{D. discoideum} pst-cell differentiation

Science of Bioresources, Functional Genomics and Biotechnology

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Abstract

*Dictyostelium discoideum*, the social amoeba, is a widely used model system for studying a variety of processes in development, including cell–cell signaling, signal transduction, pattern formation and the movement of tissue-like aggregates of cells. In multicellular organism, organiser plays an essential role in development. As a definition, it has been proposed that organiser determines cell fate through contact with other germ region. Although the social amoeba *Dictyostelium discoideum* has been known to have similar characteristics in that role of organiser since early times, its developmental function is poorly understand.

During multicellular development of the *D. discoideum*, the most anterior region of a migrating slug plays an essential role as an organiser that controls morphogenesis. The region is comprised of prestalk-cell, of which pstO-cell occupy the posterior half and pstA-cell occupy the anterior half; the organiser consists of the pstA cells. Studies of the mechanism for pstO-cell differentiation have been accumulated whereas pstA-cell differentiation is poorly understood.

In order to identify a mechanism of differentiation of a *D. discoideum* organiser, we focus on a promoter sub-fragment ecmA within ecmA (extracellular matrix A) gene, which is specifically expressed at the pstA-cell. To explore cis-regulatory elements regulating ecmA gene expression in pstA-cell, we first performed deletions of the promoter. I found CA-rich sequences (39-mer) are important for its promoter activity. Using this element as bait, I purified transcription factors by DNA affinity chromatography, and their protein identifications were determined by mass spectrometry. Several transcription factors including MybE and DimB are identified but here I focus on novel transcription factor MrfA, a *D. discoideum* protein with high sequence similarity to animal Myelin-gene Regulatory Factor (MRF)-like proteins. Also in common with MRF-like proteins, MrfA contains a strongly predicted membrane-spanning domain. MrfA displays more spatially restricted but significant sequence similarity with the DNA binding domain of the yeast Ndt80 sporulation-specific transcription factor. Also, the ecmA 39-mer shows sequence similarity to the core consensus Ndt80 binding site (the MSE) and point mutation of highly conserved arginine residues in MrfA, that in Ndt80 make critical contacts with the MSE, ablate binding of MrfA to its sites within the ecmA promoter.

When aggregates are formed, the *mrfA*- mutant exhibits a multi-tipped phenotype as a consequence of reduced "tip-dominance"; hence reduced organiser
activity. Furthermore the slugs tend to break up, resulting in small fruiting bodies. Consistent with these results, the *mrfA-* has a defect in basal disc formation, a phenotypic characteristic of DIF-signaling mutants, and *ecmB-lacZ* is not DIF-1 inducible and monolayer differentiation of stalk cells is much reduced (<10% of WT).

Hence, I propose that MrfA has a role in pstA-cell differentiation as well as in the DIF-1 signaling pathway controlling pstB-cell differentiation.
TABLE OF CONTENTS

ABSTRACT 2

CHAPTER 1: INTRODUCTION

Section 1.1
AIMS OF THIS STUDY 8

Section 1.2
THE LIFE CYCLE OF DICTYOSTELIUM DISCOIDEUM 9
  • Subsection 1.2 A; Preface 9
  • Subsection 1.2 B; Cell adhesion molecules in D. discoideum 10
  • Subsection 1.2 C; Differentiation in D. discoideum 11
  • Subsection 1.2 D; Mound, DIF-1 and birth of cell-type 11
  • Subsection 1.2 E; Slug formation 12
  • Subsection 1.2 F; culmination 13

Section 1.3
ANATOMY OF PST-CELL; ORGANISER FORMATION 14

Section 1.4
TRANSCRIPTION FACTOR REQUIRED FOR PST-CELL DIFFERENTIATION 17

CHAPTER 2: RESULTS 19

Section 2.1
IDENTIFICATION OF TRANSCRIPTION FACTOR REQUIRED FOR D. DISCOIDEUM PST-CELL DIFFERENTIATION 19
  • Subsection 2.1 A; A 39-mer from within the ecma promoter is both necessary and sufficient to direct psta-specific expression 19
  • Subsection 2.1 B; A proximal region of the ecma promoter is necessary and to direct psta-specific expression 22
  • Subsection 2.1 C; The CA-rich sequence elements are necessary for reporter expression in psta-cell 24
  • Subsection 2.1 D; Affinity chromatography using the 39-mer yields the CNS MRF orthologue MrFA 25

Section 2.2
CHARACTERISATION OF TRANSCRIPTION FACTOR (MrFA) REQUIRED TO PST-CELL DIFFERENTIATION 39
• **Subsection 2.2 A;** The MrFA gene is developmentally regulated

• **Subsection 2.2 B;** MrFA is expressed in PST-cell

• **Subsection 2.2 C;** Developing MrFA-cells exhibit delayed aggregation and increased tip-number

• **Subsection 2.2 D;** The expression of PSTA markers is highly defective in the MrFA-strain

• **Subsection 2.2 E;** The fruiting bodies of the MrFA-mutant exhibit ecMB:lacZ and ST:lacZ marker defects

• **Subsection 2.2 F;** MrFA is required for prestalk cell patterning in chimeric development

• **Subsection 2.2 G;** The predicted MrFA DNA binding domain produced in E. coli interacts with the CA-rich sequence elements in the ecMA 39-mer

• **Subsection 2.2 H;** The E. coli-derived protein MrFA binds to the 39-mer

• **Subsection 2.2 I;** MrFA is required only to bind to 39-mer

• **Subsection 2.2 J;** Mutation of two conserved residues important for specific DNA binding by yeast Ndt80 ablates binding of MrFA to the ecMA 39-mer

• **Subsection 2.2 K;** Nuclear extracts from a MrFA-null strain contain an activity that binds the 39-mer

• **Subsection 2.2 L;** Localization and potential proteolytic processing of a myc tagged form of MrFA

• **Subsection 2.2 M;** Many of cells expressing myc-MrFA show both staining puncta and nuclear

• **Subsection 2.2 N;** Cells with nuclear enriched myc-MrFA are mainly confined to the PST-cell of the slug

• **Subsection 2.2 O;** MrFA localization is developmentally regulated and required for normal morphogenesis

• **Subsection 2.2 P;** Expression of MrFA driven by actin15 promoter completely rescues the developmental phenotype of the MrFA-mutant

• **Subsection 2.2 Q;** Peptidase domain is important role of autoproteolytic cleavage reaction

• **Subsection 2.2 R;** Affinity purification of anti-MrFA antibody

• **Subsection 2.2 S;** DIF-1-dependent nuclear accumulation of DimB and STATc is normal in MrFA-mutant cells

• **Subsection 2.2 T;** The fruiting bodies of the MrFA-null mutant show a reduced number of basal disc

• **Subsection 2.2 U;** In MrFA-mutant neutral red staining of PSTB-cell is moderately reduced
• Subsection 2.2 v; MRFA-mutant cells fail to induce stalk cells and prestalk markers in response to DIF-1 82
• Subsection 2.2 w; A MRFA-null mutant shows defects in DIF responses 83

CHAPTER 3: DISCUSSION 84

Section 3.1
Summary 84

Section 3.2
Phenotype vs cell-type specific marker 85

Section 3.3
Mrfa vs 39-mer interaction 86

Section 3.4
Localisation 87

Section 3.5
Phenotypic rescue 88

Section 3.6
Processing mechanism 88

Section 3.7
Conclusion 90

CHAPTER 4: MATERIAL & METHOD 93

1. Strains and Generation of mutant 93
2. Cell culture and development 95
3. lacZ marker studies 95
4. Promoter analysis 96
5. Protein purification and mass spectrometry 98
6. DNA binding assay 10
7. WebLogo designing 11
8. Neutral red staining 11
9. QuickChange XL Site-Directed Mutagenesis 11
10. DIF responsiveness; marker induction assay in monolayer (micro-titre dish lacZ assay) 11
11. Construction of mutant rescue plasmids 11
12. Ligation 11
13. PJET CLONING (SUBCLONING) 124
14. TOPO CLONING (SUBCLONING) 125
15. IMMUNOCYTOCHEMISTRY 126
16. ANTI-MrFA ANTIBODY PURIFICATION 128
17. WESTERN TRANSFER ANALYSIS 130
18. STALK CELL DIFFERENTIATION IN MONOLAYER 136
19. IMMUNOPRECIPITATION PROTOCOL WITH DYNABEADS PROTEIN-G 137
20. IMMUNOPRECIPITATION PROTOCOL WITH PROTEIN-G-agarose beads 139
21. COLLOIDAL BLUE STAINING 140
22. SILVER STAINING 141
23. GEL PURIFICATION BY CRYSTAL VIOLET 142
24. ENZYME DIGESTION (CHECK) 142
25. ENZYME DIGESTION (PURIFICATION) 143
26. PCR 1 (IPROOF) 144
27. PCR 2 (KOD) 145
28. DIRECT PCR FOR KO CHECK (MODIFIED TETSUYA METHOD) 146
29. SYNERGY EXPERIMENT: CELL LABELING 148
30. DIF-1 INDUCTION: MrFA NUCLEAR LOCALISATION, C-TERMINAL FRAGMENT LOCALISATION CHANGE 149
31. MrFA PROCESSING CHANGE DURING DEVELOPMENT; TIME-COURSE 151
32. EDMAN DEGRADATION; BLOTTING & STAINING, DESTAINING 152
33. IMAGEJ PROCESSING 154

CHAPTER 5: REFERENCES 155

CHAPTER 6: ACKNOWLEDGEMENT 165
Introduction

Section 1.1

Aims of this thesis

The aim of these projects is identification and characterisation of transcription factors required for *D. discoideum* pst-cell differentiation. Transcription factors are well recognised as a key regulatory step in selective transcription at most eukaryotic genes. The terminal components of these signaling pathways tend to occupy the genes they regulate and modulate gene expression through activities (Sen and Baltimore, 1986; Zawel et al., 1998). In this way, external signals produce transcriptional responses that allow cells to respond to signaling molecules (cues) from their environment. A number of signaling molecules (cues) are essential for proper cell-type specification. For instance, Activin behaves as a morphogen in that it can direct amphibian blastula cells into different cell-fate pathways of the early embryo according to its concentration (Green & Smith 1990). In *D. discoideum*, the most studied is differentiation-inducing factor 1 (DIF-1) (Kay, 1998), which is essential for the specification of cell-fate, pstO-cell and pstB-cell (Keller and Thompson, 2008; Saito et al., 2008; Thompson and Kay, 2000). bZIP (DimA and DimB), myb (MybE) and STAT (STATc) family transcription factors have been identified that regulate DIF-1 responsive gene expression (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a; Fukuzawa et al., 2006). DimB is also required for DIF-1 inducibility of *ecmA* in monolayer cells and it binds to two sequence elements within the *ecmO* promoter sequence (Huang et al., 2006; Fukuzawa et al., 2006). PstO-specific gene expression is relatively well understood whereas how extracellular signals produce pstA-cell specific responses remains poorly understood. PstA-cell is of special interest in the study of *D.discoideum* development since when transplanted it exhibits some of the characteristics of an embryonic organiser region, acting to establish a secondary axis of symmetry and cause cell trans differentiation (Raper 1940).

In order to identify the signaling pathway directing pstA-cell differentiation, I have been studying the *ecmA* promoter sub-region that directs pstA expression, leading to the ability to characterise relations between such cis-regulatory elements and transcription factor(s) is an important step toward understanding how gene expression is spatial-temporal regulated in cell-fate choice.
Section 1.2
The Life-cycle of *Dictyostelium*

Preface

*D. discoideum* has been a wonderful experimental organism for developmental biologists because initially identical cells are differentiated into one of two alternative cell-types, spore and stalk. It is also an organism where individual cells come together to form a cohesive structure composed of differentiated cell-type, similar to tissue formation in more complex organisms. The aggregation of thousands of amoeba into a single organism is a feat of organisation that invites experimentation to answer questions about the mechanisms involved.

When nutrients are abundant, a *D. discoideum* cell exists as a unicellular haploid vegetative amoeba that feeds on bacteria by phagocytosis and divides by mitosis (Figure. A. 0 hr). When they have exhausted their food supply, tens of thousands of these amoebae join together to form moving streams of cells that converge at a central point (Figure. A. 4-8 hrs). What causes the amoeba to aggregate? Time-lapse movie has shown that no directed movement occurs during the first 4-5 hours following nutrient starvation. During the next 5 hours, however, the cells can be seen moving. Although the movement is directed toward a central point, it is not a simple radial movement. Rather, cells join with one another to form streams; the streams converge into larger streams, and eventually all streams merge at the aggregation center, which is the first step in the creation of multicellular organism. Bonner (1947) and Shaffer (1953) showed that this movement is due to chemotaxis: the cells are guided to aggregation centers by a soluble substance. This substance was identified as cyclic adenosine 3', 5'-monophosphate (cAMP) (Konijn et al. 1967; Bonner et al. 1969). Aggregation is initiated as each of the cells begins to synthesize cAMP. There are no dominant cells that begin the secretion or control the others. Rather, the sites of aggregation are determined by the distribution of amoeba (Keller & Segal, 1970; Tyson & Murray, 1989; Gregor, et al., 2010). Neighboring cells respond to cAMP in two ways: they initiate a movement toward the cAMP pulse, and they release cAMP of their own (Robertson et al., 1972; Shaffer, 1975). After this, the cell is unresponsive to further cAMP pulses for several minutes. The result is a rotating spiral wave of cAMP that is propagated throughout the
population of cells. As each wave arrives, the cells take another step toward the center. In addition, *D. discoideum* uses cAMP as a messenger for signaling to control cell movement in various stages of development (Firtel 1995, Williams 1995).

![Diagram of D. discoideum life cycle and corresponding anatomical structures](image)

**Figure. A. *D.discoideum* life cycle and the corresponding anatomical structures from the Dictyostelium anatomy ontology.**

Life history of Dictyostelium discoideum. Haploid spores give rise to amoeba, which can reproduce asexually to form more haploid amoeba. As the food supply diminishes, aggregation occurs at central points, and a migrating slug is formed. Eventually it stops moving and forms a fruiting body that releases more spores. The times refer to hours since nutrient starvation began the developmental sequence. 0. Vegetative amoebae. 4. Aggregation territory. 8. Streaming aggregate. 12. Tipped-mound. 16. Standing slug. 20. Migrating slug. 24. Early culminant. 28. Fruiting body.

**Cell adhesion molecules in *D. discoideum***

While growing mitotically on bacteria, *D.discoideum* amoebae do not adhere to one another. However, once cell division stops, the cells become increasingly adhesive, reaching a plateau of maximum cohesiveness around 8 hours after starvation. The initial cell-cell adhesion is mediated by a 24 kDa glycoprotein that is absent in vegetative amoebae (Knecht et al., 1987). This protein is synthesized from newly transcribed mRNA and becomes localized in the cell membranes of the amoebae. It is well known that if amoebae are treated with antibodies that bind to and mask this protein, they will not stick to one another, and all subsequent development ceases.

Once this initial aggregation has occurred, it is stabilised by a second cell adhesion molecule. This 80 kDa glycoprotein is also synthesised during the aggregation phase. It is well known that if it is defective or absent in the cells, small slugs will form, and their fruiting bodies will be only about one-third the normal size. Thus, the second cell adhesion system seems to be needed for retaining a large
enough number of cells to form large fruiting bodies (Gerisch, 1978). In addition, a third cell adhesion system is activated late in development, while the slug is migrating. This protein appears to be important in the movement of the pst-cell to the apex of the mound (Ginger et al., 1998). Thus, *D. discoideum* has evolved three developmentally regulated systems of cell-cell adhesion that are necessary for the morphogenesis of individual cells into a coherent organism.

**Differentiation in *D. discoideum***

The ability of individual cells to sense a chemical gradient (as in the *D. discoideum* amoeba’s response to cAMP and DIF-1) is very important for cell migration and morphogenesis during animal development. The biology of *D. discoideum* development is unlike metazoan development. *D. discoideum* is a "part-time multicellular organism" that does not form many cell types (Kay et al., 1989), and the more complex multicellular organisms do not form by the aggregation of formerly independent cells (Kessin, 2001). However, it is possible that pattern formation by a combination of scattered differentiation and sorting out is a common mechanism (Thompson et al., 2003). In particular, the cellular properties required to form scattered or spaced patterns (Headon and Overbeek, 1999), and for sorting out different cell-type (Xu et al., 1999), are widespread. Furthermore there are indications from chick development that these two cellular properties may be brought together in a pattern-forming process, as both the primitive streak and the limb bud apical ectodermal ridge appear to form by the recruitment of a scattered subset of migratory cells (Altabel et al., 1997; Stern and Canning, 1990).

**Mound, DIF-1 and birth of cell-type***

It is now generally accepted that the different cell-type is initially specified intermingled called mound (Figure. A. 12 hrs), without positional information, followed by subsequent cell sorting and pattern formation (Kay and Thompson, 2009; Williams et al., 1989a). Two major precursor cell-type that differentiate within the mound are the prespore (psp) cells (reproductive), which represent ~70% of the total, and prestalk (pst) cells (somatic), representing ~30% of the entire cellular population, form the tubed stalk. Differentiation into stalk or spore reflects another major phenomenon of embryogenesis. A particular cell in a vertebrate embryo, for instance,
can become either an epidermal skin cell or a neuron.

The question of how pattern arises is fundamental to our understanding of *D. discoideum* development. Although the details are not fully understood, a cell-fate appears to be regulated by certain diffusible molecules. The major candidate is differentiation inducing factor-1 (DIF-1). The chemical nature and cell culture actions of DIF-1 provide a candidate molecule for the control of *D. discoideum* patterning. DIF-1 is a chlorinated alkyl phenone produced by developing *D. discoideum* cells. DIF-1 is effective at very low concentrations and can drive amoebae to differentiate as vacuolised stalk cells (Morris et al., 1987). It also induces the expression of prestalk markers, represses prespore markers and prevents cells in culture from differentiating as spores. Consequently, DIF-1 has been considered to be a central regulator of the stalk/spore decision (Early et al., 1995; Early and Williams, 1988; Fosnaugh and Loomis, 1991; Kay and Jermyn, 1983).

However, mutants specifically defective in DIF biosynthesis have been generated, which develops relatively normally until the slug stage of development. At that stage, it makes long, thin structures compared with wild type, which later develop spores and a stalk (Thompson & Kay, 2000). These observations suggest that patterning arises by a mechanism whereby the choice between pst-cell and psp-cell fates is driven by a process similar to lateral inhibition (Clay et al., 1995; Kay et al., 1999; Leach et al., 1973; Loomis, 1993). It is proposed that as cells enter the mound, they all experience similar concentrations of DIF-1. Initial intrinsic differences between the cells distinguish between responding and non-responding populations. Such differences have been noted, and include cell cycle position and growth history (Leach et al., 1973), both of which bias cell fate choice and affect DIF-1 sensitivity (Thompson and Kay, 2000a). As some of the earliest responses to DIF-1 include the down regulation of DIF biosynthesis and up regulation of DIF breakdown (Insall et al., 1992), two populations of cells quickly emerge: DIF-1 responding (prestalk) and DIF-1 producing (prespore). Consistent with this idea, pst-cell ultimately exhibit the highest levels of DIF-1 breakdown and psp-cell the highest levels of DIF-1 biosynthesis (Kay et al., 1993; Kay & Thompson, 2001).

**Slug formation**

A spatially defined pattern of cell-type is obtained after sorting of pst-cell to the top of the mound where a tip is formed. This tip elongates and falls down the
substratum, generating a migrating slug (Figure. A. 16 h). The slug is usually 2-4 mm long and is encased in a slimy sheath. The slug begins to migrate (if the environment is dark and moist) with its anterior tip slightly raised (Figure. A. 20 h). From anterior to posterior of the slug, these are the pst-cell and psp-cell. The anterior cells (pst-cell) normally become stalk, while the remaining, posterior cells (psp-cell) are usually destined to form spores. However, surgically removing the pst-cell of the slug does not abolish its ability to form a stalk. Rather, the cells, which originally had been destined to produce spores, trans differentiation into the stalk (Raper, 1940). This ability of cells to change their developmental fates according to their location within the whole organism and thereby compensate or replenish for missing parts is called regulation. In response to intrinsic and extrinsic signals, the slug undergoes coordinated morphogenetic movements leading to the formation of a mature, differentiated fruiting body.

**Culmination & asexual cycle**

In the culmination stage, as the pst-cell differentiate, they form vacuoles and enlarge, lifting up the mass of psp-cell (Kessin, 2001). The stalk cells die, but the psp-cell, elevated above the stalk, become spore cells. These spore cells disperse, each one becoming a new amoeba (Figure. A. 24-28 hrs).

In addition to this asexual cycle, there is a possibility for sex in *D. discoideum*. Two amoebae can fuse to create a giant cell, which digests all the other cells of the aggregate. When it has eaten all its neighbors, it encysts itself in a thick wall and undergoes meiotic and mitotic divisions; eventually, new amoeba is liberated.
Section 1.3
Anatomy of Pst-cell; Organiser formation

In multicellular organism, organiser plays an essential role in development. As its name implies, the organiser is able to organise embryonic development, which is formed at the blastula stage of amphibian development and acts at the gastrula stage as a cell population capable of (1) releasing inducers to adjacent cells, (2) engaging in distinctive morphogenesis, and (3) differentiating several tissues of the embryonic body axis.

Although *D. discoideum* has been known to have similar characteristics in that role of organiser since early times, its developmental function is poorly understood. The activity of organiser in *D. discoideum* exist at least two in the morphogenesis. Firstly, it emerges in the streaming aggregate stage (Figure. A 8 hrs), which is the first step in the creation of multicellular organism. By starvation, amoeba obtains a capability of synthesizing and secreting cAMP. And then amoeba aggregates into cAMP oscillation center and forms multicellular organism, indicating that cell(s) in the cAMP oscillation center have definite activity of organiser. Secondly, cells in the most anterior region of a slug (pstA-cell) are considered to form a tip-organiser, which ensures the morphological integrity of the organism; it suppresses the formation of other tips and if a second tip is grafted onto a slug flank it will direct the formation of a secondary slug (Raper, 1940) (Figure. B). Also, a slug can neither migrate nor form a fruiting body if a laser burns out a small group of cells in the most anterior region of a slug.
Figure. B. Slug tip grafting experiment.

Early grafting experiments showed that when tips are grafted onto posterior region of a slug, they recruit slug cells from the host slug and result in the formation of an individual smaller slugs (Raper, 1940).

As previously mentioned, the developmental program is initiated by starvation and leads to the formation of a multicellular organism with a defined spatial pattern of cell types organised along an anterior/posterior (AP) axis (Loomis & Cann, 1982). From front to back of the slug, these are the pst-cell and psp-cell (Figure. C). The pst-cell can be further subdivided into pstA-cell (found at the very tip of the slug), pstB-cell (enriched at the pst/psp boundary and dotted around the psp region) and pstO-cell (a collar of cells behind the pstA region) (Williams, 2006). PstB cells that express ecmB but not ecmA is found in the basal disc and the lower cup (Dormann et al 1996, Jermyn et al 1996). These principal populations can be distinguished from one another using reporter fusions with the promotors of ecmA and ecmB; two genes that encode related extracellular matrix proteins. PstA-cell utilise cap-site proximal ecmA promoter sequences, termed ecmA sequences, while pstO-cell utilise cap-site distal sequences, the ecmO sequences (Early et al., 1993); the complete promoter of the ecmA gene is termed the ecmAO sequence.
Figure. C. Subdivisions of the multicellular organism.

The prestalk and prespore zones are recognisable from the tipped mound stage. This diagram represents the different subdivisions of the multicellular organism at the migratory slug stage. The subdivisions remain in the same relative positions and proportions until culmination.
Section 1.4
Transcription factor related to pst-cell differentiation

In eukaryotes, transcription of protein-coding genes is performed by RNA polymerase II. Genes transcribed by RNA polymerase II typically contain two distinct families of cis-acting transcriptional regulatory DNA elements: (a) a promoter, which is composed of a core promoter and nearby (proximal) regulatory elements, and (b) distal regulatory elements, which can be enhancers, silencers, insulators (Figure. D). These cis-acting transcriptional regulatory elements contain recognition sites for trans-acting DNA-binding transcription factors, which function either to enhance or repress transcription.

Figure. D. Schematic of a typical gene regulatory region.
The promoter, which is composed of a core promoter and proximal promoter elements. Distal (upstream) regulatory elements, typically spans less than 1.5 kb pairs in D. discoideum, which can include enhancers, silencers, insulators. These distal elements may contact the core promoter or proximal promoter through a mechanism that involves looping out the intervening DNA.
Transcription factors are well recognised as a key regulatory step in selective transcription at most eukaryotic genes (Roeder, 2005). The terminal components of these signaling pathways tend to occupy the genes they regulate (Sen and Baltimore, 1986; Zawel et al., 1998) and modulate gene expression through activities that include recruitment of coactivators, and activation of transcription (Clevers, 2006; Kopan and Ilagan, 2009). In this way, external signals produce transcriptional responses that allow cells to respond to signaling molecules (cues) from their environment.

A number of signaling molecules (cues) are essential for proper cell-type specification. For instance, Activin behaves as a perfect morphogen in that it can direct amphibian blastula cells into different cell-fate pathways of the early embryo according to its concentration (Green & Smith 1990) and appears to be a natural signaling molecule (Piepenburg et al. 2004). In D. discoideum, the most studied of these is differentiation-inducing factor 1 (DIF-1) (Kay, 1998), which is essential for the specification of pstO-cell and pstB-cell (Keller and Thompson, 2008; Saito et al., 2008; Thompson and Kay, 2000). bZIP (DimA and DimB), myb (MybE) and STAT (STATc) family transcription factors have been identified that regulate DIF-1 responsive gene expression (Fukuzawa et al., 2001; Fukuzawa et al., 2006; Huang et al., 2006; Thompson et al., 2004a; Fukuzawa et al., 2006). DimA, a bZIP transcription factor, is required for ecmAO::lacZ expression in pstO-cell and for the induction, by DIF-1, of ecmA expression in a monolayer assay (Thompson et al., 2004). DimB, a dimerisation partner of DimA, is also required for DIF-1 inducibility of ecmA in monolayer cells and it binds to two sequence elements within the ecmO promoter sequence (Huang et al., 2006; Fukuzawa et al., 2006). However, the capsite proximal promoter region of the ecmA gene, that directs pstA specific gene expression, is in contrast totally uncharacterised (Figure. D).

To explore transcriptional factors and regulatory elements important for differentiation of pstA-cell, we perform a promoter analysis of ecmA. I determine promoter sequences responsible for the pstA expression, by performing deletions. Finally, I identify the transcriptional factors using DNA affinity chromatography and a mass spectrometry. These analyses should uncover the part of signal transduction related to pstA-cell differentiation.
Results

Section 2.1
Identification of transcription factor required for pst-cell differentiation

A 39-mer from within the ecmA promoter is both necessary and sufficient to direct pstA-specific expression

A distal region of the ecmA promoter is active in pstA-cell but not in pstO-cell. To understand the molecular mechanisms that govern specific expression patterns, it is important to identify the transcriptional regulatory elements associated with each predicted gene.

The sequence of the 753 nucleotides immediately upstream of the ecmA coding region, extending to -493 with respect to the cap site, is shown (Figure 1). When the entire promoter is joined to the lacZ gene, with a fusion point just downstream of the initiation codon of the ecmA gene, β-gal accumulates in pstA-cell and pstO-cell (Jermyn and Williams, 1991).

Previous deletion analysis of the ecmA promoter identified a region with a 5′ end point at nt -493 (numbered relative to the cap site) that directs expression selectively in pstA-cell (Fukuzawa et al., 2006). The 117nt region adjacent to this 5′ end point is, as is typical of all D. discoideum promoters, highly AT-rich and it can be deleted, to yield construct ecmA376: lacZ, with no apparent effect on the spatial expression pattern (Figure 2). The adjacent 39 nucleotides are much less AT-rich and just a partial deletion of this, very CA-rich, region, to yield ecmA357: lacZ, abolishes expression (Figure 2). We term the sequence encompassing the entire CA-rich region the 39-mer. In detail, 39-mer is composed of two CA-rich clusters; a part of former region is 20-mer and that of latter region is 19-mer.
Figure. 1. The nucleotide sequence of the region upstream of the *ecmA* gene.
The position of the cap site is marked (+1). The end points of the 5' and 3' deletion constructs analysed are also shown.
Figure 2. Analysis of the ecmA promoter sub-region that directs expression in pstA-cell.
Schematic representation of the 5’ deletions of the ecmA promoter that define the 39-mer and expression analysis of slugs expressing them. The sequence shown in black blue is 39-mer. The position of the cap site is marked (+1). Bar represents 200 um.
A proximal region of the ecmA promoter is necessary and direct pstA-specific expression

In order to investigate the activity of the different parts of the promoter, we investigated the activity of subfragments inserted into the actin15ΔBam-gal vector (Pears and Williams, 1988; Ceccarelli et al., 1991). In this vector, an internally deleted version of the actin15 promoter is fused to the lacZ gene. Although it is inactivated by the removal of essential regulatory elements, it still provides a TATA box and cap site. In order to reduce actin 15 promoter background, we further delete its TATA box and cap site. Alternatively, I designed constructs containing authentic ecmA TATA box and cap site instead of that of actin 15. Defined fragments of the ecmA promoter were generated by PCR and inserted into this modified actin15ΔBam-gal vector. The construct showed expression in pstA-cell but not pstO-cell (Figure. 3, eA0541r). Upon removal of an additional 171 nucleotides (Figure. 3, eA05129r construct) there is a further, and much more dramatic, change in the pattern of expression. Staining is now absent from the pstA-cell of the slug (Figure. 3, eA05129r). The region between -120 and +41 is necessary for expression in pstA-cell but is not sufficient, because a fragment with a 5′ end point (Early et al., 1993). Interestingly, upon further removal of an additional 72 and 201 nucleotides (Figure. 3, eA05201r & eA330r) there is restoration in the pattern of pstA-cell specific expression (Figure. 3, eA05201r & eA330r), suggesting that existence of repressor element between -129 and -201 nucleotides.
Figure 3. Analysis of the ecmA promoter sub-region that directs expression in pstA-cell.

(A) Schematic representation of the 3' deletions of the ecmA promoter and expression analysis of slugs expressing them. The sequence shown in magenta is hypothesized essential region (Early et al., 1993). The position of the cap site is marked (+1). Scale bar represents 200 um. (B) The structure of the ecmA promoter. The region between -376 and -337 (blue) is independently active in pstA-cell. Sequences between -201 and -128 (cyan) might be repressor element in pstA-cell. The region between -120 and +41 (magenta) is essential for expression in pstA-cell.
The CA-rich sequence elements are necessary for reporter expression in pstA-cell

We next determined the effect of mutations in the CA-rich regions on the in vivo expression of ecmA376::lacZ (Figure 2). Each of the three separate block mutations that were analysed by reporter expression, only CAmt1 (left side of CA cluster in 39-mer) and CAmt3 (right side of CA cluster in 39-mer), showing the lowest relative level of expression (Figure 4 B). Interestingly, CAmt2 (middle of CA cluster in 39-mer) showed normal ecmA376 WT expression level. Thus, two CA-rich regions are needed for optimal expression in vivo.

Figure 4 Mutational analysis of the ecmA 39-mer.
(A) The CA-rich sequences (CAmt1-3) within 39-mer region of ecmA376::lacZ (Figure 1) were mutated, by C to A substitutions as indicated by small case letters, to generate CAmt1::lacZ-CAmt3::lacZ. (B) The staining patterns of a parental ecmA376::lacZ (WT) and each of the three mutant constructs described in A. They were all stained for 16 hours. CAmt1::lacZ and CAmt3::lacZ showed almost no staining by 8 hrs of incubation, at which time the WT control (ecmA376::lacZ) showed strong labeling in pstA cells. Scale bar represents 200 um.
Affinity chromatography using the 39-mer yields the CNS MRF orthologue MrfA

The 39-mer was oligomerised, bound to a matrix and used in affinity chromatography with nuclear extracts derived from slug cells. After two rounds of chromatography SDS electrophoresis resolved many stained bands (Figure 5A) and slices containing these were excised and subjected to mass spectrometry. The hits (a representative protein listed in Figure 5B) include DimB and MrfA. Since the DimB null is not defective in pstA-cell differentiation (Fukuzawa et al., 2006), we chose to determine whether MrfA plays any role in the process. We propose the more meaningful name of MrfA because there is strong similarity along most of the MrfA protein length (excepting the simple repetitive sequence) with vertebrate CNS MRF (Myelin-Gene Regulatory Factor; Emery et al., 2009).

Murine MRF is the only functionally characterised member of a family, which we will term the MRF-like family, that we find to be represented in vertebrate and invertebrate animals but not in fungi (Figure 7B). All members contain a region with sequence similarity to the DNA binding domain of yeast Ndt80 (Figure 7A). In addition, the protein contained predicted DNA binding domain homologous to the yeast transcription factor ScNdt80 (Montano et al., 2002); within this region, DdMrfA and ScNdt80 shared 29% sequence. HsMRF and ScNdt80 shared 17% sequence. DdMrfA and HsMRF shared 23% sequence.

The principal defining feature of the MRF-like family is the additional presence, nearer the C terminus, of an approximate 150 amino acid region, which we will term the peptidase domain (Figure 7B Figure 8 and Figure 9). Interestingly, the peptidase domain shows significant sequence similarity with prokaryotic proteins that include several cell wall surface anchor proteins but the significance of this is unclear (Figure 6 B; the highest scoring protein has 26% identity with MrfA). More tellingly, MrfA and the animal MRF-like proteins all contain a strongly predicted transmembrane domain situated in the same approximate position relative to their mutually conserved regions (Figure 9). The animal MRFs differ from MrfA in possessing a much longer sequence region C-terminal to the predicted transmembrane domain.

Dictyostelium discoideum have three MRF orthologues; Gene ID DDB_G0284183, Gene ID DDB_G0292186, Gene ID DDB_G0293934. A detailed explanation of composition and expression pattern of mrfA is discussed later section (Figure 15). As for DDB_G0292186, it contains unknown MRF C1 domain differently from other
orthologus (Figure. 6B a). Furthermore, DNA binding domain is located near the N-terminus differently from other orthologus (Figure. 6B b). Regarding DDB_G0293934, it has three transmembrane domains (TMD) near the C-terminus differently from other orthologus (Figure. 6C a). In addition, as analysis of spatial expression pattern of this gene had already done by in situ hybridization, its pattern shows pstO-cell, upper cup specific during late developmental stage (early culmination) (Figure. 6C c). Multiple alignment of the Dictyostelium MRF orthologues proteins show sequence similarity with the DNA binding domain (Ndt80) and C-terminal part of endoNF peptidase domain (Martina et al., 2003). We have identified several sequence homologs of DNA binding domain among three proteins. The sequence conservation (sequence identities of 14% conserved with Ndt80 DNA binding domain) is sufficiently high to suggest that the proteins are in fact structurally and functionally related (Figure. 6). However, only mrfA and DDB_G0292186 protein have two arginine residues that are essential for DNA binding of Ndt80 (Figure. 10). Alignment of peptidase domain shows sequence conservation serine (S) - lysine (K) dyad at the N-terminus (magenta asterisks) (Figure. 11). All of Dictyostelium MRF orthologues have conserved serine (S) - lysine (K) dyad amino acids, suggesting that this auto proteolysis mechanism might be conserved between prokaryote and eukaryote. When it comes to transmembrane domain (TMD), this alignment result shows large hydrophilic sequences at putative membrane-spanning domains but no sequence similarity among Dictyostelium MRF orthologus (Figure. 12).
Figure 5. Purification and identification of MrfA.

(A) Preparative SDS gel electrophoresis of the proteins purified by affinity chromatography on the 39-mer. (B) This gel image shows a list of prominent proteins identified by mass spectrometry. (C) Only the gel slices that contained known transcription factors are presented and only the hits with a "Mowse" score (Pappin, 1993) of 50 or more are listed.
A: mrfA, Chromosome 4 coordinates 1846274 to 1849299

![Diagram of mrfA coordinates]

- **GENE3D**: GDSA:2.60.40.1390
- **PANTHER FAMILY NOT NAMED**: UNCHARACTERIZED
- **PFAM**: NDT80_ProG, Peptidase_S74
- **PROFILE**: NDT80
- **SSF**: p53-like transcription factors

![Experiment Comparison for gene rcgK (DDB_G0284133)]

- **Scaled Read Counts**
- **time [hrs]**: 0 2 4 6 8 10 12 14 16 18 20 22 24
B: DDB_G0292186, Chromosome 6 coordinates 1196812 to 1200751

a

b

Experiment Comparison for gene DDB_G0292186

Legend

Show experiment selector
C: DDB_G0293934, Chromosome 6 coordinates 3520311 to 3525707

a

b

Experiment Comparison for gene DDB_G0293934

Scaled Read Counts

0 2 4 6 8 10 12 14 16 18 20 22 24

0 400 500 600 700 800 900 1000

Legend

Show experiment selector
Figure 6. Protein information of MRF orthologues (dictybase protein information).

Dictyostelium discoideum have three MRF orthologues. (A) a) MrfA contains DNA binding domain (Ndt80), peptidase domain and single-pass membrane domain (transmembrane domain). Protein molecular weight is 104 kDa. 
mrfA ORF is composed of 3,026 base pairs, which contains two introns. b) This image shows 
mrfA (rcdK) expression pattern during development. (B) a) DDB_G0292186 contains DNA binding domain (Ndt80), peptidase domain, unknown MRF C1 domain and single-pass membrane domain (transmembrane domain). Protein molecular weight is 126 kDa. DDB_G0292186 ORF is composed of 3,940 base pairs, which contains three introns. This gene null mutant shows normal WT phenotype (Strains and Phenotypes; http://dictybase.org/gene/DDB_G0292186). b) This image shows 
DDB_G0292186 expression pattern during development. (C) a) DDB_G0293934 contains 
DNA binding domain (Ndt80), peptidase domain and multi-pass membrane domain 
(transmembrane domain). DDB_G0293934 ORF is composed of 5,397 base pairs, which 
contains two introns. b) This image shows DDB_G0293934 expression pattern during development. c) This mRNA is expressed in pstO-cell and in upper cup during culmination. 
Protein molecular weight is 189 kDa. RNA-sequence data is quoted from dictyExpress 
(http://dictyexpress.biolog.sii). In situ expression pattern is quoted from In Situ Hybridization Atlas, Japanese cDNA project.
a) MrfA vs Yeast Ndt80

MrfA 362 KGSYIGGQWYCRQHFQLOHISTAYKLETQYGRHNGVTLNSRSHSDVTQPSKMTL
+GF + I +W + RR+F L T ++ +D S+ L+
Ndt80 97 RGFHIDHENWGVKSHYFTL-------------------------VSTFTANCQDFTFLKSSFDLL

MrfA 422 SGWTPQNLTLTIGKIEIMRASQDSEVEFQTSRSEKQGEHAP
+ + L + IK +A ++K+ L Q ++K+K + P
Ndt80 139 VEDESMERLNWGVYTAIKKAKHSDDTEIRLWQTMARAEQGQPFCP

b) MrfA vs Human MRF isoform 2

Ndt80 domain:

MrfA 332 WTVKSEPHWTTTFTQGDEMVPYLLNIMASKQFGSY-IGQWYIC-RRHFLDITAVYPK
W ++ W T ++ N E=+ A KGF++ +G C +NHFQ+ VY
MRF 353 WPQHQQHWMATLDANFKEPIHMLTTRVDADRGFPSLVQGALFQCQKHVFQV---TVTIG
MrfA 390 LPOETQYGHGIVTSLNSSSVDPTPSYHLSQVGYTPINMLTIGKIEIMRASQDSE
+ E + Y ++TP G+K P++ L + G+K A ++Q
MRF 410 MLHEFQY---------------VRIFCTKLPCGFLQTVKLEA----NG...-
MrfA 450 EVELFQTNSRSEKQGEKVPFAIQPG-------SVSTQRHLFQKHAEIMRASQ
+ + G + S K K+ + PV + + V+ RHEF + T NN R+ G+PHQ Q
MRF 444 SINEIQSQRDREKHPF---PVTVMLPPQVTKTVGRLHFESETANMMRKGGKHPDQPQR
MrfA 506 FNQLVSVLYGRCMQGVEVYVYSFALIVRTA
+ LVV+L Q Y + + S +IVR +
MRF 501 YPHLNLVALQAHQHYTQLAAQSERIYIRAS

MRF domain:

MrfA 725 WNSGDEKTISYLYNGVHVINPAHLSVQCTYIASEGQVYSPSRLKYDLDISDSKLG
W ++ +G+VGIN + P AL V G + + HPSDLR K ++ +D+ L
MRF 545 WQFAQPVTTVBNQGINTDPOEALVNHGIVKYGNSLHMPDSLRAREKQVEQYDTTQL
MrfA 785 DHVRMNLHLYDKHPQTRMGCGRPDQDIQGTVDIRPIAVRTPGTQKNN---VNGQ
++RhF++ YY P++ G + + GVTQ++ +LP AV+ +G+ NG+
MRF 605 KRISNLMLHLYVRKPFPAASACIEATAT---EPTVFAQVEEIRNVAVKTVGGMVANGKT
MrfA 844 IENLVLKNEALVKMTGATQELSKQHMKKLKITYE
IEN LV+ E + ME +G +E L K D ++ ++ E
MRF 663 IENLVLKvelqeALKENAGKQELCKKLTQHLETREDELE

c) MrfA and Human MRF transmembrane segments (predicted)

MrfA 895 TILLIPHTFTPLLALVHM 913
MRF 759 TITALVVVMAPIESVQGMSTL 777
Figure 7. Alignments of the MRF domain with prokaryotic proteins.

(A) a) NCBI BLAST alignment at the default settings of yeast Ndt80 (ScNdt80) and Dictyostelium discoideum Mrfa (DoMrfA). In DoMrfA sequences, 376 and 460 two arginine residues that are essential for DNA binding of Ndt80. b) NCBI BLAST alignment at the default settings of Human Myelin-gene Regulatory Factor (HsMRF) and DoMrfA. c) Manual alignment of the strongly predicted transmembrane domains of DoMrfA and HsMRF. (B) The alignment at the top is of the MRF-specific domain with Mrfa. The blocks of similarity shown in red are conserved across all MRF family members. An NCBI BLAST search was performed at default settings using the Mrfa region, with eukaryotes excluded. The Kordia and Bdellovibrio alignments are two of the top hits. The latter is annotated as a cell wall surface anchor component and several of the other hits are similarly described. Many of the lower hits are bacteriophage tail fibre components. Again, the red highlighting indicates the positions of the conserved blocks observed in the MRF alignment.
Residue composition for MrfA protein:

- %A: 2.4
- %C: 0.8
- %D: 3.1
- %E: 2.7
- %F: 2.3
- %G: 5.6
- %H: 2.9
- %I: 6.1
- %K: 3.6
- %L: 7.1
- %M: 2.3
- %N: 21.7
- %P: 5.4
- %Q: 8.7
- %R: 2.5
- %S: 9.8
- %T: 6.0
- %V: 3.3
- %W: 0.5
- %Y: 3.3

Figure. 8. MrfA domain structure.

(A, B) dictybase Gene ID: DDB_G0284183, Gene: rcdK on chromosome: 4 position 1846274 to 1849299; 3,026 base pairs, Protein length: 932 aa. N (asparagine) and Q (Glutamine) rich region is observed. MrfA was searched for conserved domains at NCBI (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi) and an NDT80/PhoG domain (magenta box, pfam05224; 369-454) was identified. magenta letters show DNA binding domain amino acid. The trans-membrane domain (blue box, letters) was predicted using TMHMM (http://www.cbs.dtu.dk/services/TMHMM-2.0/) and Uniprot (a.a. 895-915); penetrating at least one phospholipid bilayer of a membrane. May also refer to the state of being buried in the bilayer with no exposure outside the bilayer. When used to describe a protein, indicates that all or part of the peptide sequence is embedded in the membrane. Cyan box, letters show peptidase domain (MRF domain) which is spanned protein from a.a.766 to 829.

35
Figure 9. Transmembrane domain prediction of MRF-like proteins.

Transmembrane domain prediction was performed for the indicated proteins using the implementation of the "Argos" prediction method (Persson and Argos, 1994) on the "MacVector" sequence analysis package version 9.0 (MacVector, Inc). The asterisked peaks are strongly predicted transmembrane domains. Also indicated are the approximate positions of the two regions of sequence similarity, the Ndt80 (green) and the Peptidase domain (MRF domains, red), derived from an NCBI BLAST search performed at default settings using the MrfA protein as the query sequence.
Figure. 10. Sequence alignment of the DNA-binding region of Ndt80.

Comparison of DNA binding domain alignment was performed for the indicated proteins using the UniProt align program (http://www.uniprot.org). Asterisks (magenta) show three arginine residues that are essential for DNA binding of Ndt80. Green colour shows positive charge amino acid. Identity: 14%

Figure. 11. Comparison of peptidase domain (Peptidase_S74) alignment of Dictyostelium MRF orthologues.

Comparison of peptidase domain alignment was performed for the indicated proteins using the UniProt align program (http://www.uniprot.org). Proteolytic cleavage site located between serine (S) and aspartic acid (D). Asterisks (magenta) show serine (S) and lysine (K) amino acids that are essential for peptidase domain, releasing itself, via the serine-lysine dyad at the N-terminus, from the remainder of the end-tail-spike. Identity: 16%
Figure 12. Comparison of transmembrane domain (TMD) alignment of *Dictyostellium* MRF orthologues.

Comparison of TMD alignment was performed for the indicated proteins using the UniProt align program (http://www.uniprot.org/). Blue colour shows hydrophobic amino acid. Identity: 0 %
Section 2.2
Characterisation of transcription factor (MrfA) required for pst-cell differentiation

Introduction

Myelination in vertebrates has evolved to insulate axons and promote rapid propagation of action potentials (Figure. 13). Myelin, the multilayered membrane surrounding nerve axons, is produced by oligodendrocytes (OLs) in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS). Abnormal myelination during development can cause a developmental delay, functional incapacitation, and/or premature death.

Developmentally, committed OL progenitor cells (OPCs) arise from stereotyped germinal regions of the CNS, proliferate, and migrate throughout the CNS before differentiating into postmitotic premyelinating OLs (Figure. 14). These premyelinating OLs subsequently either upregulate myelin genes and ensheath adjacent axons or undergo apoptosis. MRF expression is restricted to postmitotic OLs and is required for expression of the vast majority of CNS myelin genes, interestingly but not for OL specification or differentiation per se (Ben et al., 2009). Interestingly, OL differentiation has been regarded as a default program, because OPCs cultured in defined medium without mitogens can exit the cell cycle, change shape and express myelin proteins in the absence of axons (Jang et al., 2006).

Several transcription factors are required for the specification of OPCs or their subsequent differentiation into postmitotic OLs, such as Sox10 and the bHLH proteins Olig1/2 (Fu et al., 2002; Stolt et al., 2002). However, the transcriptional control of CNS myelin gene expression is poorly understood. Recent study shows myelin gene regulatory factor (MRF) as a critical transcriptional regulator essential for oligodendrocyte maturation and CNS myelination (Ben et al., 2009).
Figure. 13. Schematic diagram; structure of a typical neuron
Myelination has evolved to insulate axons and promote rapid propagation of action potentials. Myelin, the multilayered membrane surrounding nerve axons, is produced by oligodendrocytes in the central nervous system (CNS) and Schwann cells in the peripheral nervous system (PNS).

Figure. 14. MRF is a critical transcriptional regulator essential for oligodendrocyte (OLs) maturation and myelination
OLs and neuron development is tightly regulated and the myelin sheath is constructed only when OLs reach maturity and neurons have grown appropriately. OLs are generated from migratory oligodendrocyte precursors (OPCs) that start life as pluripotent neuroepithelial precursors in the ventricular zone of the embryonic neural tube.
The *mrfA* gene is developmentally regulated

In order to check mRNA level during development, I access gene expression data from over 1,000 *D. discoideum* microarrays and *D. discoideum* RNA-Seq experiments (dictyExpress; http://dictyexpress.biolab.si). RNA-sequence, with RNA extracted during growth and at various times during development, was used to assay *mrfA* expression (Figure. 15). There is a low but finite level of expression during growth. During development there are high expression especially during aggregation.

![Graph showing developmental time course of mrfA expression.](dictyExpress (RNA-seq), Meima and Schaap 1999)

**Figure. 15. Developmental time course of mrfA expression.**

RNA-sequence analysis of a developmental time course. The morphological stages reached at the selected times indicated were: 4-8 hours streaming, 8-12 hours loose aggregates, 12-16 hours tight aggregates, 16 hours standing slugs, 16-23 hours slugs, 24 hours early-mid culminants and 28 hours mature culminants.
**mrfA is expressed in pst-cell**

As MrfA is developmentally regulated, we also tested where it was expressed in multicellular. We used upstream mrfA promoter sequences to drive lacZ expression to test whether mrfA exhibits cell type-specific developmental regulation. mrfA expression was highly enriched in the pst-cell regions at the slug stage of WT development (Figure 16). Since mrfA expression levels are much stronger compared with alternative one, I constructed unstable version. However, expression levels are still much high during development, any cells that did stain were found in the largely pst-cell (Figure 16). In addition, although we also confirmed mrfA expression in terms of spatially by in situ hybridisation, same results we got (data not shown).

![Slug](image)

**Figure 16. mrfA is expressed in pst-cell.**

Localisation of mrfA expression in the pst-cell region. Developing structures were fixed and stained for β-galactosidase expression (blue) driven by the mrfA promoter at the migrating slug stage.
Developing *mrfA*-cells exhibit delayed aggregation and increased tip-number

In order to determine the role of MrfA during development, we generated *mrfA*-mutants by homologous recombination. The mutant displays an approximate 3-5 hour delay in development that first becomes apparent at the streaming stage (Figure. 17). By the loose aggregate stage (at 12-15 hr rather than the normal 8-9 hr) the individual aggregates begin to sub-divide and by the tipped aggregate stage (at 16-18 hr rather than 12-13 hr) they form multi-tipped structures. The process of fragmentation continues during slug migration in the null mutant but it now occurs along the longitudinal axis, because the mutant slugs frequently split along their length (Figure. 17).

When grown as plaques, the *mrfA*-mutants exhibit another manifestation of the aggregation delay phenotype, whose developmental structures formation are approximate 2 days delay compared with WT (Figure. 18).

![Figure 17. Development of the *mrfA*-strain.](image)

Cells were developed on KK2 agar at a density of \(1.0 \times 10^6\) cell/cm\(^2\) for the times shown. *mrfA*-structures show a developmental delay and the mounds break up, resulting in small fruiting bodies. Scale bar: upper and middle panels, 1 mm; lower panels, 0.4 mm.
Figure 18. Developmental phenotype of WT and mrfA- mutant on bacterial lawn plate. mrfA- mutant exhibits normal proliferation, but its developmental structures formation are approximate 2 days delay compared with WT. Cells were spotted onto Klebsiella lawns and observed after 4 and 6 days growth in a humid chamber. Scale bar represents 200 um.
The expression of pstA markers is highly defective in the mrfA-strain

We first analysed expression of the ecmA376:lacZ construct in WT and mrfA-slugs (Figure 19). WT slugs expressing ecmA376: lacZ are strongly stained in the pstA region but here there is a low level of residual staining in the null mutant, predominantly restricted to the extreme anterior. WT slugs expressing multimerised 39-mer construct, 4x39-mer: lacZ, 4x20mer: lacZ (CA-rich sequences at former part of 39-mer) and 4x19mer: lacZ (CA-rich sequences at latter part of 39-mer) shows the expected strong staining, predominantly confined to the pstA region. In contrast, in the null mutant there are only a very few staining cells, scattered throughout the slug. (Figure. 19 and 20).

We speculated that the residual expression of ecmA376: lacZ in the anterior of mrfA-slugs might be due to partial redundancy with DimB, because: i) DimB is bound by the 39-mer in affinity chromatography (Figure. 5A, C) ii) the 39-mer contains several sequence tracts which resemble the sequence of R2, the highest affinity DimB binding site in the ecmO region (Fukuzawa et al., 2006) iii) an oligonucleotide containing CA3 sequences is specifically retarded in band shift analysis using recombinant DimB (unpublished results). We therefore constructed a double null strain, in which both the mrfA and dimB genes are disrupted. However, the ecmA376: lacZ construct is expressed in the double null strain at the same level as in the mrfA-strain (Figure. 19). Hence DimB is not the partially redundant partner.
Figure 19. Analysis of the ecmA promoter sub-region that directs expression in pstA-cell.

*Dictyostelium* slugs expressing ecmA376: lacZ and 4x39-mer: lacZ, a lacZ construct containing actin 15 basal promoter elements coupled to a 4-fold multimerised tandem array of the 39-mer, were stained with X-gal to detect β-galactosidase expression (blue). WT (Ax2) mrfA-null mutants and dimB-null mutants are shown. Reduction of pstA cells is seen in the mrfA-slugs. Scale bar represents 200 µm.
Figure. 20. Analysis of the artificial construct that directs expression in pstA cells.

Schematic representation and expression analysis of 4x20-mer: lacZ and 4x19-mer, a lacZ construct containing actin 15 basal promoter elements coupled to a 4-fold multimerised tandem array of the 20-mer and 19-mer. mrfA- mutant cells exhibit gene expression defects. Expression pattern of representative multimerised prestalk markers at the slug stage of development. These multimerised prestalk markers show pstA-cell region in WT cells, although expression is disappeared at the pstA-cell region in mrfA- mutant. Scale bar represents 200 μm.
The fruiting bodies of the *mrfA-* mutant exhibit *ecmB: lacZ* and *ST: lacZ* marker defects

When *ecmAO:lacZ*, *ecmO: lacZ*, *pspA: lacZ*, *ecmB: lacZ*, ST (stalk):*lacZ* and 4x28mer expression was examined in developing WT and mutant slug, no clear differences were seen (Figure. 21 and 22). Stalk tube-specific expression is regulated by sequence elements within part of the *ecmB* promoter, the stalk tube (ST) promoter region (Ceccarelli et al., 2000). Furthermore, Ceccarelli et al, also identified a GA-containing sequence element 28 nucleotide region (the 28-mer) that is required for efficient gene transcription (Ceccarelli et al., 2000). When it comes to culminating expressing *ecmB: lacZ* and *ST: lacZ*, clear differences were seen. We confirmed these reporters, which stains both the basal disc and stalk in WT culminant but much reduced in the *mrfA-* mutant (Figure. 22). In many cases the lower cup staining in *mrfA-* culminant is normal, however a poorly defined group shows much reduced lower cup staining (Figure. 22 *mrfA-:ecmB* culminant).

![Figure 21](image)

**Figure. 21.** *mrfA-* mutant exhibit normal gene expression.

Expression pattern of representative *ecmAO: lacZ*, *ecmO: lacZ* prestalk markers and *pspA: lacZ* prespore marker at the slug stage of development. There are no differences between WT and *mrfA-* mutant.
**Figure. 22.** *mrfA*-mutant cells exhibit gene expression defects.

Expression pattern of representative *ecmB*:lacZ, *ST*:lacZ and 4x28mer:lacZ prestalk marker at the slug and culminant stage of development. Note that there is some basal disc staining, but much less than the WT strain.
MrfA is required for prestalk cell patterning in chimeric development

To determine whether the developmental defects of *mrfA*- are rescued by adding WT cells, we performed chimeric development in the presence of WT cells. Relatively, development is improved with an increasing ratio of WT to *mrfA*- cells (data not shown). And also, slug split or drag phenotype of *mrfA* is rescued by adding WT cells (data not shown). To determine if the developmental defects observed in *mrfA*- cells are cell autonomous or non-cell autonomous, we demonstrated chimeric development in the presence of WT cells. A minority (10%) of WT or *mrfA*- cells were labeled by CellTracker and mixed with unlabeled cells. *mrfA*- cells preferentially localise to the rear of the prespore zone when mixed with WT cells (Figure 23 upper right). By contrast, WT cells localised throughout structure when mixed with *mrfA*- mutant cells (Figure. 23 lower left), suggesting that MrfA is required for prestalk cell patterning.

![WT (10%) labeled vs mrfA- (10%) labeled](image)

**Figure. 23. mrfA- mutant exhibits cell-autonomous defects.**
A minority (10%) of WT or *mrfA*- mutant cells were labeled by CellTracker and mixed with unlabeled cells. Upper right: *mrfA*- mutant cells preferentially localise to the rear of the prespore zone when mixed with WT cells. Lower left: WT cells localised throughout structure when mixed with *mrfA*- mutant cells.
The predicted MrfA DNA binding domain produced in *E. coli* interacts with the CA-rich sequence elements in the *ecmA* 39-mer.

Web LOGO shows yeast middle sporulation element consensus, which is bound to yeast Ndt80, suggesting that CA-rich sequences are highly-consensus between MrfA binding motif and middle sporulation element (Figure 24 lower).

**39-mer**

```
TAA	CA1
ACACTTT	CA2
TTCAAAAT
```

**20-mer**

```
AGCATACCACACCAC
```

**19-mer**

```
AGCATACCACACC
```

**Figure. 24. De novo motif analysis of MrfA-bound promoters.**

Upper panel shows MrfA binding site at *ecmA* promoter fragment 39-mer. Lower panel shows *Saccharomyces cerevisiae* middle sporulation element (MSE) consensus, which is bound to Ndt80, is shown on the bottom of the logo. CA-rich sequences are highly-conserved between MrfA binding motif and MSE.
The *E. coli*-derived protein MrfA binds to the 39-mer

In order to determine whether MrfA bind to 39-mer *in vitro*, we expressed Ndt80 in *E. coli* BL21 Codonplus RIL under the regulation of the phage T7 promoter. A segment of MrfA, containing amino acids 294-546 and spanning the predicted DNA binding domain (Ndt80), was oligo-histidine tagged and expressed in *E. coli*. Following induction of the cultures with IPTG, no protein of the size expected for Ndt80 was produced in *E. coli* carrying the mock vector (Figure. 25A). By contrast *E. coli* carrying the Ndt80 plasmid produced protein migrating through SDS polyacrylamide gels with mobility consistent with a 28 kDa protein (Figure. 25A).

The protein isolated by the chromatography purification procedure (see material & method) was used in an electrophoretic gel mobility shift assay (EMSA) to test the ability of the recombinant MrfA to bind a 39-mer *cis*-element from the *ecmA* promoter fragment (Figure. 25B).

In the absence of specific competitor there is a retarded band (Figure. 25B). The core consensus binding site for the interaction of yeast Ndt80 with sporulation-specific promoters is CRCAAA (Jolly et al., 2005).

The 39-mer contains three discrete CA-rich cluster regions, CA1, CA2 and CA3 (Figure. 25B), that possess similarity with this sequence. As previously mentioned that each of the three separate block mutations that were analysed by reporter expression, only CAmt1 (left side of CA cluster in 39-mer) and CAmt3 (right side of CA cluster in 39-mer), showing the lowest relative level of expression (Figure. 4B).

These regions (CA1-CA3) were mutated, separately and in combination, and the 39 nt sequences containing them were used as competitors in band shift assays (Figure. 25B). The separate mutations produce only relatively small effects on the efficiency as a competitor. However, at concentrations where the unmutated 39-mer acts as a potent competitor, simultaneous mutation of all three regions eliminates competition (Figure. 25B).
A

Vector construction

Expressed in *E. coli*

Protein purification

EMSA

B

Competitor 39-mer (ng):

<table>
<thead>
<tr>
<th>Competitor 39-mer (ng)</th>
<th>WT</th>
<th>CA1m</th>
<th>CA2m</th>
<th>CA3m</th>
<th>CA123m</th>
</tr>
</thead>
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<tr>
<td>CA1</td>
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<td>10</td>
<td>50</td>
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<td>50</td>
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<tr>
<td>CA3</td>
<td>10</td>
<td>50</td>
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</tbody>
</table>

39-mer:
- gatccTAACACACTTTTACCCAAAATTATACACCCACCCATAATc
- gatccTAACACAaATTTCCCAAAATTATACACCCACCCATAATc
- gatccTAACACCTTTaAaAAATTATACACCCACCCATAATc
- gatccTAACACCTTTTAAaAAAATTATACACCCACCCATAATc
- gatccTAACACAaTTTaaAAAAATTATACACCCACCCATAATc
Figure. 25. *In vitro* binding of recombinant MrfA to the ecmA 39-mer

(A) It was expressed in *E. coli* strain BL21 Codonplus RIL (Stratagene), as a His-fusion protein, and purified using TALON metal affinity resin (Clontech). The histidine tagged MrfA DNA binding domain was effectively isolated from the soluble fraction by chromatography. Coomassie stained SDS-polyacrylamide gel showing cell extracts from *E. coli* expressing a MrfA protein. Molecular weight (MW) markers are shown at the left, molecular weights are indicated in kDa. (B) Bacterially expressed MrfA protein region 294-546, a region that spans the predicted DNA-binding domain Ndt80, was used in band shift assay. Protein amounts were 0.5 μg per reaction. Parental (WT) and mutated 39-mer sequences are shown below. The C-rich sequences (CA1, CA2 and CA3) are underlined and C to A substitutions introduced into the mutant competitors are shown in small case.
MrfA is required only to bind to 39-mer

As previously mentioned that we speculated that the residual expression of ecmA::lacZ in the anterior of mrfA-376 slugs might be due to partial redundancy with DimB, because: i) DimB is bound by the 39-mer in affinity chromatography (Figure 5A, C) ii) the 39-mer contains several sequence tracts which resemble the sequence of R2, the highest affinity DimB binding site in the ecmO region (Fukuzawa et al., 2006). This suggests independent binding to the three regions and this is consistent with the smearing to higher mobility of the retarded products observed with the individually mutated competitors.

In order to determine whether MrfA binds to individually CA-rich sequences in 39-mer, we performed band shift assays using ecmO R2 (22mer) (Fukuzawa et al., 2006), CA1+CA2 (20-mer) and CA3 (19-mer) as a probe. Surprisingly, MrfA fail to bind to 22mer, 20-mer and 19-mer (Figure 26), suggesting that MrfA recognises only long version of CA-rich sequences, 39-mer.

![Figure 26. In vitro binding of recombinant MrfA to the ecmA 39-mer](image)

Bacterially expressed MrfA protein region 294-546, a region that spans the predicted DNA-binding domain Ndt80, was used in band shift assay. Protein amounts were 0.5, 1 and 1.5 µg per reaction. ecmO 22mer, 20mer, 19mer and 39mer were used for band shift assay as a probe. Protein amounts are expressed as the number of µl used in each reaction and the fusion protein amounts were normalized to approximately 0.5 µg by parallel gel electrophoretic analyses.
Mutation of two conserved residues important for specific DNA binding by yeast Ndt80 ablates binding of MrfA to the ecmA 39-mer

The functional homology of Ndt80 and MrfA was validated, at the protein level, utilizing known structural and DNA binding information for the yeast protein. This was done by inserting point mutations in MrfA, to replace two residues known to be critically important in the interaction of Ndt80 with the MSE. Two arginine residues, R111 and R177 DdMrfA; R376 and R460, respectively interact with the first and second C-G base pairs in the core consensus MSE (Figure. 7A). Mutation of either of these two base pairs leads to a 20-fold decrease in Ndt80-binding affinity and a greater than 100-fold decrease in biological activity (Fingerman et al., 2004). We mutated each equivalent residue in MrfA to alanine, to create R376A and R460A and we also constructed the double mutant R376A+R460A. The protein isolated by the chromatography purification procedure (see material & method) was used in EMSA to test the ability of the recombinant MrfA (WT, R376A, R460A and double mutant) to bind a 39-mer cis-element from the ecmA promoter fragment (Figure. 27A).

The proteins were expressed in E. coli and used in assay with the 39-mer as probe. Increasing amounts of protein, normalized by concentration estimates from aliquots resolved on an SDS gel, were used in the band shift (Figure. 27B). The unmutated protein retarded the probe at all concentrations tested while the two single mutant proteins and the double mutant were inactive (Figure. 27B).
A

Substitution R to A

Expressed in *E. coli*

Protein purification

EMSA

B

Probe: 39-mer

<table>
<thead>
<tr>
<th>Protein (ul)</th>
<th>WT</th>
<th>R376A</th>
<th>R460A</th>
<th>R376A+R460A</th>
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</tr>
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<tr>
<td>2</td>
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</tbody>
</table>

MW kDa 1 2 3 4

1. WT
2. R376A
3. R460A
4. Double mutant
Figure 27. Functional analysis of regions of MrfA essential for binding the ecmA 39-mer

(A) It was expressed in E. coli strain BL21 Codonplus RIL (Stratagene), as a His-fusion protein, and purified using TALON metal affinity resin (Clontech). The histidine tagged MrfA DNA binding domain was effectively isolated from the soluble fraction by chromatography. Coomassie stained SDS-polyacrylamide gel showing cell extracts from E. coli expressing a MrfA protein. Molecular weight (MW) markers are shown at the left, molecular weights are indicated in kDa. (B) Mutant proteins that contain R to A substitutions in two residues essential for DNA binding of Ndt80 (Figure, 6) were prepared as his fusions in E. coli and assayed for binding to the 39-mer. Protein amounts are expressed as the number of μl used in each reaction and the fusion protein amounts were normalized to approximately 0.5 μg by parallel gel electrophoretic analyses.
Nuclear extracts from a *mrfA* null strain contain an activity that binds the 39-mer

When the 39-mer is used as a probe in EMSA with nuclear extracts prepared from WT (Ax2) and *mrfA*- mutant slug cells, one major retarded complex is observed. Unlabeled 39-mer, used as a competitor, inhibits formation of the complex. Variants of the 39-mer, containing the point mutations analysed in the biological experiments described above (Figure. 4 and Figure. 25B), are effective as competitors for the complex (Figure. 28). Hence, it might be existence another its binding partner.

**Figure. 28. In vitro binding of nuclear extract to the 39-mer.**

EMSA using the 39-mer as probe. A partially purified nuclear extract from WT (lane 1-6) and *mrfA*-(lane 7-12) slug stage cells was bound to Cy-5 labeled, 39-mer oligonucleotide (Figure. 18B) with: lane 1-3, no competitor (-) and 39-mer WT 10, 50 pmol competitors; lane 4-6, no competitor (-) and 39-mer CA123m 10, 50 pmol competitors; lane 7-9, no competitor (-) and 39-mer WT 10, 50 pmol competitors; lane 10-12, no competitor (-) and 39-mer CA123m 10, 50 pmol competitors. CA123m mutant forms of the 39-mer (Figure. 18 B as indicated. The major complex is efficiently competed by the 39-mer WT and CA123m oligonucleotide.
Localisation and potential proteolytic processing of a myc tagged form of MrfA

In order to characterise MrfA further, a myc tag was added to either its N or C terminus under control of the actin15 promoter. When transformed into the mrfA-strain the resultant myc-MrfA construct "rescued" normal development (data not shown). Two transformant clones in a WT background, 1 and 2, were analysed at the slug stage by Western transfer using the 9E10 anti-myc monoclonal antibody. Interestingly, only N-terminal fusion revealed a single species with an apparent molecular weight of 85 kDa (Figure. 29).

![Figure. 29. MrfA C-terminus might be cleaved by secretase](image)

Full length MrfA, with a myc tag at the N and C-terminus, was expressed under the semi-constitutive actin15 promoter (Myc-MrfA and MrfA-Myc). Cells were developed to the slug stage, lysed and subjected to Western transfer using the 9E10 myc monoclonal antibody.
Many of cells expressing myc-MrfA show both staining puncta and nuclear

When vegetative cells harboring myc tag at N-terminus are stained with 9E10 the all of cells do not stain (data not shown). When cells are dissociated at the slug stage and stained with 9E10 the majority of cells do not stain (Figure 30). Many of those that do stain display diffuse, or sometimes particulate, staining throughout the cytoplasm. The other staining cells show strong nuclear fluorescence (Figure 30).

Figure 30. MrfA localisation in dissociated cells

Full length MrfA, with a myc tag at the N-terminus, was expressed under the semi-constitutive actin15 promoter (myc-MrfA). Dissociated slug cells were fixed and stained with the 9E10 myc monoclonal antibody. The phase contrast image of the selected field is shown with the fluorescence image. The arrow points out a cell with stained granular structures.
Cells with nuclear enriched myc-MrfA are mainly confined to the pst-cell of the slug

When whole-mount slugs are stained the pattern is variable between individual slugs on a plate. In many cases the strongest nuclear-localised staining is in the rearguard region (Figure. 31 upper): a poorly defined group of prestalk cells. However, in such slugs, and in slugs where the rearguard is not stained (Figure 31 lower), there is also nuclear staining in cells in the anterior of the prestalk zone; the size of the zone of anterior staining is variable between slugs but falls within the range seen for ecmA::lacZ expressing cells.

Figure. 31. Whole mount staining of slugs expressing myc-MrfA
Cells expressing Myc-MrfA were developed to the slug stage, fixed as whole mounts and stained with 9E10. Two slugs are shown to represent the variable staining patterns that are observed.
MrfA localisation is developmentally regulated and required for normal morphogenesis

To better understand MrfA behavior from vegetative cells to multicellular state, we performed immunohistochemical staining using monoclonal c-myc antibody (Figure. 32). We found that levels of nuclear accumulation of myc-MrfA are lowest in vegetative cells (Figure. 32A), but gradually increase during the first starved 6 hrs of development (Figure. 32B). Levels are highest around the mound stage then decrease dramatically (Figure. 32C lower and D, E). During development there are two peaks of expression, one during mound (Figure. 32 C lower) the second during mid culmination (data not shown). These findings demonstrate that MrfA nuclear accumulation is regulated during multicellular development.

A. Vegetative cells

<table>
<thead>
<tr>
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<th>myc-MrfA</th>
<th>Merge</th>
<th>Phase</th>
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<td><img src="image7.png" alt="Image" /></td>
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</table>
B. Starved cells 6 hrs

C. Streaming aggregate, loose mound and mound
Figure 32. Confocal image; developmental time-course expressing myc-MrfA.

(A) Vegetative cells; Most of cells show cytoplasmic localisation of myc-MrfA.

(B) Starved cells at 6 hrs; A few cells show nuclear accumulation of myc-MrfA.

(C) Streaming aggregate (upper); 30% of cells show nuclear accumulation of myc-MrfA.

Loose mound (middle); Nearly 50% of cells show nuclear accumulation of myc-MrfA.

Mound (lower); >80% of cells show nuclear accumulation of myc-MrfA.

(D) Tipped mound; A few cells at apical region of the tipped mound show nuclear accumulation of myc-MrfA. Most of cells at bottom of the tipped mound show nuclear accumulation.

Numbers and circles represent magnified region of interest.

(E) Slug; There is staining in the pstA and pstO-cell region.
Expression of MrfA driven by actin15 promoter completely rescues the developmental phenotype of the mrfa- mutant

In order to 1) rescue mrfa- phenotype, 2) follow localisation of C-terminal fragment, 3) determine cleavage site, we made overexpression construct under the control of actin15 semi-constitutive promoter, which have N-terminus c-myc and C-terminus FLAG-tagged (MrfA FL) (Figure. 33A). Furthermore, in order to understand transmembrane domain (TMD) function, we made TMD deletion construct (MrfA ΔTMD) (Figure. 33A). When transformed into the mrfa- strain the resultant MrfA FL construct "rescued" normal development (Figure. 34). However, MrfA ΔTMD did partially complementation (Figure. 34). The transformants pool, 1 and 2 (MrfA FL) and pool 1 (MrfA ΔTMD), were analysed at the slug stage by Western transfer using the c-myc monoclonal antibody (CRUK; 9E10) and the FLAG monoclonal antibody (SIGMA; M2). Using c-myc antibody, both cell-lines showed single species with an apparent molecular weight of 85 kDa (Figure. 33B left). On the other hand using FLAG antibody, MrfA FL showed two species; one is a molecular weight of 19 kDa and the other is a >100 kDa (Figure. 33B right). In the case of MrfA ΔTMD, it also showed two species; one is a molecular weight of 14 kDa and the other is a nearly 100 kDa (Figure. 33B right). These results indicate that both fusion proteins were functional because MrfA FL in the mrfa- mutant or MrfA ΔTMD expression in the mrfa- mutant rescued mrfa- phenotype (MrfA ΔTMD partially rescued) (Figure. 34).
Figure 33. MrfA is cleaved at near C-terminus.

(A) Schematic of the MrfA composition and strategy of the tagged-fusion vector. The predicted MrfA FL protein (upper) contains 932 amino acids. The predicted ΔTMD (lower) protein contains 927 amino acids. From TMD site to C-terminus is nearly 5 kDa. Each c-myc and FLAG tag is nearly 1 kDa, respectively. Dictyostelium MrfA contains an Ndt80 DNA binding domain (magenta) and a C-terminal peptidase domain (cyan) and transmembrane (blue). MrfA FL and MrfA ΔTMD construct contain an N-terminal c-myc tag and a C-terminal FLAG tag. These constructs were electroporated into mrfA- mutant cells and the resultant transformants selected at 20 mg/ml G418. (B) Cells were developed to the slug stage, lysed and subjected to Western transfer using the c-myc monoclonal antibody and FLAG monoclonal antibody. Left side numbers represent molecular weight.
Figure 34. MrfA FL rescues mrfA- phenotype.

*mrfA-* mutants exhibit delayed culmination and abnormal mound and slug morphology. MrfA has been shown to function in the pst-cell of the slug, as expression of full-length MrfA FL under the control of an actin15 promoter is able to completely rescue the *mrfA-* mutant developmental delay and morphology phenotype. However, MrfA ΔTMD did partially complementation. These cells were developed on KK2 agar plates until appropriate stage.
Peptidase domain is important role of autoproteolytic cleavage reaction

As previously observed by Western transfer (Figure. 29), MrfA is cleaved near C-terminus, we check sequences of MrfA using bioinformatics. Interestingly, there is a conserved peptidase domain near the C-terminus, which is the very C-terminal, chaperone, domain of the bacteriophage protein endosialidase (Stummeyer et al., 2005). It releases itself, via the serine (S) lysine (K) dyad at the N-terminus, from the remainder of the end-tail-spike (Figure. 35A). Threonine 910 introduces a kink into the backbone, in consequence serine 911 comes into close proximity of lysine 916, which activates serine 911. Serine 911 attacks the peptide bond either directly or mediated by a water molecule (Knitting and snipping: chaperones in b-helix folding). The endosialidase protein forms homotrimeric molecules in bacteriophages. The catalytic dyad allows this portion of the molecule to be cleaved from the more N-terminal region such that the latter can fold and presumably bind to DNA.

To further characterise the MrfA, firstly I performed immunoprecipitation experiments from vegetative cell extracts using FLAG antibodies. As shown in Figure 33B right, C-terminal cleaved fragment was very efficiently isolated. Now I analyse mass spectrometry data (Figure. 36A, B). Secondly, I performed mutation analysis of peptidase domain in MrfA. Schulz et al., showed that cleavage is inhibited by the alanine substitution of serine 911 and lysine 916 in endoNF, respectively (Schulz et al., 2010). Therefore, it is possible to identify residues apparently involved in the autocatalytic cleavage reaction in Dictyostelium MrfA. To confirm this idea, the location of cleavage site conserved residues preserved in bacteriophages endoNF, the critical residues serine 767 and lysine 772 in peptidase domain of MrfA were substituted by alanine individually. Schulz et al., reported that the self-cleavage reaction depends on the catalytic residue serine 911 in bacteriophage. The WT and mutants form were expressed in Dictyostelium cells and performed western transfer with cell lysate from cells expressing MrfA FL and each mutant form (Figure. 37A). The mutant proteins behave like WT form in terms of expression level and maturation by mutation of proteolytic cleavage site (Figure. 37B), indicating that neither the folding nor the overall stability of MrfA is affected by the mutations. As expected, substitution of serine 767 to alanine inhibits cleavage activity (Figure. 37B). In addition, Schulz et al., reported that activation of serine 911 depends on the presence of lysine 916, as indicated by substitutions for alanine (K916A) (Schulz et al., 2010). In Dictyostelium MrfA, mutation of lysine 772 to alanine (K772A) shows that this residue is crucial for the cleavage reaction, as substitution of the residue
leads to uncleaved (Figure. 37B). These results strongly suggested that this auto proteolysis mechanism is also conserved in lower eukaryote Dictyostelium discoideum.
Figure. 35. Comparison of the peptidase domain.

(A) Multiple sequence alignment of conserved peptidase domain (peptidase S74) found in a database search. Proteolytic cleavage site located between serine (S) and aspartic acid (D). Asterisks (magenta) show serine (S) and lysine (K) amino acids that are essential for peptidase domain, releasing itself. An NCBI BLAST search was performed at default settings using the peptidase domain region. Alignment was performed by UniProt align program (http://www.uniprot.org/). GI number; Dictyostelium discoideum MrfA (DDB_G0284183), Enterobacteria phage K1F endoN (YP_338127), Enterobacteria phage K1E endoN (YP_425027), Escherichia coli eliminase (CAA65353), Bacillus phage GA-1 neck appendage protein precursor (NP_073695), Homo sapiens MRF isoform2 (NP_001120864), Caenorhabditis elegans PQN47 (CAA88602) (B) a) MrfA ΔTMD sequence is 913 residues, the protein weighs 102.03 kDa (including FLAG). b) Cleaved results for 134 residues, the protein weighs 15.69 kDa (including FLAG). SD (magenta) represents predicted cleavage site between S and D. EOKLl8EEDLNK (green) represents c-myc tag sequences. DYKDDDDK (cyan) represents FLAG tag sequences. Asterisk shows TAA stop codon.
Figure 36. Immunoprecipitation of soluble extracts from \textit{mrfA-} cells expressing MrfA \textit{ΔTMD}.

(A) Immunoprecipitations were performed using anti-FLAG antibodies (Wako) on total cell extracts collected vegetative cells. Immunoprecipitation experiments were performed essentially as previously described (Tsuyoshi's protocol). Arrow indicates region of interest containing cleaved fragment (14 kDa), which were excised and the proteins identified by mass spectrometry. (B) Purified proteins by immunoprecipitation from \textit{mrfA-} cells expressing MrfA \textit{ΔTMD} was analysed by western transfer using the FLAG antibody. Lane 1 shows purified proteins without FLAG antibody. Lane 2 shows purified protein with FLAG antibody. Arrow indicates signal of cleaved fragment.
A

- MrfA FL
- MrfA ΔTMD
- MrfA ΔNdt80

B

- c-myc
- FLAG

<table>
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<tr>
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Figure 37. Effects of two point mutations (S767A and K772A) on autoproteolytic cleavage reaction in *Dictyostelium* MrfA protein. (A) Schematic of the MrfA composition and strategy of the deletion mutant of DNA binding domain and point mutation tagged-fusion vector. Serine 767 in cyan box is estimated catalytic residue. Lysine 772 in cyan box is estimated essential residue for activation of catalytic serine. (B) Cell lysate from *mrfA*-cells expressing MrfA FL and ΔTMD with point mutation (S767A and K772A) was analysed by western transfer using the c-myc and FLAG antibody. DNA binding domain deletion form (ΔNd180 DBD); Translation 673 amino acids. Molecular weight =75 kDa. Left panel shows western transfer results with c-myc antibody. Right panel shows western transfer results with FLAG antibody. Red arrow (1) indicates signal of non-cleaved MrfA FL product (104 kDa). Red arrow (2) indicates signal of non-cleaved MrfA ΔTMD product (99 kDa). Red arrow (3) indicates signal of cleaved N-terminal MrfA FL and MrfA ΔTMD products (85 kDa). Blue arrow (4) indicates signal of cleaved C-terminal MrfA FL fragment (19 kDa). Blue arrow (5) indicates signal of cleaved C-terminal MrfA ΔTMD fragment (14 kDa).
Affinity purification of anti-MrfA antibody

In order to determine whether MrfA shows nuclear accumulation in slug stage, we performed immunohistochemical staining using anti-MrfA polyclonal antibody (Figure. 38 B). WT, mrfa- and dimB-/mrfA- cells at the slug stage were dissociated, treated with anti-MrfA antibody. There is lots of non-specific signal by Western transfer analysis, and that similar results were obtained with both WT and mrfa- strains (Figure. 38A), suggesting that Anti-MrfA does not work in this experiment. But, interestingly, dimB-/mrfA- double mutant shows weak nuclear accumulation of MrfA in dissociated cells derived from slug (Figure. 38B).

A

EQKLI SEEDLNKMDGYNQQQQQQQQQQQQQQQQQQQHQMLGGILSHPQQQQQQQQQQQQQQQQQQQQQQQQQQQQQPMGSLNNLLGNSNGFGTMLQQSAPINIQGSNSFSTFTPLSADDSNLKKRK
IKNEDGTYSEIPSPNLSVGSNGGLDSSLMLQQQLODQQQQIAQFNSVNSPSFSLNSSQVNLLLLNNINNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNNN
Figure 38. MrfA localisation in dissociated cells in WT and mrfA- mutant cells.

(A) Schematic representation of MrfA (includes c-myc and FLAG) coding sequences (ΔTM). Red letters show anti-MrfA antibody amino acids (93-105). From initiation codon to antibody is nearly 10 kDa. (B) Western transfer analysis by anti-MrfA antibody. Cells were allowed to develop until slug stage, then lysed with SDS sample buffer. (C) Dissociated mound or slug cells were fixed and stained with the MrfA polyclonal antibody.
DIF-1-dependent nuclear accumulation of DimB and STATc is normal in mrfA- mutant cells

In order to determine whether DimB or STATc in mrfA- exhibits normal nuclear accumulation after treated with DIF-1, we examined the behaviour of these transcription factors. DimB (driven by actin15 promoter, G418 resistance #489 Dundee) or STATc (driven by actin15 promoter, Hygromysin resistance #73 Hirosaki) were constitutively expressed as GFP fusion proteins in cells starved for 6 hours. After DIF-1 treatment, the number of cells with even staining in cytoplasm and nucleus rapidly decreased with a concomitant increase in the number of cells with stronger staining in the nucleus compared with cytoplasm. DimB and STATc in mrfA- rapidly accumulate in the nucleus of DIF-1-treated cells. No change in distribution was observed in untreated control cells (Figure. 39). These results suggest that the behavior of DimB and STATc transcription factor activities with DIF-1 treated in mrfA- are unaffected by DIF-1 and downstream of the signal.

Figure. 39. DIF-1-dependent nuclear accumulation of DimB and STATc.

DimB and STATc rapidly accumulate in the nucleus of DIF-1-treated cells. DimB and STATc were constitutively expressed as GFP fusion proteins in cells starved for 6 hrs. After DIF-1 treatment, the number of cells with even staining in cytoplasm and nucleus rapidly decreased with a concomitant increase in the number of cells with stronger staining in the nucleus compared with cytoplasm. No change in distribution was observed in untreated control cells.
The fruiting bodies of the *mrfA*-null mutant show a reduced number of basal disc

Saito et al. (2008) and Yamada et al. (2010) have identified that the basal disc is almost entirely absent from the PKS null mutant and DIF-1 signaling mutant fruiting bodies and the few that were scored as present were very defective (Saito et al., 2008; Yamada et al., 2010). In order to further characterise the role of MrfA and the correlation of DIF-1 signaling, we counted the number of basal disc formed by *mrfA*-cells. *mrfA*-fruiting bodies are very untidy, and we found only 36% of *mrfA*-fruiting bodies had a clear basal disc, compared with 94% for the WT parent (Figure 40 and Table 1).

The basal disc formation was also recognised in mid-culminants of strains expressing ecmB:/lacZ after lacZ staining. Only 38% of *mrfA*-fruiting bodies show ecmB:/lacZ expression, compared with 100% for the WT parent (Figure 41, Table 2), suggesting that MrfA might be involved in DIF-1 signaling.

![Image of fruiting bodies](image)

**Figure. 40. The fruiting bodies of the *mrfA*-mutant lack a basal disc.**

Whole fruiting body structure of (left) *dimB*-mutant strain; (middle) WT; (right) *mrfA*-mutant. The stalk of the *dimB*- and *mrfA*-mutant lies partially on the substratum. Arrows (cyan; *dimB*-; magenta; WT, black; *mrfA*-) represent basal disc formation.

<table>
<thead>
<tr>
<th>Table 1. Basal disc formation</th>
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<tr>
<td>Wild-type</td>
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<tr>
<td>Basal disc</td>
</tr>
</tbody>
</table>

Table 1

Basal discs were scored microscopically on mature fruiting bodies; scoring was conservative, so that even a small gathering of cells at the base of a stalk was not scored as a basal disc. Results are given with SEM and number of experiments.
Figure. 41. The ecmB staining of lower cup and basal disc are occasionally missing in mrfA- mutant fruiting bodies.

Typical staining pattern of ecmB-lacZ in culminants: (left) WT, (right) mrfA- mutant (note that there is some lower cup and basal disc staining, but much less than the WT). The basal disc and lower cup staining was also recognised in mid-culminants of strains expressing ecmB: lacZ after lacZ staining. Arrows indicate the basal disc staining.

Table 2. ecmB-gal staining

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>mrfA-</th>
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<tbody>
<tr>
<td>Lower cup</td>
<td>100% (n=50)</td>
<td>70% (n=50)</td>
</tr>
<tr>
<td>Basal disc</td>
<td>100% (n=50)</td>
<td>38% (n=50)</td>
</tr>
</tbody>
</table>

Table 2. The ecmB staining of lower cup and basal disc are occasionally missing in mrfA- mutant fruiting bodies.

The lower cup and basal disc staining was also recognised in mid-culminants of strains expressing ecmB: lacZ after lacZ staining. Results are given with SEM and number of experiments.
In *mrfA*- mutant neutral red staining of pstB-cell is moderately reduced

In order to extend the above correlations by genetic analysis, vital dye staining was performed on *mrfA*- mutant. Neutral red staining of parental WT slugs revealed the expected pattern; staining in cells throughout the anterior, prestalk zone and a band of strongly stained cells, located at variable positions along the slug and closely apposed to the ventral surface (Figure. 42). In the *dimB*- slugs anterior staining is retained but the neutral red staining band is either absent or in some cases greatly reduced. In *mrfA*- mutant there is a little neutral red stained cells in ventral surface. There are, however, in some structures unstained, apparently undifferentiated, cells situated at the position normally occupied by the lower cup and at the base of the stalk tube.

![Figure. 42. Parental (WT), mrfA- and dimB- mutant slugs stained with neutral red.](image)

WT, *mrfA*- and *dimB*- cells stained with neutral red were incubated under unidirectional light till 20 hrs of development then migrating slugs were visualised from the side by light microscopy.


**mrfA- mutant cells fail to induce stalk cells and prestalk markers in response to DIF-1**

In order to directly test whether MrfA is required for DIF-1 responses, we examined the behaviour of *mrfA*- mutant cells in the cAMP removal monolayer assay. In this assay, cells are initially induced by cAMP to become competent to respond to DIF-1. After removal of cAMP and addition of DIF-1, WT cells differentiate as stalk cells. The DIF-1 non-responsive mutant, *dimB-* , does not produce stalk cells in response to DIF-1 in this assay (Huang et al., 2005). Similarly, we found that *dimB-* and most of *mrfA*- mutant cells also failed to respond to DIF-1 (Figure. 43). Almost *mrfA*- mutant cells remained as amoebae, demonstrating that MrfA, like DimB, is required for normal responses to DIF-1. In addition, unlike in the case of *dimB-* mutant cells, *mrfA*- mutant cells do not exhibit non-vacuolar cell death (NVCD) (Figure. 43 right).

![Image of WT, dimB-, and mrfA- cells](image_url)

**Figure. 43. Most mrfA- mutant cells fail to respond to DIF-1.**

At this stage, in the DIF-containing chamber, most WT cells differentiated to “stalk” cells (highly vacuolated). Almost no vacuolated cells should be seen in the *mrfA*- mutant cells.