A *mrfA*- null mutant shows defects in DIF responses

Previously, Huang et al. (2005) have demonstrated that DimB is required for both the induction of prestalk markers and repression of prespore markers under these conditions (Huang et al., 2005). We compared the behaviour of *mrfA*- mutant cells using strains transformed with the prestalk marker ecmAO:*lacZ*, ecmO:*lacZ*, ecmB:*lacZ*, ST:*lacZ* and the prespore marker pspA:*lacZ*. Unlike *dimB*- mutant cells, *mrfA*- mutant cells showed normal induction of ecmAO:*lacZ* and ecmO:*lacZ* in the presence of DIF-1. However, we found that *mrfA*- mutant cells showed little or no induction of ecmB:*lacZ* and ST:*lacZ* over a 24-hour period in the presence of DIF-1 when compared with WT cells (Figure. 44). Despite this, we found that repression of the prespore marker pspA:*lacZ* by DIF-1 was unaffected (Figure. 44).

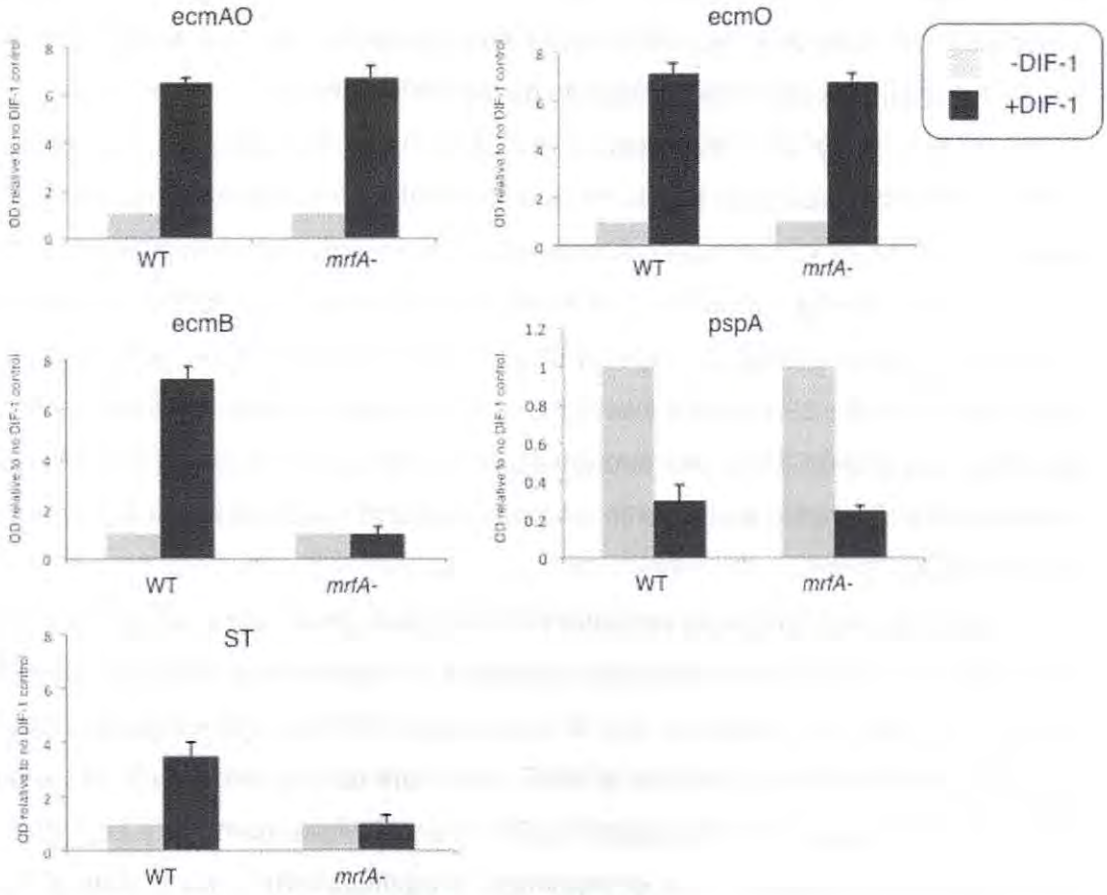


Figure. 44. *mrfA*- cells fail to induce ecmB markers.

In the *mrfA*- mutant, ecmB and ST:*lacZ* was non responsive to DIF-1, whereas a prestalk marker ecmAO:*lacZ* and ecmO:*lacZ* was responsive to DIF-1. And also, a prespore marker pspA:*lacZ* in the *mrfA*- mutant was repressed as in the WT.

Discussion

Section 3. 1

Summary

In most cases, multicellular development begins with a single cell which divides mitotically to produce all the cell-type. These developmental patterning processes require some cells adapt to changes in their environment by activating signal transduction pathways that alter gene expression. Transcriptional activity is greatly stimulated by a second class of factors, termed activators. In general, activators are sequence-specific DNA-binding proteins whose recognition sites are usually present in sequences upstream of the core promoter (Ptashne & Gann 1997). Many classes of activator(s), discriminated by different DNA binding domains, each associating with their own class of specific DNA sequences. Here we summarise how novel transcription factor MrfA impinge on pst-cell differentiation.

In this study we have defined the DNA target of presumptive signaling pathway; the 39 nucleotide region of ecmA, identified as essential by mutational analysis (Figure. 2), directs strong ecmA-specific expression when multimerised and drive actin 15 minimal promoter. The 39-mer therefore contains all the information necessary for pstA-specific expression (Figure. 19). We also identify a transcription factor that binds the 39-mer and that is therefore a potential mediator of extracellular signaling. The protein, MrfA, has strong sequence similarity with vertebrate MRF and a predicted DNA binding domain with similarity to that of the yeast transcription factor Ndt80 (Figure. 7).

MRF is a transcriptional regulator of CNS myelin gene expression and plays a role in the CNS. MRF is not detectably expressed by oligodendrocyte progenitor cells (OPCs), neurons, or astrocytes and is upregulated strongly upon oligodendrocyte (OL) differentiation. In the absence of MRF, OPCs are able to withdraw from the cell cycle and differentiate into premyelinating OLs but are unable to mature, express the full complement of myelin genes, or myelinate. In addition, MRF's role is distinct from that of other transcription factors (Olig1/2 and Sox10) previously implicated in the OL lineage. Although MRF is specific to OLs within the CNS, it is also expressed in other tissues such as lung and pancreas. Embryos lacking MRF in all tissues from germline deletion of MRF via a ubiquitous Cre driver failed to survive past ~E12.5 (Ben et al., 2009). This indicates that MRF has additional roles unrelated to myelin

gene expression outside the CNS. In *D.discoideum* we have shown similarity data that provides MrfA might involve in pstA-cell and pstB-cell differentiation.

Further analysis of *mrfA*- mutant and MrfA function will provide insights into cell-type specification as well as its binding specificity and regulatory action.

Section 3. 2

Phenotype vs cell-type specific marker

The transcription factors have been impinged on the proliferation and differentiation of many cell-type as master regulators. Our findings provide a crucial mechanistic advance in our understanding of "Tip-dominance" by pstA-cell differentiation and activities of transcription factors in *D. discoideum*. The basic *D.discoideum* body plan is established in the mound, intermingled with prestalk and prespore cells. It is now generally accepted that this period is characterised by the formation of the anterior-posterior (AP) axes, the development of the two precursor cells, the specification of terminal progenitors, and the complex morphogenetic movements of cells (Kay and Thompson, 2009; Williams et al., 1989a). We find that MrfA is a key regulator of pstA-cell differentiation. The phenotype of the *mrfA*- mutant is consistent with a role in prestalk differentiation. The mutant cells aggregate with initially normal kinetics but then later, starting at the streaming stage, there is a delay in their development (Figure. 17). At the mound stage the mutant structures sub-divide and go on to form multi-tipped mounds that yield small slugs (Figure. 17). Such multi-tipping behavior is presumably the result of lowered "Tip-dominance" and the tip-organiser cells, that mediate "Tip-dominance", are a sub-set of the pstA-cell (Fukuzawa et al., 1997; Gaudet et al., 2008). Later, as the mutant slugs migrate, they continuously divide in two along their length and, although DIF-1 is not the inducer of pstA-cell differentiation, this is also a hallmark of mutants defective in DIF-1 signaling (Huang et al., 2006; Thompson et al., 2004; Fukuzawa et al., 2006). Consistent with "RNA-seq Expression profile" data during development (Figure. 15), there is peak of expression, early during aggregation, subsequently moderate reduction is seen from mound to culminant.

The effect is also observed for *lacZ* reporter analysis. When 39-mer is multimerised 4 times (4x39-mer:*lacZ*), it is expressed in pstA-cell. However, the 4x39-mer:*lacZ* is completely inactive in *mrfA*- mutant (Figure. 19). For the ecmA-376:*lacZ*, there is a very low residual level of anterior expression in the *mrfA*- mutant

(Figure. 19). This is we suggest the result of partial redundancy with one or more other transcription factors that recognise elements of the 39-mer region. We also surmise that redundancy explains why the full length, ecmAO, promoter is active in pstA-cell of the *mrfA-* mutant. We examined the possibility that DimB might be the partially redundant partner but it is not (Figure. 19). There are, however, several other proteins in *D, discoideum* with strong sequence similarity to MrfA, so one of these might be the redundant partner. If this hypothesis is true, one expectation is that 4x39-mer:*lacZ* in *mybE-* mutant also exhibits abnormal expression while recombinant MybE fail to bind to 39-mer in band shift assay. These results suggest that MybE might be a co-activator of MrfA to regulate pstA-cell differentiation.

Section 3. 3

MrfA vs 39-mer interaction

DNA–protein interactions are fundamental to the existence of life forms, providing the key to the genetic plan as well as mechanisms for its maintenance. The study of these interactions is therefore fundamental to our understanding of growth, development, differentiation and disease. The DNA-binding sites for activators (also called transcription factor-binding sites) are generally small, in the range of 6–12 bp, although binding specificity is usually dictated by no more than 4–6 positions within the site. We found two linked CA-rich sequences (20-mer and 19-mer in 39-mer) within the ecmA promoter sub-region (Figure. 25A), which is composed of a three distinct CA-rich, CA1-3, sequences that impinge on pstA expression (Figure. 4). The core consensus sequence for binding of Ndt80 to sporulation-specific genes, CRCAAA, has sequence similarity to the CA-rich blocks (Figure. 24). Simultaneous mutation of all three sequences ablates competition activity in a band-shift assay using recombinant MrfA (Figure. 25B). We also confirmed a functional homology to Ndt80 by analyzing the effect of point mutations in MrfA of two residues known to be critical for the interaction of Ndt80 with the two core sequence C-G base pairs (Figure. 27B). Again, in vivo assay, mutation of either CA1 or CA3 alone greatly reduces ecmA expression in the pstA-cell, suggesting that, although the band shift assay denies independent binding of separate 20-mer or 19-mer in vitro (Figure. 26), in vivo functioning requires the co-operative interaction of multiple MrfA molecules. These hypothesis support that Montano et al. (2002) have shown the protein–protein interactions of Ndt80 are distinct in the two crystal lattices and are not consistent with

a biological dimer (Montano et al., 2002). Hence, MrfA might need spacing as scaffolds to recognise long version of CA-rich sequence 39-mer.

As previously mentioned above, MybE, which fail to bind to 39-mer in vitro (data not shown), might impinge on pstA specific expression. Because 1) *mybE-* slugs show strong ecmAO:*lacZ* expression in the very front, pstA-cell, of the slug. (Fukuzawa et al., 2006). 2) *mybE-* slugs showed abnormal 4x39-mer expression (data not shown). This is hypothesis whereas data are accumulating in favor of a DNA-looping model, whereby the activator(s), enhancer(s) and core promoter are brought into close proximity by "looping out" the intervening DNA. A number of studies suggest that the DNA-looping model may in fact be a general mechanism by which activators and enhancers function (Vilar et al., 2005). This would allow for more precise control of the spatial and temporal of transcription activation, and may be more defines many types of cellular differentiation.

Section 3. 4

Localisation

It is well known that spatial and temporal expression of specific transcription factors defines many types of cellular differentiation. We find that a novel behaviour of transcription factor MrfA in *D. discoideum* regulates pst-cell differentiation. The structure of MrfA provides a potential insight into its activation mechanism. MrfA contains a strongly predicted membrane-spanning domain located very near its C terminus (Figure. 7A and Figure. 9). Several transcription factors are known to localise to membrane structures within cells, where their activity is regulated by a proteolysis mechanism called regulated intramembrane proteolysis (Rip). Transcription factors regulated by Rip commonly contain hydrophobic transmembrane domains, which anchor the proteins to intracellular membranes. Through Rip, membrane- bound transcription factors are cleaved, allowing proteolytic fragments to enter the nucleus and regulate gene transcription. In mammalian cells, only four transcription factors, SREBP1, SREBP2, ATF6, and CREB-RP, are known to undergo Rip. Two related bZIP transcription factors, SREBP-1 and SREBP-2, that normally reside in the ER and the Golgi (Brown et al., 2000). When cellular sterol concentrations fall they are released from the membrane by the sequential action of two proteases. They migrate to the nucleus and activate expression of cholesterol uptake and bio-synthesis genes. In addition, yeast has an Sre1, which is orthologue of mammalian SREBP. Under low oxygen, Sre1 is proteolytically cleaved and the N-

terminal transcription factor domain (Sre1N) enters the nucleus and upregulates genes essential for low oxygen growth (Hughes et al., 2005). Several other transcription factors possess a similar configuration of domains and a similar activation mechanism, including ATF6 and Notch (Haze et al., 1999). ATF6 utilises the same two proteases for processing as SREBPs while Notch uses two different enzymes (Ye et al., 2000; Weinmaster et al., 2000). One of the two Notch proteases, γ-secretase, catalyses intramembranous proteolysis. There are orthologus for each γ- secretase component in *D.discoideum* and Amyloid Precursor Protein (APP), a mammalian substrate, is cleaved correctly by the *D.discoideum* enzyme (McMains et al. 2010).

Section 3. 5

Phenotypic rescue

The parental construct rescues normal timing of development in the mutant while the ΔTMD deletion construct partially does (Figure. 34), suggesting that transmembrane domain of MrfA is essential for its biological activity. Ben et al., showed that overexpression of a truncated form of MRF protein lacking the DNA binding domain (Ndt80) in cultured cells failed to induce marker expression (data not shown). Follow this idea, to evaluate whether the Ndt80 domain is necessary for activating gene related pstA-cell differentiation, I construct artificial MrfA protein lacking the DNA binding domain. Although I confirmed expression level of MrfA Δ Ndt80 in transformants (deletion of DNA binding domain did not lead to auto-cleavage activity, Figure. 37B), phenotypic rescue remain to be determined.

Section 3. 6

Processing mechanism

MRF exhibits nuclear localisation and conservation of the DNA binding domain with the yeast transcriptional activator Ndt80. Consistent with this role, overexpression of a truncated form of MRF protein lacking the DNA binding domain in cultured OPCs failed to induce myelin basic protein (MBP) expression. However, its binding *cis*-element and translocation behaviour, which includes proteolysis mechanism, remain to be determined. Ben et al. (2009) has been indicated whether MRF function as a bona fide transcriptional activator is an important question for future studies.

We have identified, in part at least, proteolysis mechanism of MrfA. Direct, preliminary evidence in support of the notion that MrfA is a membrane- tethered transcription factor comes from the Western Transfer analysis of myc tagged MrfA. This yielded an apparent molecular weight of 85 kDa (Figure. 29). Since the predicted molecular weight of myc-tagged MrfA is 105 kDa and the tag is at the N terminus this disparity would be consistent with a proteolytic processing event that removes 20 kDa from the C terminus. One possible explanation is that there is very rapid processing to generate the 85 kDa protein but some of the MrfA protein remains in the membrane and is clipped at the N terminus, removing the tag. It has been reported that vertebrate full-length Notch is a short-lived species constituting less then 1% of the total Notch surface protein at any time (Kidd & Lieber 2002, Logeat et al. 1998). To access this problem; 1) need to higher expression level, 2) double tag, N-terminal and C-terminal, respectively, I construct high expression, double tag MrfA vector. As expected, using c-myc antibody, both cell-lines showed a 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 20 kDa and the other is a >100 kDa (Figure. 33B right). A database search using the peptidase domain (peptidase S74) sequence as reference revealed a high number of homologous proteins. Notably, homologous Cleavage-proteins are found not only in bacteriophages but also in several bacteria such as *Vibrio cholerae* (Schulz et al., 2010). Furthermore, animal MRF sequence is also contained this conserved region. Analysis of flanking sequences in these bacterial genomes shows that the genes containing a C-terminal intramolecular chaperone domain (CIMCD) are most likely phage related (Schulz et al., 2010). CIMCD is removed after proper assembly of the triple β-helix by autoproteolytic, ATP- independent cleavage (Schwarzer et al., 2007). The first report that the intramolecular chaperone plays a crucial role in protein folding that leads to functionally active conformation was based on studies of proteases (Ikemura et al., 1987). Unlike the molecular chaperone, the intramolecular chaperone is encoded in the primary sequence of the protein as an N-terminal or a C-terminal sequence extension and is usually termed propeptide or prosequence. Although, it is not part of the functional domain and does not contribute to the protein function, it is essential for the folding of the functional protein (Inouye et al., 1991). Indeed, all these intramolecular chaperones are removed upon the completion of protein folding either by autoprocessing in the case of proteases or by an exogenous protease in the case of non-protease proteins. On the basis of their roles in protein

folding, intramolecular chaperones can be classified into two categories (Chen et al., 2008). The type I intramolecular chaperones include those that assist tertiary structure formation and mostly are produced as the N-terminal sequence extension, and the type II intramolecular chaperones are those that are not directly involved in tertiary structure formation but guide the assembly of quaternary structure to form the functional protein complex and are mostly located at the C-terminus of the protein. Hence MrfA might be classified in type II protein.

Section 3. 7

Conclusion

The fact that many of the cells that accumulate MrfA display particulate cytoplasmic staining while others display nuclear staining is also consistent with some form of processing and release from a membranous compartment. If this is true one expectation is that induction to become a pstA-cell might trigger proteolytic processing of a membrane localised MrfA pro-protein. Yet pstA-cell inducer polyketide(s), which is not DIF-1, remain to be determined. MrfA driven by semi-constitutive promoter is indeed nuclear localised in the pstA-cell region but is often also nuclear localised in cells located in the rearguard. This could indicate a function for MrfA in the rearguard but the population could equally well be pstA-cell in process of being sloughed off into the slime trail. Consistent with their not being pstB-cell in the slug. However their localisation exhibit upper, lower-cup and basal disc in culminant. In addition, the *mrfA*- mutant exhibits moderately reduction basal disc formation, a phenotypic characteristic of DIF-signaling mutants, and it also shows an aberrant ecmB:*lacZ* expression pattern in the fruiting body; staining in the basal disc is moderately reduced. Furthermore, in *mrfA*- mutant ecmB-*lacZ* is not DIF-1 inducible (Figure. 44) and monolayer differentiation of stalk cells is much reduced; instead remain as viable amoebae (<10% of WT) (Figure. 43). These results suggest that MrfA has a role in pstA-cell differentiation as well as pstB-cell differentiation in the DIF-1 signaling pathway. Consistent with this, Hong (University of Dundee) separately isolated MrfA as a transcription factor that binds to an activator element in the stalk-tube (ST) region of the ecmB promoter. However, recombinant MrfA fail to bind CA-rich sequence similarity with 39-mer in ST region of ecmB promoter (data not shown).

One intriguing possibility, therefore, is that GataC may directly affect the activity of other DIF response regulators such as DimA, DimB or MybE. Indeed,

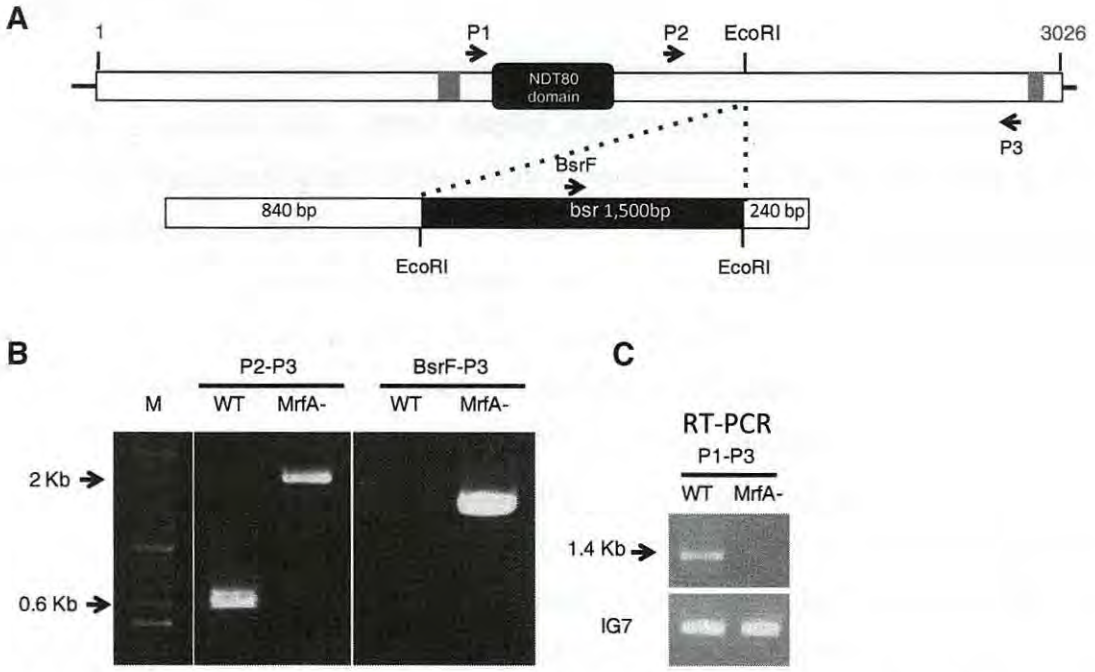
regulatory protein complexes between GATA, bZIP and myb family transcription factors have previously been described and shown to regulate mammalian hematopoietic and adipocyte differentiation (Takahashi et al., 2000; Tong et al., 2005).

Further investigation of the mechanism of activation and mode of action of MrfA is needed to clarify these issues. Given the structural similarity to vertebrate CNS MRF, the results of such studies could also extend mechanistic understanding of a critical regulator of oligodendrocyte differentiation.

Material & Method

Strains and Generation of mutant

Dictyostelium discoideum strain Ax2c (Cambridge, UK, RR Kay's strain) was used as a wild-type (WT) and a parental strain for various transformants and mutants. Several *mrfA-* clones were obtained by gene disruption and one representative clone was used for analysis (Fig. S1). *mybE-/mrfA-* double mutant was further created by disruption of *mrfA* in *mybE-*.



(A) At the top is a schematic representation of the knockout construct used for targeted mutagenesis of the mrfA gene. The coding region of mrfA is boxed, with two introns (gray boxes) and the Ndt80 homologous do- main (black box). The knockout construct was based on a cDNA clone obtained from NBRP, Japan (clone SSA107) as a template. A blasticidin (bsr) cassette (black box; not to scale) was inserted at the unique *EcoR*I site of SSA107, as described in methods. (B) Confirmation of gene targeting by PCR. Genomic DNA was extracted from Ax2 (lane WT) and the null strain (lane mrfA-) and PCR was performed with primer pairs P2-P3 or BsrF-P3. The P2-P3 primer pair generates a 611 bp product for WT and 2,111 bp for mrfA-. This increased size is as expected if the blasticidin cassette is indeed inserted into the mrfA gene by homologous recombination. The BsrF (Bsr F4) -P3 (rcdK01) primer pair generates a 1,725 bp product for mrfA- but not for WT DNA.

Since this uses the same WT DNA as is used for the P2 (rcdK10f)-P3 (rcdK09r) PCR reaction the lack of signal in the WT lane cannot be due to template degradation. (C) Confirmation of gene inactivation by RT-PCR. Total RNA was extracted from the Ax2 strain (lane marked WT) and the null strain (lane marked MrfA-). After synthesis of cDNAs PCR was performed with the P1 (rcdK08f)-P3 (rcdK09r) primer pair, which generates a 1,545 bp product only in the WT lane. IG7 was analysed in the same reaction as a ubiquitously expressed control.

rcdK08fBamHI;

cgggatccCCAGTACCACTTTTAAATATTATGGCA

rcdK10f;

GTTAACAAGTAGTAGTTTATTAAATGG

rcdK09r;

AAGTTCTTGAGTTGCACCAATTGTTTC

BsrF4;

CATTTCGGCAGTACATATTGAAGC

Cell culture and development

Dictyostelium discoideum strains were grown in axenic medium (HL5) (Sussman, 1987) or on Klebsiella lawns. For development, axenically growing cells were washed in KK2 (16.1 mM $KH2PO_4$, 3.7 mM $K2HPO_4$) and plated at $1X10^6$ (in 10 µl cell suspension) cells per 1-cm^2 1.5% KK2 agar plate or membrane filter and left in the dark or light chamber.

lacZ marker studies

A 1.5 kb fragment upstream of the mrfA transcription start site was amplified with a *Xba*I sequence for the 5' primer and *Bgl*II sequence for the 3' primer. After digestion with *Xba*I and *Bgl*II, the fragment was cloned into *Xba*I/*Bgl*II cut actin15ΔBam-gal vector (Pears and Williams, 1988; Ceccarelli et al., 1991) to make mrfA promoter *lacZ* construct. This construct and ecmO-*lacZ*, ecmB-*lacZ*, pspA-*lacZ* and ecmAO-*lacZ*, ST-*lacZ* were electroporated into Ax2 and mutant cells and the resultant transformants selected at 10 mg/ml G418. *lacZ* whole-mount staining was then performed as previously described (Dingermann et al., 1989) as well as measurements of β-galactosidase activity upon DIF-1 induction in monolayer culture, as described (Thompson et al., 2004a). For each experiment, several thousand transformants were pooled and the result confirmed with triplicate electroporations.

Promoter analysis

For 5' and 3' deletions the ecmA promoter sequence was amplified by PCR using primers corresponding to each deletion point with a *Bam*HI site at the 5' end, each of which was pared with a reverse primer containing a start codon and a restriction sequence to add a *Bgl*II site at the 3' end of the PCR product. After digestion with *Bam*HI and *Bgl*II, the fragment was cloned into *Bam*HI/*Bgl*II-cut Actin15deltaBam:gal vector, which provides basal transcription sequences (Fukuzawa et al., 2006). For mutations, nucleotide substitution(s) were introduced into each 5' primer, with a *Bam*HI cohesive end. Various deleted and mutated promoter constructs were grown in the presence of G418 (50 μg/ml) and developed either on water agar plates or nitrocellulose filters until slug stage. Fixation and staining for β-galactosidase were performed as described. The deletion analysis, R3R4 (39mer) WT and a variety of mutant oligonucleotides, that were used as competitors, were:

ecmA05;
cgggatccCTATTTTCAAACACACACATGTGA
ecmA376;
cgggatccTAACACACTTTCACCAAAATTATAC
ecmA374;
cgggatccACACACTTTCACCAAAATTATAC
ecmA357;
cgggatccTTATACACCACCACCATAAATAA
ecmA284;
cgggatccATAATGAAACATTGGGGTTGTAT
ecmA245;
cgggatccAAAATACATACATACACATATTA
ecmA200;
cgggatccTTTATATTGCGTAATGGTTTTGC
ecmA01r;
gaagatctCATTTTCAACGTTATAATTTTTAAACTAATG
ecmA41r;
gaagatctCCAATTTAAAAGTTAATTTTTG
ecmA129r;
cgggatccATAATTGGATTGTCGATCATATTTG
ecmA201r;
cgggatccATATACATTTTTTTAACAATAAT

ecmA330r;

cgggatccTTTTATTATTTATGGTGGTGGT

ecmA376WT;

gatccTAACACACTTTCACCAAAATTATACACCACCACCATAAATg;

ecmA376CAmt1;

gatccTAACAaAaTTTCACCAAAATTATACACCACCACCATAAATg;

ecmA376CAmt2;

gatccTAACACACTTTaAaaAAAATTATACACCACCACCATAAATg;

ecmA376CAmt3;

gatccTAACACACTTTCACCAAAATTATAaAaaAaaACCATAAATg;

2xR3R4 U;

GATCCTAACACACTTTCACCAAAATTATACACCACCACCATAAATAACACACTTTCA

CCAAAATTATACACCACCACCATAAATA

2xR3R4 L;

GATCTATTTATGGTGGTGGTGTATAATTTTGGTGAAAGTGTGTTATTTATGGTGGTG

GTGTATAATTTTGGTGAAAGTGTGTTAG

Protein purification and mass spectrometry

Nuclear extract derived from $3.5x10^{11}$ slug cells were purified by precipitation with 40 % (w/v) ammonium sulfate. It was then loaded onto a DNA affinity column bearing an oligonucleotide corresponding to the R3R4 (39-mer).

This was synthesized as a duplicate copy, annealed with the complementary strand, multimerised by ligation and coupled to sepharose beads. Bound proteins were eluted with 0.4 M KCl. The eluted proteins were further purified through a second round of binding on the affinity column, concentrated and loaded onto an SDS-polyacrylamide gel. After staining with Colloidal Blue Staining Kit (Invitrogen), protein bands were excised from the gel and digested, in-gel, with trypsin. The resulting peptides were analysed by Q-TOF (Quadrupole) mass spectrometry.

Phosphorylation of oligos

Materials

10x Kinase buffer

0.5 M	Tris 7.2
100 mM	MgSO4
1 mM	DTT

T4 PNK

USB/Amersham E70031X (high conc. At –30 U/µl)

Normally the final coupling is like 100 µg of DNA/ml of resin. 1 g of dried resin will give ca 3.5-4 ml of resin, it is better idea to start with 400 µg of DNA. Synthesize 0.04 OD of oligos which would be at least 300-400 µg of DNA for both up and down. Just in case 0.2 OD would be ideal for synthesis. This method is a modified version of Handa's condition. DNA ligase works in Kinase buffer with ATP. However, most cases addition of ligase buffer (NEB) to the reaction would greatly help ligation (recommended).

1. Resuspend at 100 pmol/µl in T buffer (QIAGEN's elution buffer is fine)
2. Mix 200 µl each and put on a heat block at 80 c (or 100 c) for 10 min

3. Switch off and allow cooling at RT for overnight

Kinase reaction

• 10x Kinase buffer	50	µl
• 0.1 M ATP	5	µl
• Kinase (30 U/µl)	20	µl
• Water	20	µl
o Total	500	µl

Incubate at 37 c for 2 hrs

Ligation

Ligase reaction

• 10x ligase buffer	60	µl
• Ligase (6 u/µl)	20	µl
• Water	20	µl
o Total	600	µl

Incubate at 16 c overnight plus a few days more.

Check 2 µl on a gel.

Note:
Addition of the ligase buffer is necessary in this big prep. Ligation should be done at 16 c for 2-3 days which will produce ladder on a gel. If ligation is not efficient, ethanol precipitate the DNA and start from Kination. This will yield much longer product.

Nick column

1. Ethanol or isopropanol-precipitate the ligated sample and dissolve in 100 µl of MilliQ
2. Wash a NICK column (17-0855-01) with two fills of MilliQ.

3. Load 100 µl of the sample then 400 µl of MilliQ; this fraction does not contain DNA.
4. Elute with 400 µl of MilliQ; this contains most of DNA.
5. Another 400 µl to get trace amount of DNA; these three fractions should be checked on a gel (2 µl per lane).

Note;
Determine the DNA amount. It would be roughly 1 µg/µl. Use the second fraction straight for coupling.

Coupling to CNBr-activated S4B

Materials

Storage buffer

10 mM	Tris pH7.6
0.3 M	NaCl
1 mM	EDTA
0.02%	NaN3

CNBr-activated sepharose 4B
Amersham 17-0430-01.

Note;
Prepare 200 ml of 1 mM HCl by adding 17µl into 200 ml of MilliQ (conc HCl; 11.46N). 10 mM K-P buffer pH 7.5, 100 ml. According to the manual pH 8.0-8.3 is the best but pH 7.5 works. The following steps should be done within 10 min to allow maximum coupling efficiency.

1. Weigh 1 g of dried beads in a 50-ml falcon. Add 1 mM HCl to immerse. This will give 3.5 ml of gel
2. Transfer on a funnel and wash with 200 ml of 1 mM HCl
3. Wash with 50 ml of cold MilliQ

4. Wash with 50 ml of 10 mM K-P buffer. Transfer in a 15 ml falcon and suspend to 5 ml
5. Add 4 µl of 1M K-P buffer to 400 µl of the DNA. Add to resin and shake vigorously
6. Rotate overnight at RT
7. Ctf at 2,000 rpm for 2 min and take sup for checking remaining DNA on a gel
8. Wash with MilliQ once
9. Wash with 1M ethanolamine pH8.0 twice; 1 M Tris pH 8.0 works
10. Ethanolamine to 10 ml and incubate at RT for at least 2 hrs
11. Transfer on a funnel and wash with 30 ml of 10 mM K-P buffer
12. Wash with 30 ml of 1 M K-P buffer
13. Wash with 30 ml of 1 M KCl
14. Collect resin with storage buffer and store at 4 c; DNA-beads is stable for long time

Midi scale nuclear extract

Nuclear extracts

For the preparation of nuclear extracts the cells are gently lysed in a NP-40 containing lysis buffer while the nuclei remain intact. After a washing step, the nuclei are suspended in a hypotonic "low salt 2M KCl 20 µl = 20 mM": the nuclei swell. Then a "high salt 2 M KCl 480 µl = 0.4 M" is added slowly: the nucleoplasm is extracted into the buffer while the nuclear envelop stays intact and retains the genomic DNA. The extract is separated from the nuclear envelop/DNA by ctf.

Note; mini or large scale prep could be done in similar way.

- Cells in 500 ml culture at 5-10x10^6 cells/ml or total 2x10^9 cells
- Wash and plate on a 24x24 agar plate
- Cells are allowed to develop until slug stage

1. Collect cells into a 4x50 ml falcon and ctf at 600 g for 3 min. Slugs could be collected with this high ctf
2. Add 4 ml of lysis buffer
3. Add 1/4 vol (1 ml) of 10% NP40; final 2%. Triturate several times. Stand on ice for 5 min. * avoid whipping the lysate
4. Ctf at 15,000 rpm, 4 c for 10 min. * Spin nuclei down for 5 min at 500 g, 4 c
5. Remove sup (maybe keep this as cytosolic fraction, after ctf). You should see white nuclei ppt
6. Wash nuclei once with lysis buffer (optional)
7. Resuspend the pellet in the lysis buffer to 2 ml. Add total 1/4 vol (500 µl) of 2M KCl (final conc. 0.4 M) and mix and stand on ice for 10 min. * First, add 20 µl of 2M KCl, then finger vortex. Be sure that all liquid remains at the bottom of the tube. 2nd, add 480 µl of 2 M KCl very slowly (possibly in small aliquots) while mixing with the pipet tip
8. Ctf at 15,000 rpm, 4 c for 15 min. Sup is 'crude nuclear extract'

$AmSO_4$ precipitation

Materials

K-P buffer (pH 7.5)

1 M K_2HPO_4	83.4 ml
1 M KH_2PO_4	16.6 ml

DB 0 (10 L)

K-P buf (1 M; pH7.5)	500	ml	50	mM
Glycerol	1000	ml	10	%
EDTA (0.5 M)	10	ml	0.5	mM
$MgCl_2$	1	ml	0.1	mM

$ZnCl_2$ (1 M)	1	ml	0.1	mM
30% Brig35	3.33	ml	0.01	%

Add DTT (1 M) to 1 mM.

Store at 4 c. After adding DTT, discard after 1 months.

KCl, 74.55 g/L to make 1 M (DB1000). Mix 0 M and 1 M, to make appropriate concentration of KCl (DB100, etc).

Nuclear Lysis solution

50 mM K-P buffer, pH7.5

2 mM $MgCl_2$

10% Glycerol

1 mM DTT added freshly

Store at 4 c.

Saturated ammonium sulfate solution in PBSA (4.1 M at 25°C)

ammonium sulfate	761	g
20x PBS	10	ml
MilliQ (not a final v.)	1,000	ml

Stir for a day at RT, then bring pH to 7.2-7.5 with 1 N NaOH.

1M PMSF

3.45 g/20 ml of DMSO

1M Benzamidine

1.57g/10 ml of MilliQ

1. Add saturated $AmSO_4$ to make 40% (450 µl per 1 ml of sample); 1,300 µl to 2.5 ml of this sample

2. Let stand on ice for 20 min.
3. Ctf at 4 c for 10 min and collect ppt; the pellet may be floated as a lid. Remove solution carefully
4. Dissolve the pellet into 3 ml of DB100 (500 µl per 200 ml of cultured cells).
5. Dialyse against 500 ml of DB100 at 4 c for 3 hrs then, change the DB100 and dialyse for overnight; contamination of $AmSO_4$ will result in bad gel separation
6. Ctf at 15,000 rpm, 4 c for 5 min, to remove precipitates during dialysis

- Check protein concentration of samples. This will help to access activity
- Examine by gel shift assay. Store samples in aliquots in liqN2 or -80 c

Add saturated $AmSO_4$ to make 20% $AmSO_4$ as follows

To 1 ml nuclear-KCl extract add;

1. Add 149.8 µl AmSO4 to get 20%
2. Stand on ice for 20 min
3. Ctf at 14,000 rpm, 4 c and collect sup
4. Add 362.6 µl (in addition to the above) to get 60 %
5. Stand on ice for 20 min
6. Ctf at 14,000 rpm, 4 c and collect ppt * The pellet may be floated as a lid

DNA affinity column

Binding and elution

Before binding, preincubation of $AmSO_4$ fraction with dA/dT and ssDNA (salmon sperm DNA; sonicated) is necessary.

- $AmSO_4$ fraction 20 mg of protein

- dA/dT 300 µg
- ssDNA 1,000 µg

Incubate on ice for 30 min

Ctf at 20,000 g for 15 min

Note;

Flow rate should be 0.1 ml/min. This must be checked before loading, and adjust using stop-cock attached on the 1-ml syringe column.

1. Take the sup and load onto the DNA column
2. Wash with 10 vols of DB100
3. Put one column vol of the buffer and elute
4. Repeat 10 times and collect fractions into epps. Alternatively, if you do not need these fractions, just collect all eluates into one tube.

Elution of the bound protein

Put a half vol of the column of DB400 and take fractions using repeated elution with DB400. Take 20 fractions.

Concentrate protein

Materials

Amicon µ ltra 0.5 ml 10 K; Millipore

See product protocol

DNA binding assay

The region of mrfA-encoding the Ndt80 domain (amino acids 294-546) was cloned in pET15b (+) *NdeI/BamHI* site (Novagen). It was expressed in *E. coli* strain BL21 Codonplus RIL (Stratagene), as a His-fusion protein, and purified using TALON metal affinity resin (Clontech). Point mutations (R376A, R460A) were introduced into the region of mrfA-encoding the Ndt80 domain (amino acids 294-546) of the bacterial expression pET15b (+) (Novagen) by using the QuikChange mutagenesis kit (Stratagene). Substituted proteins were expressed and purified same as above procedure.

Bacterially expressed His-tag protein (TALON resin)

1xEquilibration/Wash buffer

- Tis-HCl, pH 7.0 50 mM
- NaCl 300 mM
- Protease inhibitor tablet 1 per 50 ml
- DNase small amount

❖ No EDTA, DTT for TALON purification

Bacteria culture

Use BL21-CodonPlus (DE3)-RIL

1. Preincubation (100 ml culture for overnight) in Terrific Broth (TB) at RT
2. Inoculate 50 ml. into 500 ml culture (in 2 L flask) in TB at RT
3. Shaking culture at RT (until OD600 > 0.6)
4. Incubation with 0.1 mM IPTG at RT for 4-5 hrs
5. Harvest cells and put into 2x50 ml falcon tubes (ctf 5,000 rpm for 15 min)
6. Add 10 ml of 1xEquilibration/Wash buffer
7. Sonicate with big tip and Maximum setting for 45 sec~1 min x 3 times on ice

8. Add 10 ml 1xEquilibration/Wash buffer and put into one tube (Total 40 ml)
9. Ctf at 15,000 rpm for 20~30 min
10. Collect Supernatant and filtrate with Ministart High flow with 20 ml syringe
11. Freeze down in Dry ice and keep at -80 c or proceed following purification steps

TALON resin preparation

1. Thoroughly resuspended the TALON resin (Clonetech)
2. Immediately transfer 2 ml (packed vol.) of resin suspension to a sterile tube that will accommodate 10-20 time the resin bed volume
3. Ctf at 700 g for 2 min to pellet the resin
4. Remove and discard the supernatant
5. Add 10 bed volumes of 1xEquilibration/Wash buffer and mix briefly to pre-equilibrate the resin
6. Re-ctf at 700 g for 2 min to pellet the resin, discard the buffer
7. Repeat steps 4 and 5
8. Discard the buffer as much as possible

1xEquilibration/Wash buffer + PMSF

- Tris-HCl, pH 7.0 50 mM
- NaCl 30 mM
- PMSF 1 mM
- Protease inhibitor 1 tablet per 50 ml

His-tagged protein purification

1. Add sup to TALON resin
2. Gently agitate at 4 c for 2 hrs on a platform shaker to allow the polyhistidine-

tagged protein to bind the resin

3. Ctf at 400 g for 5 min
4. Carefully remove as much supernatant as possible without distribution the resin pellet
5. Wash the resin by adding 10-20 bed volumes of 1xEquilibration/Wash buffer
6. Gently agitate the suspension at 4 c for 10 min on a platform shaker to promote though washing
7. Ctf at 700 g for 5 min
8. Remove and discard the supernatant
9. Repeat steps 4 to 8
10. Add 1 bed volume of the 1xEquilibration/Wash buffer to the resin, and resuspend by voltex
11. Transfer the resin to a gravity-flow column with an end-cap in place, and allow the resin to settle out of suspension
12. Remove the end-cap, and allow the buffer to drain until it reaches the top of the resin bed. (Making sure no air bubbles are trapped in the resin bed)
13. Wash column once with 5 bed volumes of 1xEquilibration/Wash buffer
14. [Optional]; If necessary, repeat step 8 (TALON resin preparation) under more stringent condition using 5-10 mM imidazole in 1xEquilibration/Wash buffer
15. Elute the polyhistidine-tagged protein by adding 5 bed volumes of 1xEquilibration/Wash buffer to the column. Collect the elute in 500 µl fractions
16. Use spectrophotometric and SDS/PAGE analyses to determine which fractions contain the bulk of the polyhistidine-tagged protein

1xElution buffer

- Tris-HCl, pH 7.0 50 mM
- NaCl 30 mM
- Imidazole 150 mM

- PMSF 1 mM
- Protease inhibitor 1 tablet per 50 ml

Dialysis

Materials

- Dialysis membrane; Snakeskin (Thermo scientific)
- DB100 (+DTT); recipe is previously shown
- Clip (x2)
- 15 cm dish
- Stirer

1. Cut the Snakeskin appropriate size
2. Wash twice with MilliQ
3. Wash twice with DB100 and immerse it into Snakeskin
4. Fasten up the bottom of the Snakeskin with clip
5. Load sample into Snakeskin
6. Fasten up the top of the Snakeskin with clip
7. Put sample into flask containing DB100
8. Dialyse against 1,000 ml (sample x100 volume) of DB100 at 4 c for 3 hrs
9. Change the DB100 to the new one
10. Let rotate at 4 c for overnight

The fluorescent labeled probe, non-labeled competitors and EMSA was performed as described (Yamada et al., 2008). The R3R4 (39mer) WT and a variety of mutant oligonucleotides, that were used as competitors, were:

39-mer WT

gatccTAACACACTTTCACCAAAATTATACACCACCACCATAAATg

CAmt1

gatccTAACAaAaTTTCACCAAAATTATACACCACCACCATAAATg

CAmt2

gatccTAACACACTTTaAaaAAAATTATACACCACCACCATAAATg

CAmt3

gatccTAACACACTTTCACCAAAATTATAaAaaAaaACCATAAATg

CAmt123

gatccTAACAaAaTTTaAaaAAAATTATAaAaaAaaACCATAAATg

Labeled probe and unlabeled competitors preparation

Material

- Electrophoresis device (Invitrogen)
- Novex DNA retardation gels (Invitrogen)
- Odyssey Infrared Imaging System (LI-COR Biosciences)
- Cy5-dCTP (GE Healthcare)
- Mobi-spin (Mo Bi Tec)
- Exonuclease I (NEB)
- Klenow fragment (NEB)
- 2xannealing buffer; 0.25 M Tris-HCl (pH 7.5), 0.1 M EDTA, 0.2 M NaCl, MilliQ

Annealing oligos

100 µM = 100 pmol/µl

• Oligo U (100 µM)	10, 25	µl
• Oligo L (100 µM)	10, 25	µl
• 2xannealing buffer	50, 55	µl
• MilliQ	30, 5	µl
o Total	100, 105	µl

Put samples on the water bath at 95 c for 5min, then turn off the switch of water bath. Store samples at water bath for O/N.

Cy5 labeled probe for EMSA

• Annealed oligo	4	µl
• 10xNEB2 buffer	2	µl
• dNTPs (-dCTP)	2	µl
• Klenow fragment	0.5	µl
• Cy5-dCTP	2	µl
• MilliQ	9.5	µl
o Total	20	µl

Incubate at 37 c for 2 hrs

Heat inactivation at 75 c for 20 min

Remove 1st strand DNA

To remove noreaction 1st strand DNA, add 1 µl of Exonuclease I to the samples

• Annealed oligo or labeled probe	100 or 20	µl
• 10x Exonuclease buffer	15 or 3	µl
• MilliQ	35 or 7	µl
• Exonuclease I	2	µl
o Total	150 or 30	µl

Mix samples and store at 37 c for 1 hr

Heat inactivation at 80 c for 20 min

Remove short and sheered nucleotide

- Purified with Mobi-spin
- Dilute 1 : 20–50 with T10 buffer (elution buffer)
- Store at -20 c until use for assay (light shielding)

EMSA (Band shift assay)

Binding reaction (make sure to do on ice)

• Recombinant protein	1	µl (0.3 µg/µl)
• DB100	4	µl
o Sub total	5	µl
• dA dT (250ng / μ l) or BSA (1 mg/ml)	1	µl
• Competitor (cold)	1	µl (10– 50 pmol)
• MilliQ	2 or 3	µl

Incubate on ice for 30 min

• Probe (hot Cy5-labelled)	1	µl
o Total	10	µl

Incubate at room temperature for 30 min

Electrophoresis (make sure to do at 4 c cold room)

Prerun	50 V, 20–30 min at 4 c cold room
Run	150 V, 60 min at 4 c cold room
Running buffer	0.5 x TBE (store at 4 c room)

Detection

Gels bearing products with a Cy5-dCTP-labelled probe were scanned at 700 nm wave-length using the Odyssey Infrared Imaging System (LI-COR Biosciences).

WebLogo designing

In order to evaluate MrfA bindig *cis*-element 39-mer consensus among yeast MSE, we use WebLogo (http://code.google.com/p/weblogo/), which is a tool for creating sequence logos from biological sequence alignments. The R3R4 (39mer) and a variety of MSE consensus sequences (Ozsarac et al., 1996) that were used as alignment

```
sps4;
GCGCCACAAAA
sps19;
ACGTCACAAAA
spR2;
TAGCCACAAAA
spR2;
GGGACACAAAA
DIT2;
GCGTCGCAAAA
SMK1;
GTGTCACAAAT
CDC10;
AGACCACAAAT
```

Neutral red staining

For neutral red staining cells were suspended in KK2 (20mM K2HPO4/KH2PO4 pH 6.2) containing 0.008% neutral red and then washed with KK2 three times.

QuikChange XL Site-Directed Mutagenesis

Prepare the sample reaction(s) as indicated below:

- 37 µl of ddH2O to a final volume of 50 µl
- 5 µl of 10 × reaction buffer
- 1 µl of dNTP mix
- 3 µl of QuikSolution
- 1 µl of PfuTurbo DNA polymerase (2.5 U/µl)
- 1 µl (10 - 50 ng) of dsDNA template
- 1 µl (10 pmol; 125 ng) of oligonucleotide sense (S) primer
- 1 µl (10 pmol; 125 ng) of oligonucleotide antisense (AS) primer

- Segment 1; cycle 1
- 95 c 1 min
- Segment 2; cycle 18
- 95 c 50 sec
- 60 c 50 sec
- 68 c 9 min
- Segment 3; cycle 1
- 68 c 7 min

* For example, a 5-kb plasmid requires 5 minutes at 68 c per cycle.

Dpn I Digestion of the Amplification Products

1. Add 1 µl of the DpnI restriction enzyme (10 U/µl) directly to each amplification reaction

2. Gently and thoroughly mix each reaction mixture by pipetting the solution up and down several times. Spin down the reaction mixtures in a microcentrifuge for 1 min, then immediately incubate the reactions at 37 c for 1 hr to digest the parental (i.e., the nonmutated) supercoiled dsDNA.

Transformation of XL-1 Blue competent Cells

• Follow the sample reaction(s) same as usual TF protocol

1. Gently thaw the XL10-Gold ultracompetent cells on ice. For each sample reaction to be transformed, aliquot 45 µl of the ultracompetent cells to a prechilled epp.
2. Add 2 µl of the β-ME mix provided with the kit to the 45 µl of cells. (Using an alternative source of β-ME may reduce transformation efficiency.)
3. Swirl the contents of the epp gently. Incubate the cells on ice for 10 min, swirling gently every 2 min
4. Transfer 2 µl of the Dpn I-treated DNA from each control and sample reaction to separate aliquots of the ultracompetent cells

DIF responsiveness; Marker induction assay in monolayer (micro-titre dish lacZ assay)

Monolayer and dissociated cell assays were performed as described (Berks and Kay, 1990; Huang et al., 2006).

Cell density;

Collect vegetative cells growing in 1.5 x 10^6 cells per 10 ml of HL-5 medium.

2.5 x 10^5 cells per well (96 wells plate)

Incubation time;

20-24 hours at 22 c in a humid box without the lid

Lysis and detection

1. The next day, remove incubation medium and add lysis buffer with DTT/protein inhibitor cocktail to each well. Incubate for 15 min on ice.
2. Add 1 mM CPRG in lysis buffer.
3. Incubate at 37 C. Incubation time varies from 30 min to overnight, depending on the sensitivity of the strain. Usually needs over 2 hrs incubation.
4. Read A_{575} using plate reader.

Reagent

100 nM of DIF-1, 5 mM of cAMP, 50 μM of cerulenin. 1 mM CPRG (chlorophenolred-β cD-galactopyranoside)

Lysis buffer;

100mM HEPES, 1 mM $MgSO_4$, 2 % Triton X-100, pH 8.0

(5 mM of DTT and protease inhibitors mix just before use)

Stalk Medium;

10 mM MES (K+) pH6.2, 1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, 200 μ /ml Streptomycin

Construction of mutant rescue plasmids

A cDNA covering the whole length of *mrfA* coding region was obtained by screening a Phage cDNA library prepared from slug stage cells.

1. N-terminal myc-tagged MrfA full-length

The ORF of *mrfA* was amplified using the *mrfA* cDNA with a *Kpn*I site and a myc sequence for the 5' primer and *Xba*I site for the 3' primer. After digestion with *Kpn*I and *Xba*I, the fragment was cloned into *Kpn*I/ *Xba*I-cut pDXA-3C vector (Manstein, GENE 162, 129, 1995) to make N-terminal myc-tagged MrfA construct.

* Magenta colour shows c-myc and FLAG sequences
* Cyan colour shows stop codon sequences
* Small characteristics show restriction enzyme sequences

```
rcdK02mycKpnI;
      ggggtaccGAACAAAAATTAATTTCAGAAGAAGATTTAAATAAAATGGATGGGTATA
      ACCAACAGCAACAG

rcdK01XbaI;
      gctctagaTTAATTATCATTATTATCAAAATTATGAC
```

2. N-terminal myc, C-terminal FLAG-tagged MrfA full-length

The ORF of *mrfA* was amplified using the *mrfA* cDNA with a *Cla*I site and a myc sequence for the 5' primer and *Xho*I site and a FLAG sequence for the 3' primer. After digestion with *Cla*I and *Xho*I, the fragment was cloned into *Cla*I/*Xho*I-cut pA15GFPS65T (Heim R, Cubitt AB, Tsien RY: Improved green fluorescence. Nature 1995, 373: 663-664) via TOPO sub-cloning vector to make N-terminal myc, C-terminal FLAG-tagged MrfA construct.

```
mrfA02mycClaI;
      atcgatGAACAAAAATTAATTTCAGAAGAAGATTTAAATAAAATGGATGGGTATAAC
      CAACAGCAACAG

mrfA01FLAGXhoI;
      ctcgagTTATTTATCATCATCATCTTTATAATCATTATCATTATTATCAAAATTATG
      AC
```

3. N-terminal myc, C-terminal FLAG-tagged MrfA Δtransmembrane domain (ΔTMD)

The ORF of *mrfA* was amplified using the *mrfA* cDNA with a *Cla*I site and a myc sequence for the 5' primer and *Xho*I site and a FLAG sequence for the 3' primer. After digestion with *Cla*I and *Xho*I, the fragment was cloned into *Cla*I/*Xho*I-cut pA15GFPS65T via TOPO sub-cloning vector to make N-terminal myc, C-terminal FLAG-tagged MrfA ΔTMD construct.

mrfA02mycClaI;

atcgatGAACAAAAATTAATTTCAGAAGAAGATTTAAATAAAATGGATGGGTATAAC
CAACAGCAACAG

mrfA11rFLAGXhoI;

ctcgagTTATTTATCATCATCATCTTTATAATCTTGATTTTTTGATTTCTTTTTTAA
ATTTTTTAATTTTGATTCATAGG

4. N-terminal myc, C-terminal FLAG-tagged MrfA ΔNdt80 DNA-binding domain (Δ Ndt80 DBD); Translation 673 a.a. MW=75 kDa

The starting construct myc-MrfA contains full-length of MrfA coding region cloned into ClaI/XhoI site of the pA15GFPS65T vector via sub-cloning TOPO or pJET vector.
In order to delete orthologous sequence to the Ndt80 DNA-binding domain, PCR was performed on using 2 set of primers. This deletes a.a. 286 to 546 from MfrA coding region. 3 pieces ligation method was used for construction.

To amplify amino acid from 1 to 285

mrfA02mycClaI;

atcgatGAACAAAAATTAATTTCAGAAGAAGATTTAAATAAAATGGATGGGTATAAC
CAACAGCAACAG

mrfA25r PstI;

ctgcagTGATGGATTTGGTGATATACTACGTGG

To amplify amino acid from 547 to stop codon

mrfA24f PstI;

ctgcagTCACCTGTTCATACCCCTGATTATGC

mrfA01FLAGXhoI;

ctcgagTTATTTATCATCATCATCTTTATAATCATTATCATTATTATCAAAATTATG
AC

5. N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation full-length

N-terminal myc, C-terminal FLAG-tagged MrfA full-length vector is used for point mutation construction

1. This vector is digested by *ClaI*/*XhoI* (full-length) this digested product was cloned into *ClaI*/*XhoI*-cut pJET; a) N-terminal myc, C-terminal FLAG-tagged MrfA full-length in pJET
2. S767A point mutation PCR was performed on original N-terminal myc, C-terminal FLAG-tagged MrfA full-length vector with a set of primers; rcdK 22f SpeI - mrfA S767A antisense, mrfA S767A sense – mrfA01 FLAG XhoI
3. rcdK 22f SpeI - mrfA S767A antisense PCRed products and mrfA S767A sense – mrfA01 FLAG XhoI PCRed products is diluted (50 dilution), then mixed up as a next PCR templates
4. PCR with rcdK22f SpeI – mrfA FLAG XhoI primer
5. This S767A PCRed fragment was cloned into pJET vector; b) MrfA S767A *SpeI*/*XhoI* digested fragment in pJET
6. Check sequence by DNA Sequencing & Services (http://www.dnaseq.co.uk)
7. b) based S767A point mutation vector was digested by *SpeI*/*Bam*HI, this digested product was cloned (substituted into alternative WT fragment) into a) based *SpeI*/*Bam*HI -cut pJET vector

8. Check sequence by DNA Sequencing & Services (http://www.dnaseq.co.uk)
9. N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation full-length in pJET vector was digested by *ClaI*/*XhoI*, this digested products was cloned into *ClaI*/*XhoI*-cut pA15GFPS65T vector.
10. Check sequence by DNA Sequencing & Services (http://www.dnaseq.co.uk)

```
rcdK22f SpeI;
      actagtCAAGGTCAAACAAATATTTTACGTT

mrfA S767A antisense
      CTTAAATCagcTGGATGATAAAC

mrfA S767A sense
      GTTTATCATCCAgctGATTTAAG

mrfA01 FLAG XhoI;
      ctcgagTTATTTATCATCATCATCTTTATAATCATTATCATTATTATCAAAATTATG
      AC
```

6. N-terminal myc, C-terminal FLAG-tagged MrfA K772A point mutation full-length

N-terminal myc, C-terminal FLAG-tagged MrfA full-length vector is used for point mutation construction

- Same as procedure N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation full-length (different primer set was used, indicate below)

```
rcdK22f SpeI;
      actagtCAAGGTCAAACAAATATTTTACGTT

mrfA K772A antisense
      CGATTTCAAATCATAtgcTATTCTTAAATC

mrfA K772A sense
```

GATTTAAGAATAgcaTATGATTTGAAATCG

mrfA01 FLAG XhoI;

ctcgagTTATTTATCATCATCATCTTTATAATCATTATCATTATTATCAAAATTATG
AC

7. N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation Δ transmembrane domain (ΔTMD)

N-terminal myc, C-terminal FLAG-tagged MrfA Δ transmembrane domain (Δ TMD) is used for point mutation construction

- Same as procedure N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation full-length (different primer set was used, indicate below)

rcdK22f SpeI;

actagtCAAGGTCAAACAAATATTTTACGTT

mrfA S767A antisense

CTTAAATCagcTGGATGATAAAC

mrfA S767A sense

GTTTATCATCCAgctGATTTAAG

mrfA11r FLAG XhoI;

ctcgagTTATTTATCATCATCATCTTTATAATCTTGATTTTTTGATTTCTTTTTTAA
ATTTTTTAATTTTGATTCATAGG

8. N-terminal myc, C-terminal FLAG-tagged MrfA K772A point mutation Δ transmembrane domain (Δ TMD)

N-terminal myc, C-terminal FLAG-tagged MrfA Δ transmembrane domain (Δ TMD) is used for point mutation construction

- Same as procedure N-terminal myc, C-terminal FLAG-tagged MrfA S767A point mutation full-length (different primer set was used, indicate below)

rcdK22f SpeI;

actagtCAAGGTCAAACAAATATTTTACGTT

mrfA K772A antisense

CGATTTCAAATCATAtgcTATTCTTAAATC

mrfA K772A sense

GATTTAAGAATAgcaTATGATTTGAAATCG

mrfA11r FLAG XhoI;

ctcgagTTATTTATCATCATCATCTTTATAATCTTGATTTTTTGATTTCTTTTTTAA

ATTTTTTAATTTTGATTCATAGG

Ligation

Materials

- High concentration (HC) T4 DNA ligase (Promega; M1794)
- 2X rapid Ligation buffer (Promega; M8221)

Assemble the following reaction in a sterile microcentrifuge tube:

• Insert (fragment)	2	µl
• Vector	0.5	µl
• 2x buffer (rapid)	3	µl
• T4 ligase (HC)	0.5	µl
o Total	6	µl

1. Incubate the reaction at RT for 5 min for cohesive-ended ligations, or 15 min for blunt-ended ligations.
2. Use the ligation mixture directly for transformation; XL1-Blue, DH5α

pJET cloning (subcloning)

Materials

- CloneJET™ PCR Cloning Kit (Fermentas; #K1231)

Assemble the following reaction in a sterile microcentrifuge tube:

• 2x reaction buffer	5	µl
• Purified PCRed product	2	µl
• pJET vector	0.5	µl
• MilliQ	2	µl
• T4 ligase	0.5	µl
o Total	10	µl

1. Incubate at RT for 5 min
2. Use the ligation mixture directly for transformation; XL1-Blue, DH5α

TOPO cloning (subcloning)

Materials

- Zero Blunt® TOPO® PCR Cloning (Invitrogen; # 450245)

Assemble the following reaction in a sterile microcentrifuge tube:

• Salt solution	1	µl
• Purified PCRed product	3.3	µl
• TOPO vector	0.3	µl
o Total	4.6	µl

1. Incubate at RT for 5 min
2. Use the ligation mixture directly for transformation; XL1-Blue, DH5α

Immunocytochemistry

The N-terminal myc-tagged MrfA construct was transformed into Ax2 or *mrfA*- cells and they were selected at 20 µl/ml of G418. Transformant mounds or slugs were dissociated and fixed as described previously (Fukuzawa, 2001). Cell samples were treated with 20,000x diluted mouse anti-c-myc monoclonal antibody (mAb9E10), followed by 2,000x diluted Alexa488-conjugated goat anti-mouse IgG. Samples were visualized with a (Leica microscope) confocal microscope.

Standard immunofluorescent (IF) staining (dissociated cells)

Cell density; each 10 µl of cell suspension ($1x10^7$ cells/ml in KK2)

1. Develop on HA filter for appropriate time (10 hrs for mound)
2. Collect cells with HA filter into 2 ml epp containing 1ml of KK2 PB and vortex vigorously then, ctf at 5,000 rpm for 1 min
3. Remove HA filter and dissociate cells from developing structures by titration through a syringe needle (23 G) MAKE SURE TO DO ON ICE
5. Add same volume of absolute MtOH (final 50 % MtOH) and ctf at 5,000 rpm for 1 min
6. Resuspend cells in absolute MtOH and incubate on ice for 10 min
7. Ctf at 5,000 rpm for 1 min
8. Resuspend cells in 51 µl of absolute MtOH
9. Spread 3 µl of cell-suspension on glass-well, let stand at RT for 1 min
10. Add appropriate volume of PBS into the well
11. Let stand at RT for 5 min
12. Remove PBS and incubate with primary antibody in PBS
    (20,000 dilution; 0.25 µl in 5,000 µl for c-myc Ab) at 4 c for O/N
13. Wash 3 in PBS
14. Incubate with secondary Ab in PBS
    (2,000 dilution 0.5 µl in 1,000 µl) at RT for >2 hrs
15. Wash 3 times in PBS
16. Mount with Gelvatol

Standard immunofluorescent (IF) staining (slug)

Cell density; each 10 µl cell suspension ($1x10^7$ cells/ml in KK2)

1. Develop on 1.5 % water agar for appropriate time (13-15 hrs for slug)

2. Cut out 3 or 4 spots (with agar) and place on dry plastic dish
   Put these spots close together for one 22 x 22 mm coverslip

3. Put MilliQ on these spots (tipped mounds or slugs float up)

4. Transfer tipped mounds or slugs to coverslip by touching on the MilliQ surface

5. Dip into cold 50 % MtOH in MilliQ for 5min

6. Fix in cold absolute MtOH for 10 min
   * At this step, you can store samples at -20 c

7. Post-fix with cold 50 % MtOH in MilliQ for 5-10 min

8. Post-fix with cold 20 % MtOH in PBS for 5-10 min

9. Wash 3 in cold PBS

10. Incubate with primary antibody in PBS
    (20,000 dilution; 0.5 µl l in 10,000 µl for c-myc Ab) at 4c for O/N

11. Wash three times in PBS

12. Incubate with secondary Ab in PBS
    (2,000 dilution; 1 µl in 2,000 µl) at 4 c for O/N

13. Wash three times in PBS

14. Mount with 20 % of Gelvatol

Anti-MrfA antibody purification

Polyclonal antibodies ELISA & synthesis report

- Hapten; 'N' – ADDSNLKKRKIKNC (peptide for immunisation)
- Amount; 5 mg
- Length; 14 a.a.
- Mw; 1,633
- Form; Lyophilised
- Antigen; KLH-peptide conjugate

- Lyophilised antiserum; Rabbit 3.7 g (from 50 ml), reconstitute each vial with 50 ml of sterile deionized H_2O, stored at -80 c

Affinity resin purification of polyclonal antibody

Materials

- Affi-Gel 10 (BIO-RAD); wash and stored at -20 c
- Glass wool

Buffers

- Wash buffer 1; 10 mM Tris 7.5
- Wash buffer 2; 10 mM Tris 7.5, 0.5 M NaCl
- Elution buffer; 0.1 M Glycine 2.5 w/HCl
- Equilibration buffer; 1 M Tris 8.0

Coupling peptides

- Peptide; 5 mg/ml in PBS
- 2 mM solution of 14 a.a. peptide might be 3.5 mg/ml
- 10 µl of this solution per 100 µl of Affi-Gel to couple

1. Make 1:1 slurry resin (50 %) with PBS. Use fresh gel for the coupling. Take 200 µl of slurry (100 µl of resin).

2. Add 35 µl (2 mM; 10 µl of 3.5 mg/ml of peptide solution) in PBS and top-up to 400 µl with PBS.

3. Rotate 2 hrs at 4 c

4. Add 50 µl of 1 M ethanolamine (pH 8.0) to block the unreacted residue. Rotate for overnight.

Affinity purification of antibodies

1. Make a tip column. Take a gel-loading tip and attach on a stand. Put a glass wool ball and pour 50 µl of affinity gel. Attach a 1 ml tip cut at the end to fit the 200 µl tip (as a reserver), and wash with PBS to equilibrate

2. Pass crude serum 1 ml at least 5 times over 1 hr to bind antibody at RT

3. Wash with buffer 1 once, wash with buffer 2, 3 times

4. Place a eppie with 150 µl of the equilibration buffer. Elute the bound antibody into this with 50 µl of the elution buffer three times

5. Micro-dialyse against PBS at 4 c

Western Transfer Analysis

Material

- Transfer device; XCell II Blot Module (Invitrogen) EI0002
- Electrophoresis device; XCell superLock Mini-Cell (Invitrogen) EI0002
- Protein gel; 4–12% BisTris precast 12 well gel (Invitrogen) NP0321BOX
- Transfer membrane; Hybond-C extra nitrocellulose (GE Healthcare), cut into 8.5 x 8.0 cm
- Blotting pad (x6); sponge (Unknown product)
- Filter paper; 17 Chr (GE Healthcare), cut into 9.0 x 8.0 cm

- Transfer buffer;
    1. 20x NuPAGE Transfer Buffer (Invitrogen protocol);
       Bicine (N, N, Bis 2-hydroxyethyl glysine) 10.2 g, Bis-Tris (free base) 13.1 g, EDTA 0.75 g, Chlorobutanol (Trichloro-2-methyl-2-propane) 0.025 g, adjust the volume to 125 ml with DW

    2. 1x NuPAGE Transfer buffer;
       NuPAGE Transfer buffer (20x) 100 ml, Methanol 400 ml (final 20 %), DW 1,500 ml

- Protein marker; See blue plus 2 Pre-Stained Standard (Invitrogen)

- 2xSDS sample buffer;
  0.125 M Tris-HCl (pH 6.8), 10 % 2-mercaptoethanol, 4 % SDS, 10 % Sucrose, 0.01 % BPB, MilliQ

- NuPAGE® MOPS SDS Running Buffer (for Bis-Tris Gels only) (20X);
  MOPS 209.2 g (1.0 M), Tris base 121.2 g (1.0 M), SDS 20 g (69.3 mM), EDTA free acid 6.0 g (20.5 mM), DW to 1.0 liter final concentration. 1X buffer should be pH 7.7 (do not adjust with acid or base)

- TBST buffer;
  10xTBS buffer:
  NaCl 80 g, KCl 2 g, Tris base 30 g and 800 ml of MilliQ

1. Dissolve all dry reagents together in 800 ml of MilliQ
2. Adjust the pH to 7.4 using HCl
3. Add MilliQ to a final volume of 1 L
4. Sterilize by autoclaving

- 1xTBST buffer;
  20 % Tween-20 2.5 ml, 10xTBS 50 ml and MilliQ 447.5 ml
  1. Add 50 ml of 10xTBS to the 447.5 ml of MilliQ
  2. Add 2.5 ml of 20 % Tween-20 and mix

- 5 % skim milk
- Polypack
- Monoclonal c-myc antibody (9E10) CRUK; 1.6 mg/ml
- Monoclonal FLAG antibody (Wako); 0.5 mg/ml
- Polyclonal FLAG antibody (M2) SIGMA; 3.8 mg/ml
- Goat anti-mouse IgG (H+L) – HRP conjugate (BIO-RAD)
- SuperSignal West Pico Chemiluminescent (Thermo scientific)

Sample preparation

Harvest growing transformant cells from HL-5 medium. Ctf and wash twice with KK2. Cells were allowed to develop until mounds or slugs onto Millipore HA filter on non-nutrient agar. The multicellular structures were dissociated in 1 x SDS sample buffer. Cell samples were boiled at >100 c for 8 min on heat-block. Stored at -20 c until use.

Running NuPAGE Gels

1. Select the desired running buffer (MOPS works for >200 to 14 kDa and MES for 60 to 2.5 kDa) and make up 800 ml using the 20x stocks stored at 4 c
2. Remove precast gel from bag, rinse with DW.
3. Peel off tape on back of gel and remove comb.
4. Optional procedure; wash out wells a total of three times with 1x running

buffer using a pasteur pipette. Fill the sample wells with buffer and remove all air bubbles

5. Set up XCell II mini cell apparatus as per instructions
6. Fill the center buffer chamber with about 200 ml MOPS running buffer and check for leaks. If OK, add the remainder of MOPS buffer to the outer buffer chamber
7. Load 15 µl of total lysate protein per lane and 7.5 µl of protein marker
8. For MOPS buffer, firstly run at 50 V constant voltage for 40 min, then run at 150 V constant voltage for ~80 min. Expect 100-115 mA current at the beginning and 60-70 mA at the end

Western transfer, antibody treatment & detection

For Western analysis, samples were separated by SDS-PAGE on a 4–12 % BisTris gel, blotted onto Hybond nitrocellulose membrane, and probed with anti-myc monoclonal antibody (1.3 mg/ml) or Wako FLAG monoclonal antibody (0.5 mg/ml). Horseradish peroxidase-coupled goat anti-mouse IgG secondary antibodies were used accordingly, with ECL detection.

1. Separate tagged fusion proteins from sample lysates using a standard SDS-PAGE protocol (see above running NuPAGE gels section)
2. Soak Hybond membrane and filter paper (17 Chr, x2) in appropriate volume of transfer buffer in square dish individually
3. Soak the blotting pads (x6) in Pyrex glass container containing transfer buffer (no air bubbles); put 2 blotting pads in transfer device, the others (blotting pads are subdivided into 2 sets) are positioned aside in transfer buffer (make sure to reduce air bubble as much as possible)
4. After opening the gel cassette, remove the wells with the knife
5. Place a presoaked Hybond on the gel (no air bubbles)

6. Drop the structure (gel and Hybond membrane) into square dish containing transfer buffer
7. Cut the foot off the gel using a knife.
8. Place a piece of presoaked 17 Chr on top of the blotting pads.
9. Place the structure (gel and Hybond membrane) onto 17 Chr; make sure to touch gel surface to 17 Chr
10. Place 17 Chr onto Hybond membrane (no air bubbles)
11. Place 4 blotting pad onto 17 Chr
12. Assemble the sandwich in the transfer apparatus as shown in the NOVEX instructions * see XCell II™ Blot Module Catalog no. EI9051, page. 12-13
13. Fill the chamber with ~200 ml transfer buffer until the gel/membrane is just covered
14. Fill the outer buffer chamber with 650 ml DW (necessary to dissipate heat during the transfer).
15. Run the unit at 30 V constant voltage for 90 min to 2 hr
16. Wash the Hybond membrane in at least three times with TBST for 5 min interval by mild agitation
17. Block the Hybond membrane with 20 ml of TBST (per membrane) with 5 % skim milk, for 30 min at RT with agitation
18. Remove the blocking agent, then transfer membrane to the falcon or polypack
19. Add antibody to the Hybond membrane in at least 2 ml of TBST with 5 % skim milk and incubate at 4 c for overnight
20. Next day, remove the primary antibody solution
21. Wash three times with TBST for 5 min interval by mild agitation
22. Add secondary anti-body to TBST with 5 % skim milk

23. Incubate the blots with shaking at RT for 90 min
24. Wash the blot three times with TBST for 5 min interval
25. Develop the blots with the appropriate substrate for 5 min maximum
26. Expose light film to the blot. Exposure times range from 5 sec to 10 min

Note;

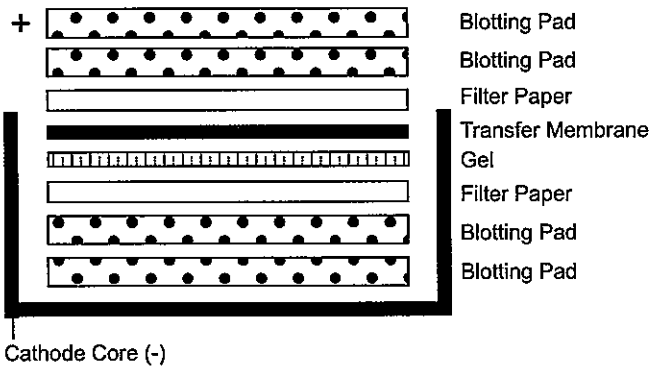
It is best to do a quick exposure of 10–30 seconds to determine what exposure time is needed. If the signal is too intense even at the short exposure times let the signal decay from 1–8 hrs or longer if necessary and then re-expose the film.

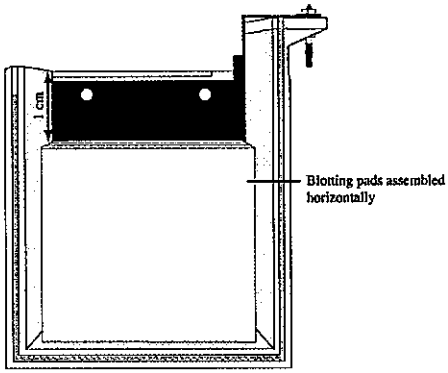
Electrophoresis

- MOPS buffer
- Protein gel
- 50 V, 30 min, then 120 V, 120 min
- 7.5 µl of marker
- 15 µl of 1 x SDS boiled sample

Transfer

- Transfer device
- Transfer buffer
- Transfer membrane
- Blotting pad (x6)
- Filter paper (x2)
- 30 V, 120 min (almost 100 mA/gel)





XCell IITM Blot Module Catalog no. EI9051, page. 12-13

Antibody treatment

- myc, FLAG and secondary antibody in 5 % skim milk, respectively
- myc (50,000 dilution)
- FLAG (50,000 dilution)
- Secondary antibody (2,000 dilution)
- Skim milk in TBST

Stalk cell differentiation in monolayer

Materials

- 3.5 cm Nunc dish (9.6 cm2)
- Stalk Medium; 10 mM MES (K+) pH6.2, 1 mM $CaCl_2$, 2 mM NaCl, 10 mM KCl, 200 µg/ml Streptomycin
- 5 mM cAMP (stock)
- 100 nM DIF-1 (50 µM stock)

1. Collect vegetative cells growing in HL-5 medium in late log phase (i.e., no more than 2.0×10^6 cells/ml) and ctf at 5,000 rpm for 1 min
2. Wash twice with stalk medium, count
3. To each of two Lab-Tek chambers, add 3.0×10^5 cells in 1 ml of stalk medium containing 5 mM cAMP.
4. Incubate for 8 h at 22 c. Most (>80 %) cells should adhere firmly to the bottom slide. Because of the high concentration of cAMP, almost no aggregation should be seen at this stage, and the cells should be randomly scattered and isolated
5. Carefully remove the liquid and wash once with 1 ml of stalk medium
6. Replace with:
7. 1 ml of stalk medium containing 100 nM DIF-1 in one chamber or 1 ml of stalk medium containing no DIF-1 in the chamber
8. Incubate for 24 hrs at 22 c

Immunoprecipitation protocol with Dynabeads Protein-G

Materials

Lysis buffer; mNP40–lysis buffer (1x)

- 50 mM TrisHCl pH8.0
- 150 mM NaCl
- 1.0 % NP40
- 50 mM NaF
- 2 mM EDTA pH 7.2 or 8.0
- 2 mM Na-pyrophosphate (tetrasodium)

Add following inhibitors just before use

Complete EDTA-free proteinase inhibitor

Immunoprecipitation

1. Take $5.0x10^8$ cells in 50 ml tube, harvest via ctf, and resuspend ppt in 5 ml of mNP40-lysis buffer
2. Lyse on ice for 10 min, with occasional inversion
3. Ctf 14,000 rpm for 10 min at 4 c
4. Transfer supernatant to a new tube
5. Add 5 µl of anti-FLAG antibody (Wako)
6. Incubate with rotation at 4 c for 30 min

7. Wash twice in Dynabeads Protein-G with mNP-40 lysis buffer; 200 µl slurry in a new tube (for 1 sample)
8. After short ctf, transfer sample-antibody solution to Dynabeads tube
9. Incubate with rotation at 4 c for 2 hrs
10. Wash beads five times in mNP40-lysis buffer
11. Remove excess buffer as much as possible
12. Add 400 µl of 1xSDS sample buffer, mix (ctf) and boil for 8 min

Acetone precipitation

1. Add 1,600 µl of acetone to the 400 µl of boiled sample, then vortex
2. Incubate at -20 c for overnight or -80 c for 2 hrs
3. Ctf at >10,000 rpm at 4 c for 30 min
4. Let stand for 1 hr at RT for air dry (you can see white pellet bottom of the epp)
5. Add 30 µl of 1xSDS sample buffer, mix (ctf) and boil for 8 min
6. Load 30 µl of sample for electrophoresis

Immunoprecipitation protocol with ProteinG-agarose beads

Materials

Lysis buffer; mNP40--lysis buffer (1x)

- 50 mM TrisHCl pH8.0
- 150 mM NaCl
- 1.0 % NP40
- 50 mM NaF
- 2 mM EDTA pH 7.2 or 8.0
- 2 mM Na-pyrophosphate (tetrasodium)

Add following inhibitors just before use

Complete EDTA-free proteinase inhibitor

- 1 tablet in 50ml of mNP40 lysis buffer

Immunoprecipitation

1. Take $2.0x10^7$ cells in 50 ml tube, harvest via ctf, and resuspend ppt in 1ml of mNP40-lysis buffer
2. Lyse on ice for 10 min, with occasional inversion
3. Ctf 14,000 rpm for 10 min at 4 c
4. Transfer supernatant to a new tube
5. Using a tip which the bottom has been cut off, add 10 µl of *prewashed ProteinG-agarose beads [Roche]; *Prewash = wash in mNP40-lysis buffer

several times (ctf at 400 x g) & resuspend in mNP40-lysis buffer to make a 1:1 slurry; can prepare larger stock & store at 4 c

6. Rotate for 30 min at 4 c
7. Ctf at 400 x g for 2 min at 4 c, transfer sup to new tube
8. Add 0.5 µl of anti-FLAG antibody (Wako)
9. Incubate with rotation at 4 c for 30 min
10. Add 20 µl of new prewashed ProteinG-agarose beads (1:1 slurry)
11. Incubate with rotation at 4 c for 1. 2 hrs, 2. O/N
12. *Wash beads three times in mNP40-lysis buffer; *wash = resuspend beads in 500 µl of mNP40-lysis buffer on ice for 2 min, then ctf at 400 x g for 2 min at 4 c and discard buffer
13. After final wash step, ctf again (400 x g, 2 min), remove as much liquid as possible
14. Add 15 µl of mNP40-lysis buffer and 25 µl of 2x SDS sample buffer, mix (ctf) and boil for 8 min
15. Load up to 15 µl per lane of gel

Colloidal Blue Staining

Refer to Colloidal Blue Staining Kit user protocol (Invitrogen)

Stain NuPAGE® Novex® Bis-Tris Gels

1. Shake the gel in the Fixing Solution for 10 minutes at RT
2. Shake the gel in the Staining Solution without Stainer B for 10 min at RT
3. Add Stainer B to the existing Staining Solution in the proper volume as shown in the following table
4. Shake the gel in Staining Solution for a minimum of 3 hrs and a maximum of 12 hrs

- Note: Protein bands begin to appear in 2–5 min. Staining intensity does not vary significantly if the gel is left in stain for 3 hrs or 12 hrs

5. Decant the Staining Solution and replace it with 200 ml of deionized water per gel. Shake gel in water for at least 7 hrs
6. The gel will have a clear background after 7 hrs in water

- Note: Gels can be left in water for up to 3 days without significant change in band intensity and background clarity

7. For long-term storage (over 3 days), keep the gel in 20% ammonium sulfate solution at 4 c

CBB staining solution 1

• DW	20	ml
• Methanol	25	ml
• Acetic acid	5	ml
o Total	50	ml

Destaining solution

• DW	27.5	ml
• Methanol	10	ml
• Stainer A	10	ml
• Stainer B	2.5	ml
o Total	50	ml

Silver staining

Refer to SilverQuest™ Silver Staining Kit user protocol (Invitrogen)

Gel purification by Crystal Violet method (Recommend 50 V running)

Materials

- Crystal Violet (2 mg/ml)
- 6xCrystal Violet loading buffer; 30 % glycerol, 20 mM EDTA, 100 µg/ml Crystal Violet
- 0.5 or 1xTAE running buffer
- 20 µl of Crystal Violet solution in 25 ml of 0.8 % agarose gel

Enzyme digestion (check)

Materials

- Restriction enzyme
- Buffer (NEB, TaKaRa, Roche and TOYOBO)
- 10 dilutioned BSA (1 mg/ml)

Assemble the following reaction in a sterile microcentrifuge tube:

• MilliQ	8	µl
• DNA	2	µl
• Buffer (10x)	1.2	µl
• (10D BSA	1.2	µl)
• Enzyme(s)	0.5	µl
o Total	13	µl

Incubate at 37 c for >1 hr

Enzyme digestion (purification)

Materials

- Restriction enzyme
- Buffer (NEB, TaKaRa, Roche and TOYOBO)
- 10D BSA(1 mg/ml)

Assemble the following reaction in a sterile microcentrifuge tube:

• MilliQ	if necessary	
• DNA	20	µl
• Buffer (10x)	2.5	µl
• (10D BSA	2.5	µl)
• Enzyme(s)	2	µl
o Total	27	µl

Incubate at 37 c for >2 hr

PCR 1 (iproof)

Materials

- Commercial PCR kit (iproof) proof-reading
- Loading buffer (0.5xTAE)
- 1.0 % agarose gel
- Gene Ruler DNA Ladder Mix (Fermentas; #SM0331)

Assemble the following reaction in a sterile microcentrifuge tube:

- 37 µl of MilliQ to a final volume of 50 µl
- 10 µl of 5 × reaction buffer
- 1 µl of dNTP mix (10 mM each)
- 0.5 µl of i-proof DNA polymerase
- 1 µl (10 - 50 ng) of dsDNA template
- 1 µl (10 pmol) of forward (F) primer
- 1 µl (10 pmol) of reverse (R) primer

- Segment 1; cycle 1
  - 95 c 3 min
- Segment 2; cycle 18
  - 95 c 10 sec
  - 58 c 30 sec
  - 68 c 1 min
- Segment 3; cycle 1
  - 68 c 5 min
- Segment 3; cycle 1
  - 22 c 1 min

* For example, a 5-kb plasmid requires 2.5 minutes at 68 c per cycle.

PCR 2 (KOD)

Materials

- Commercial PCR kit (KOD) proof-reading
- Loading buffer (0.5xTAE)
- 1.0 % agarose gel
- Gene Ruler DNA Ladder Mix (Fermentas; #SM0331)

Assemble the following reaction in a sterile microcentrifuge tube:

- 30 µl of MilliQ to a final volume of 50 µl
- 5 µl of 10 x reaction buffer
- 5 µl of dNTP mix (2 mM each)
- 6 µl of $MgSo_4$
- 1 µl of KOD Taq
- 1 µl (10 - 50 ng) of dsDNA template
- 1.5 µl (10 pmol) of forward (F) primer
- 1.5 µl (10 pmol) of reverse (R) primer

- Segment 1; cycle 1
  - 95 c 3 min
- Segment 2; cycle 30
  - 95 c 20 sec
  - 58 c 20 sec
  - 71 c 30 sec
- Segment 3; cycle 1
  - 22 c 1 min

* For example, a 5-kb plasmid requires 2.5 minutes at 71 c per cycle.

Direct PCR for KO check (modified Tetsuya method)

Template prep from HL5

1. Culture cells in 24-well plate
2. Take 100-200 µl cells into PCR tube
3. Ctf 2,000-3,000 rpm for 2 min
4. Wash with MilliQ or KK2
5. Ctf 5000 rpm for 1 min
6. Resuspend cell pellet in 20 µl of ProK solution
7. 56 c for 45 min (Thermal cycler)
8. 95 c for 10 min (Thermal cycler)
9. Use 2 µl as PCR template

Template prep from plaque

1. Spread cells on SM agar plate with Ka
2. Pick the cells using yellow tip (as bacteria pick) and mix into 20 µl of ProK solution (in PCR tube)
3. 56 c for 45 min (Thermal cycler)
4. 95 c for 10 min (Thermal cycler)
5. Use 2 µl as a PCR template

- After heat up steps, genome DNA solution can be kept in freezer (-80 c, but not before heat up.)

ProK solution (per one sample)

• 10 x PCR buffer (minus MgCl2)	2.0	µl
• 25 mM MgCl2	1.4	µl
• 10 % NP-40	1.0	µl
• 10 mg/ml ProK	0.1	µl
• MilliQ	15.5	µl

(*) If $MgCl_2$ is contained in PCR buffer, you do not need to add it.

PCR reaction

- Use 2 µl templates in 10 µl PCR reaction
- Using ExTaq (TaKaRa) is strongly recommended
- This method can amplify 3-4 kb products
- Good elongation temperature is 65 c

Recommended PCR condition for 3 kb

94 c 2 min

(30-32 cycle)

94 c 15 sec

52 c 20 sec

65 c 3 min 30 sec

Synergy experiment: Cell labeling

To label cells with CellTracker (Cat. No. PA-3011; Solvent for stock: DMSO), place 1 ml of the cell suspension in a 1.5-ml microcentrifuge tube and add 2 µl of the 10 mM CellTracker stock solution (1:500-1,000 dilution). Wrap tube with tinfoil and mount horizontally on a shaker (160 rpm) at 22 c for 45 min. Cells were washed twice in KK2 by pelleting the cells (5 sec at 20,000 g of microcentrifuge) and resuspending them in KK2 buffer. Resuspend cells after final wash in 1 ml of KK2 (cell density 1 × 10^7 cells/ml). A minority (10%) of labeled WT or *mrfA*- cells were mixed with unlabeled cells. Cells (labeled vursus unlabeled) were allowed to develop on water agar until slug stage. Slugs were lift up and observed by fluorescent microscopy.

DIF-1 induction: MrfA nuclear localisation, C-terminal fragment localisation change

In the assay cells are starved in shaken suspension for 4 hrs and then are induced with DIF-1 at 100 nM. Cells were collected by ctf and resuspending them in 1xSDS sample buffer. Samples were boiled at >100 c for 8 min.

Materials

- DIF-1 (final 100 nM)
- EtOH (absolute)
- Flask (20 ml)
- c-myc antibody (previously shown)
- FLAG antibody (SIGMA)
- Electrophoresis set (previously shown)
- Western transfer set (previously shown)

Western transfer; DIF-1 induction (20 March 2012)

- $5.0x10^6$ cells in 200 µl of 1xSDS sample buffer
- 19/3/12 sample preparation
- 20 dilution sample loaded

1. N-terminal c-myc, C-terminal FLAG double Tag MrfA FL (FL)
2. N-terminal c-myc, C-terminal FLAG double Tag MrfA Δ TM (TMd)
3. Non-Transformed cells (*mrfA*-); negative control (N)

- D; DIF-1 added
- E; EtOH (Absolute) added
- D5, 15; after 5 or 15 min of induction with DIF-1
- E5, 15; after 5, 15 min of induction with EtOH

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Time	M	0	D5	D15	E5	E15	M	0	D5	D15	E5	E15
Name	-	FL	FL	FL	FL	FL	-	TMd	TMd	TMd	TMd	TMd
Ab	0.1 µl in 4,000 (40,000 D) c-myc											

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Time	M	0	D5	D15	E5	E15	M	0	D5	D15	E5	E15
Name	-	FL	FL	FL	FL	FL	-	TMd	TMd	TMd	TMd	TMd
Ab	0.125 µl in 4,000 FLAG (SIGMA)											

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Time	M	0	D5	D15	E5	E15	M	0	D5	D15	E5	E15
Name	-	N	N	N	N	N	-	N	N	N	N	N
Ab	c-myc						FLAG					

MrfA processing change during development; time-course

Vegetative cells were collected by ctf (Do not use KK2; keep vegetative state) and resuspending them in 1xSDS sample buffer. Cells were allowed to develop on HA filter until desired stage and resuspending them in 1xSDS sample buffer. Samples were boiled at >100 c for 8 min. Adjust concentration of each cell lysate by SDS-PAGE and Colloidal Blue Staining.

Materials

- MF- membrane filters; 0.45 μm HA (CST No. HAWP04700, MILLIPORE)
- c-myc antibody (previously shown)
- FLAG antibody (SIGMA)
- Electrophoresis set (previously shown)
- Western transfer set (previously shown)

Western transfer; 27 March 2012

- $5.0x10^6$ cells in 200 μl of 1xSDS sample buffer
- N-terminal c-myc, C-terminal FLAG double Tag MrfA FL (FL)
- N-terminal c-myc, C-terminal FLAG double Tag MrfA ΔTM (TMd)
- Non-Transformed cells (*mrfA*-); negative control (N)
- 15,13/3/12 sample preparation
- Vegetative cells, 4 hrs; 20 dilution sample loaded
- Stream ; 5 dilution sample loaded
- Mound, slug ; 10 dilution sample loaded

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Strain	M	Veg	4	St	M	S	M	Veg	4	St	M	S
Name	-	FL	FL	FL	FL	FL	-	TMd	TMd	TMd	TMd	TMd
Ab	0.1 μl in 4,000 (40,000D) c-myc											

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Strain	M	Veg	4	St	M	S	M	Veg	4	St	M	S
Name	-	FL	FL	FL	FL	FL	-	TMd	TMd	TMd	TMd	TMd
Ab	0.125 μl in 4,000 FLAG (SIGMA)											

	1	2	3	4	5	6	7	8	9	10	11	12
Protein	7.5	15	15	15	15	15	7.5	15	15	15	15	15
Strain	M	Veg	4	St	M	S	M	Veg	4	St	M	S
Name	-	N	N	N	N	N	-	N	N	N	N	N
Ab	c-myc						FLAG					

Edman degradation; blotting & staining, destaining

1. Pre-wetting PVDF for 30 sec in absolute MtOH
2. Rinse PVDF briefly in Milli-Q
3. Soak PVDF in transfer buffer for 5-10 min
4. Transfer samples to PVDF using NuPAGE transfer buffer at 30 V for 2 hrs
5. Wash twice with Milli-Q
6. Stain with CBB staining solution at RT for 1 min
7. Wash twice with Milli-Q
8. Destain with destaining solution three times, each 5 min interval
9. Wash with Milli-Q
10. Take image of blot for reference
11. Excise required bands carefully and transfer to new 9 cm dish
12. Wash with Milli-Q extensively at least 10 times, each 10 min interval
13. Air dry blot at RT on filter for appropriate time
14. Bands place in marked Eppendorf tube

CBB staining solution

•	Coomassie R-250	0.4	g (0.1 %)
•	Methanol	200	ml (50 %)
•	Acetic acid	4	ml (1 %)
•	DW	196	ml
o	Total	400	ml

Destaining solution

•	Methanol	200	ml (40 %)
•	Acetic acid	50	ml (10 %)
•	DW	250	ml
o	Total	500	ml

Materials

NuPAGE MES/SDS buffer (1 liter);

•	MES	195.2	g (1 M)
•	Tris base	121.2	g (1 M)
•	SDS	20	g (69.3 mM)
•	EDTA free acid	6.0	g (20.5 mM)

Ultrapure water to 1.0 liter final concentration

1X buffer should be pH 7.3 (do not adjust with acid or base).

NuPAGE transfer buffer (20 % MtOH)

NuPAGE® Novex® Bis-Tris 4-12% Gel

ImageJ processing

Images were compiled and analyzed using in ImageJ. To prepare Figures for publication, Crop tool was used to magnify region of interest in some images, and brightness and contrast were adjusted for easy visibility. No other manipulations were performed.

1. Crop images (30Hx30W), Image > Crop
2. Image > Colour > Channels tool
3. More in Channels tool > Split Channels
4. More in Channels tool >select appropriate colour against channels
5. More in Channels tool > Merge Channels
6. Select appropriate colour in Colour Merge tool
7. Untick all of check boxes; Create composite, Keep source images and Ignore source LUTs
8. File > Save as > Tiff
9. More in Channels tool > Split Channels
10. More in Channels tool > select appropriate colour against channels
11. File > Save as > Tiff

References

Altabef, M., Clarke, J. D. W., Tickle, C. (1997). Dorso-ventral ectodermal compartments and origin of apical ectodermal ridge in developing chick limb. *Development* 124, 4547–4556.

Berks, M. and Kay, R. R. (1990). Combinatorial control of cell differentiation by cAMP and DIF-1 during development of *Dictyostelium discoideum. Development* 110, 977-984.

Brown, M. S., Ye, J., Rawson, R. B. and Goldstein, J. L. (2000). Regulated intramembrane proteolysis: a control mechanism conserved from bacteria to humans. *Cell* 100, 391-8.

Ceccarelli, A., Mahbubani, H. and Williams, J. G. (1991). Positively and negatively acting signals regulating stalk cell and anterior-like cell differentiation in *Dictyostelium. Cell* 65, 983-989.

Mohanty, S., Jermyn, K. A., Early, A., Kawata, T., Aubry, L., Ceccarelli, A., Schaap, P., Williams, J. G. and Firtel, R. A. (1999). Evidence that the *Dictyostelium* Dd-STATa protein is a repressor that regulates commitment to stalk cell differentiation. *Development* 126, 3391-3405

Chu, S., DeRisi, J., Eisen, M., Mulholland, J., Botstein, D., Brown, P. O. and Herskowitz, I. (1998). The transcriptional program of sporulation in budding yeast. *Science* 282, 699-705.

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

Dormann D, Weijer CJ. 2003. Chemotactic cell movement during development. *Curr Opin Genet Dev* 13: 358 – 364.

Durston AJ. 1976. Tip formation is regulated by an inhibitory gradient in the *Dictyostelium discoideum* slug. *Nature* 263: 126–129.

Dormann, D., Siegert, F. and Weijer, C. J. (1996). Analysis of cell movement during the culmination phase of *Dictyostelium development. Development* 122, 761-769.

DA Knecht, WF Loomis. Antisense RNA inactivation of myosin heavy chain gene expression in *Dictyostelium discoideum. Science*, 1987 May 1987: Vol. 236 no. 4805 pp. 1081-1086

Early AE, Williams JG. 1988. A Dictyostelium prespore- specific gene is transcriptionally repressed by DIF *in vitro. Development* 103: 519–524.

Early, A. E., Gaskell, M. J., Traynor, D. and Williams, J. G. (1993). Two distinct populations of prestalk cells within the tip of the migratory *Dictyostelium* slug with differing fates at culmination. *Development* 118, 353-362.

Early A. E, T Abe, J Williams. Evidence for positional differentiation of prestalk cells and for a morphogenetic gradient in *Dictyostelium. Cell*, 1995

Emery, B., Agalliu, D., Cahoy, J. D., Watkins, T. A., Dugas, J. C., Mulinyawe, S. B., Ibrahim, A., Ligon, K. L., Rowitch, D. H. and Barres, B. A. (2009). Myelin gene regulatory factor is a critical transcriptional regulator required for CNS myelination. *Cell* 138, 172-85.

Eike, C., Schulz, Achim Dickmanns, Henning Urlaub, Andreas Schmitt, Martina Muhlenhoff, Katharina Stummeyer, David Schwarzer, Rita Gerardy-Schahn & Ralf Ficner. (2010). Crystal structure of an intramolecular chaperone mediating triple– β -helix folding. *Nature Structural & molecular biology* 17:210-216.

Fingerman, I. M., Sutphen, K., Montano, S. P., Georgiadis, M. M. and Vershon, A. K. (2004). Characterization of critical interactions between Ndt80 and MSE DNA defining a novel family of Ig-fold transcription factors. *Nucleic Acids* Res 32, 2947-56.

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

Fukuzawa, M., Araki, T., Adrian, I. and Williams, J. G. (2001). Tyrosine phosphorylation-independent nuclear translocation of a *Dictyostelium* STAT in response to DIF signaling. *Mol. Cell* 7, 779-788.

Fukuzawa, M., Zhukovskaya N. V., Yamada, Y., Araki T, Williams, J.G. (2006). Regulation of *Dictyostelium* prestalk-specific gene expression by a SHAQKY family MYB transcription factor. *Development* 133, 1715-1724.

Fosnaugh, K. L., Loomis, W. F. (1993). Enhancer regions responsible for temporal and cell-type-specific expression of a spore coat gene in Dictyostelium. *Dev. Biol.* 157, 38–48.

Gale NW, Dominguez MG, Noguera I, Pan L, Hughes V, et al. (2004). Haploinsufficiency of Delta-like 4 ligand results in embryonic lethality due to major defects in arterial and vascular development. *Proc. Natl. Acad. Sci.* USA 101:15949–54

Gaudet, P., Williams, J. G., Fey, P. and Chisholm, R. L. (2008). An anatomy ontology to represent biological knowledge in *Dictyostelium discoideum. BMC Genomics* 9, 130.

Green J. B. A., Smith J. C. (1990) Graded changes in dose of a *Xenopus* activin A homologue elicit stepwise transitions in embryonic cell fate. *Nature* 347, 391–39

Gerisch,G., Hagmann,J., Hirth,P., Rossier,C., Weinhart,U. and Westphal,M. (1985) *Cold Spring Harbor Symp. Quant. Biol.*, 50, 813-822.

Ginger, R. S., Drury, L., Baader, C., Zhukovskaya, N. V. and Williams, J. G. (1998). A novel *Dictyostelium* cell surface protein important for both sell adhesion and cell sorting. *Development* 125, 3343-3352

Glenn A. Maston, Sara K. Evans, and Michael R. Green. (2006). Transcriptional Regulatory Elements in the Human Genome. *Annu. Rev. Genomics Hum. Genet.* 2006. 7:29–59

Haze K, Yoshida H,Yanagi H,Yura T,Mori K. (1999). Mammalian transcription factor ATF6 is synthesized as a transmembrane protein and activated by proteolysis in response to endoplasmic reticulum stress. *Mol Biol Cell* 1999;10:3787–3799.

Headon and Overbeek, D.J. Headon, P.A. Overbeek. (1999) Involvement of a novel TNF receptor homolog in hair follicle development. *Nat. Genet.,* 22 (1999), pp. 370–374

Holstege, F.C. and Clevers, H. (2006). Transcription factor target practice. *Cell* 124: 21–23.

Huang, E. Y., Blagg, S. L., Keller, T., Katoh, M., Shaulsky, G. and Thompson, C. R. L. (2006). bZIP transcription factor interactions regulate DIF responses in *Dictyostelium. Development* 133, 449-458.

Insall, R., Nayler, O. and Kay, R. R. (1992). DIF-1 induces its own breakdown in *Dictyostelium. EMBO J.* 11, 2849-2854.

Ikemura H, Takagi H, Inouye M Z (1987). Requirement of Pro-sequence for the Production of Active Subtilisin E in Escherichia coli. *J Biol Chem* 262:7859-7864.

Jang, W., Kim, K.P., and Schwob, J.E. (2007). Nonintegrin laminin receptor precursor protein is expressed on olfactory stem and progenitor cells. *J. Comp. Neurol.* 502, 367–381.

Jermyn KA, Duffy KTI, Williams JG. 1989. A new anatomy of the prestalk zone in *Dictyostelium. Nature* 340: 144–146.

Jermyn, K. A. Traynor, D. and Williams, J. G. (1996). The initiation of basal disc formation in *Dictyostelium discoideum* is an early event in culmination.

Development 122, 753-760.

Jolly, E. R., Chin, C. S., Herskowitz, I. and Li, H. (2005). Genome-wide identification of the regulatory targets of a transcription factor using biochemical characterization and computational genomic analysis. *BMC Bioinformatics* 6, 275.

J.T. Bonner, D.S. Barkley, E.M. Hall, T.M. Konijn, J.W. Mason, G. O'Keefe, P.B. Wolfe. (1969) Acrasin, acrasinase, and the sensitivity to acrasin in *Dictyostelium discoideum. Developmental Biology*
Volume 20, Issue 1, July 1969, Pages 72–87

Kay RR, Jermyn KA. 1983. A possible morphogen controlling differentiation in *Dictyostelium. Nature* 303: 242 – 244.

Kay RR, Thompson CRL. 2001. Cross-induction of cell types in Dictyostelium: Evidence that DIF-1 is made by prespore cells. *Development* 128: 4959–4966.

Kay, R. R. and Thompson, C. R. (2009). Forming patterns in development without morphogen gradients: scattered differentiation and sorting out. *Cold Spring Harb Perspect Biol 1*, a001503.

Keller, T. and Thompson, C. R. (2008). Cell type specificity of a diffusible inducer is determined by a GATA family transcription factor. *Development* 135, 1635-45.

Leach, C. K., Ashworth, J. M. and Garrod, D. R. (1973). Cell sortingout during the differentiation of mixtures of metabolically distinct populations of *Dictyostelium discoideum. J. Embryol. Exp. Morphol.* 29, 647-661.

Kessin RH. *Dictyostelium*: evolution, cell biology, and the development of multicellularity. *Cambridge (UK): Cambridge University Press*; 2001.

Kessin RH, Gundersen GG, Zaydfudim V, Grimson M, Blanton RL. How cellular slime molds evade nematodes. *Proc Natl Acad Sci* USA 1996;93:4857-4861

Konijn, T. M., van de Meene, J. 0. C., Bonner, J. T, and Barkley, D. S. (1967). *Proc.*

Natl. Acad. Sci. USA 58, 1152-1154.

Kopan, R., and Ilagan, M.X. (2009). The canonical Notch signaling pathway: unfolding the activation mechanism. *Cell* 137, 216–233.

Kuspa, A. and Loomis, W. F. (1996). Ordered yeast artificial chromosome clones representing the *Dictyostelium discoideum* genome. *Proc. Natl. Acad. Sci.* USA 93, 5562-5566.

Lamoureux, J. S., Stuart, D., Tsang, R., Wu, C. and Glover, J. N. (2002). Structure of the sporulation-specific transcription factor Ndt80 bound to DNA. *EMBO J* 21, 5721-32.

Loomis, W. F. (1993). Lateral inhibition and pattern formation in *Dictyostelium. Curr. Topics Dev. Biol.* 28, 1–46.

Leigh Zawel, Jia Le Dai, Phillip Buckhaults, Shibin Zhou, Kenneth W Kinzler, Bert Vogelstein, Scott E Kern. (1998) Human Smad3 and Smad4 Are Sequence-Specific Transcription Activators. *Molecular cell.* Volume 1, Issue 4, March 1998, Pages 611–617

Malik S, Roeder RG. (2005). Dynamic regulation of polII transcription by the mammalian Mediator complex. *Trends Biochem. Sci.* 30:256–63

Maeda M, Haruyo S, Maruo T, Ogihara S, Iranfar N, Fuller D, Morio T, Urushihara H, Tanaka T, Loomis WF. 2003. Changing patterns of gene expression in prestalk cell subtypes of Dictyostelium recognised by in situ hybridisation with genes from microarray analyses. *Eukaryot Cell* 2: 627–637.

Manstein, D. J., Schuster, H. P., Morandini, P. and Hunt, D. M. (1995). Cloning vectors for the production of proteins in *Dictyostelium discoideum. Gene* 162, 129-134.

MORRIS, H. R., TAYLOR, G. W., MASENTO, M. S., JERMYN, K. A. AND KAY, R. R. (1987). Chemical-structure of the morphogen differentiation inducing factor from

Dictyostelium discoideum. *Nature* 328, 811-814.

Qi, Y., Cai, J., Wu, Y., Wu, R., Lee, J., Fu, H., Rao, M., Sussel, L., Rubenstein, J., and Qiu, M. (2001). Control of oligodendrocyte differentiation by the Nkx2.2 homeodomain transcription factor. *Development* 128, 2723–2733.

Morimoto, M., Liu, Z., Cheng, H.T., Winters, N., Bader, D., and Kopan, R. (2010). Canonical Notch signaling in the developing lung is required for determination of arterial smooth muscle cells and selection of Clara versus ciliated cell fate. *J. Cell Sci.* 123, 213–224.

Muler,K. and Gerisch,G. (1978) *Nature*, 274, 445-449.

Ohta, Y,, Hojo, H., Aimoto, S,, Kobajashi, T., Zhu, X., Jordan, F,, and Inouye, M. (1991). *Mol Microbioi* 5: 1507- 1510,

Pascale Gaudet, Jeffery G Williams, Petra Fey and Rex L Chisholm (2008). An anatomy ontology to represent biological knowledge in *Dictyostelium discoideum*. *BMC Genomics* 2008, 9:130

Pears, C. J. and Williams, J. G. (1988). Multiple copies of a G-rich element upstream of a cAMP-inducible *Dictyostelium* gene are necessary but not sufficient for efficient gene expression. *Nucl. Acids Res.* 16, 8467-8486.

Piepenburg O, Grimmer D, Williams PH, Smith JC. (2004). Activin redux: specification of mesodermal pattern in *Xenopus* by graded concentrations of endogenous activin B. *Development* 131:4977–86

Ptashne, M., and Gann, A. (1998). Imposing specificity by localization: mechanism and evolvability. *Curr. Biol.* 8, R812–R822.

Russel, S., Frand, A. R. and Ruvkun, G. (2011). Regulation of the *C. elegans* molt by *pqn-47*. *Dev Biol.* 360, 297-309.

RAPER, K. B. (1940). Pseudoplasmodium formation and organisation in

Dictyostelium discoideum. J. Elisha Mitchell *Scient. Soc.* 56, 241-282.

Robertson, Smeaton, T. C. & Durnford, R. (1972) A method for the extraction, separation and estimation of unconjugated. estrone estradiol-17 β and estradiol in plasma. *Steroids*, 20, 651.

Redmond L, Oh SR, Hicks C, Weinmaster G, Ghosh A. (2000). Nuclear Notch1 signaling and the regulation of dendritic development. *Nat. Neurosci.* 3:30–40
Sherwin P. Montano, Marie L. Cote, Ian Fingerman, Michael Pierce, Andrew K. Vershon, and Millie M. Georgiadis. (2002). Crystal structure of the DNA-binding domain from Ndt80, a transcriptional activator required for meiosis in yeast. *Proc. Natl. Acad. Sci. USA* 99, 14041–14046

Staropoli, J. F., C. McDermott, C. Martinat, B. Schulman, E. Demireva, and A. Abeliovich. (2003). Parkin is a component of an SCF-like ubiquitin ligase complex and protects postmitotic neurons from kainite excitotoxicity. *Neuron* 37:735–749.

Saito, T., Kato, A. and Kay, R.R. (2008). DIF-1 induces the basal disc of the *Dictyostelium* fruiting body. *Dev Biol.* 317, 2): 444-53.

Sen and Baltimore. (1986), Inducibility of K Immunoglobulin Enhancer-Binding Protein NFKB by a Posttranslational Mechanism. *Cell.* 47, 921-926

Serafimidis, I. and Kay, R. R. (2005). New prestalk and prespore inducing signals in *Dictyostelium. Dev. Biol.* 282, 432-441.

Schwarzer D, Stummeyer K, Gerardy-Schahn R, Muhlenhoff M. (2007) Characterization of a novel intramolecular chaperone domain conserved in endosialidases and other bacteriophage tail spike and fiber proteins. *J Biol Chem* 2007, 282:2821-2831.

SMITH, J. C , COOKE, J., GREEN, J. B. A., HOWES, G. AND SYMES, K. (1989). Inducing factors and the control of mesodermal pattern in *Xenopus laevis. Development* 107, Supplement 149-160.

Stolt CC, Rehberg S, Ader M, Lommes P, Riethmacher D, Schachner M, Bartsch U, Wegner M: Terminal differentiation of myelin-forming oligodendrocytes depends on the transcription factor Sox10. *Genes Dev* 2002, 16:165-170.

Schulz EC, Neumann P, Gerardy-Schahn R, Sheldrick GM, Ficner R (2010). Structure analysis of endosialidase NF at 0.98 A resolution. *Acta Crystallogr D Biol Crystallogr* 2010, 66:176-180.

Schulz EC, Dickmanns A, Urlaub H, Schmitt A, Muhlenhoff M, Stummeyer K, Schwarzer D, Gerardy-Schahn R, Ficner R (2010). Crystalstructure of an intramolecular chaperone mediating triple-beta-helix folding. *Nat Struct Mol Biol* 2010, 17:210-215.

Shearer, I. J., Purvis, K., Jenkin, G. & Haynes, N. B. (1972). Peripheral plasma progesterone and oestradiol-17 β levels before and after puberty in gilts. J. Reprod. Fert. 30, 347.

Stern, C. D., Canning, D. R. (1990). Origin of cells giving rise to mesoderm and endoderm in chick embryo. *Nature* 343, 273–275.

Takahashi, T., Suwabe, N., Dai, P., Yamamoto, M., Ishii, S. and Nakano, T. (2000). Inhibitory interaction of c-Myb and GATA-1 via transcriptional coactivator CBP. *Oncogene* 19, 134-140.

Traynor D, Kessin RH, Williams JG. 1992. Chemotactic sorting to cAMP in the multicellular stages of *Dictyostelium* development. *Proc Natl Acad Sci* 89: 8303–8307.

Thompson, C. R. L., Fu, Q., Buhay, C., Kay, R. R. and Shaulsky, G. (2004). A bZIP/bRLZ transcription factor required for DIF signaling in *Dictyostelium. Development* 131, 513-523.

Thompson, C. R. L. and Kay, R. R. (2000). The role of DIF-1 signaling in *Dictyostelium* development. *Mol. Cell* 6, 1509-1514.

Thomas Gregor, Koichi Fujimoto, Noritaka Masaki, Satoshi Sawai. (2010). The Onset of Collective Behavior in Social Amoebae. *Science* 328, 1021 (2010); DOI: 10.1126/science.1183415

Tong, Q., Tsai, J., Tan, G., Dalgin, G. and Hotamisligil, G. S. (2005). Interaction between GATA and the C/EBP family of transcription factors is critical in GATA-mediated suppression of adipocyte differentiation. *Mol. Cell. Biol.* 25, 706-715.

TYSON, J. J. ANDMURRAY, J. D. (1989). Cyclic AMP waves during aggregation of *Dictyostelium* amoebae. *Development* 106, 421-426.

Umeda T, Inouye K. 2004. Cell sorting by differential cell motility. A model for pattern formation in *Dictyostelium*. *J Theor Biol* 226: 215–224.

Vilar JM, Saiz L. (2005). DNA looping in gene regulation: from the assembly of macromolecular complexes to the control of transcriptional noise. *Curr. Opin. Genet. Dev.* 15:136–44

Watts, D. J. and Ashworth, J. M. (1970). Growth of myxamoebae of the cellular slime mold *Dictyostelium discoideum* in axenic culture. *Biochem. J.* 119, 171-174.

Williams, J. G., Duffy, K. T., Lane, D. P. et al. (1989). Origins of the prestalk-prespore pattern in Dictyostelium development. *Cell* 59, 1157–1163.

Xu, Q., Mellitzer, G., Robinson, V., Wilkinson, D. G. (1999). In vivo cell sorting in complementary segmental domains mediated by Eph receptors and ephrins. *Nature* 399, 267–271.

Yamada Y, Sakamoto S, Ogihara S, Maeda Y. (2005). Novel patterns of the gene expression regulation in the prestalk region along the antero-posterior axis during multi- cellular development of *Dictyostelium*. *Gene Expr Patt* 6: 63–68.

Yamada, Y., Nunez-Corcuera, B. and Williams, J. G. (2011). DIF-1 regulates *Dictyostelium* basal disc differentiation by inducing the nuclear accumulation of a

bZIP transcription factor. *Dev Biol* 354, 77-86.

Yu-Jen Chen and Masayori Inouye. (2008). The intramolecular chaperone-mediated protein folding. *Current opinion in Structural Biology*, 18:765-770.

Zawel, L., Dai, J.L., Buckhaults, P., Zhou, S., Kinzler, K.W., Vogelstein, B., and Kern, S.E. (1998). Human Smad3 and Smad4 are sequence-specific transcription activators. *Mol. Cell* 1: 611–617.

Zhukovskaya, N. V., Fukuzawa, M., Yamada, Y., Araki, T. and Williams, J. G. (2006). The *Dictyostelium* bZIP transcription factor DimB regulates prestalk-specific gene expression. *Development* 133, 439-48.

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