Pleiotropic Roles of a Ribosomal Protein in *Dictyostelium discoideum*

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**Abstract**

The cell cycle phase at starvation influences post-starvation differentiation and morphogenesis in *Dictyostelium discoideum*. We found that when expressed in *Saccharomyces cerevisiae*, a *D. discoideum* cDNA that encodes the ribosomal protein S4 (DdS4) rescues mutations in the cell cycle genes *cdc24*, *cdc42* and *bem1*. The products of these genes affect morphogenesis in yeast via a coordinated moulding of the cytoskeleton during bud site selection. *D. discoideum* cells that over- or under-expressed DdS4 did not show detectable changes in protein synthesis but displayed similar developmental aberrations whose intensity was graded with the extent of over- or under-expression. This suggested that DdS4 might influence morphogenesis via a stoichiometric effect – specifically, by taking part in a multimeric complex similar to the one involving Cdc24p, Cdc42p and Bem1p in yeast. In support of the hypothesis, the *S. cerevisiae* proteins Cdc24p, Cdc42p and Bem1p as well as their *D. discoideum* cognates could be co-precipitated with antibodies to DdS4. Computational analysis and mutational studies explained these findings: a C-terminal domain of DdS4 is the functional equivalent of an SH3 domain in the yeast scaffold protein Bem1p that is central to constructing the bud site selection complex. Thus in addition to being part of the ribosome, DdS4 has a second function, also as part of a multi-protein complex. We speculate that the existence of the second role can act as a safeguard against perturbations to ribosome function caused by spontaneous variations in DdS4 levels.

**Introduction**

The origin of heterogeneity within groups of cells that are (to begin with) identical in their genotype and phenotype is a fundamental issue in developmental biology. Mutational studies that explore the links between genes, proteins and phenotypes rarely go beyond the single gene-single protein-single trait framework. The social amoeba *Dictyostelium discoideum* (Dd) provides an experimentally tractable system that is both simple and sufficiently intricate to enable an exploration of multifunctional roles of proteins during development.

Under laboratory conditions, genetically identical amoebae that have been raised in a common environment come together and, via complex shape changes, construct a polarised motile structure, the slug, which is made up of two spatially patterned cell types [1]. The slug differentiates into a fruiting body consisting of viable spores and dead stalk cells [2]. Three sources of pre-aggregation bias can bear on post-aggregation cell fate: the nutritional status of a cell, the phase of the cell cycle and the level of cellular calcium [3,4,5]. Calcium levels vary in a cell cycle phase-dependent manner [6]. The present study was initiated with a view to exploring the cell cycle-calcium link further. The genetics of the cell cycle in the yeast *Saccharomyces cerevisiae* (Sc) has been studied intensively and we decided to take advantage of the fact that the sequences of some *S. cerevisiae* and *D. discoideum* genes and proteins are rather similar [7]. Cross-complementation can take place between them: for example the *Ddcdc2* gene of *D. discoideum* can rescue the *cdc28* mutant phenotype in *S. cerevisiae* and *Ddycdc2* can substitute for *yak1* in *S. cerevisiae* [8,9]. The other way round, the *clu1* gene of *S. cerevisiae* complements the *clu1* mutation in *D. discoideum* [10].

In yeast, the morphogenetic process of bud initiation is an essential event of the cell division cycle. Many inputs go into the process of bud site selection – in particular, for the symmetry-breaking steps that lead to bud initiation [11,12,13]. ScCdc24p, ScCdc42p and ScBem1p function in concert as part of a complex. ScBem1p acts as a scaffold protein and mobilises the concerted and location-specific activities of ScCdc42p (a GTPase) and ScCdc24p (a guanine-nucleotide exchange factor). We began by looking for a gene known to be important in regulating the yeast cell cycle that also had a ‘calcium link’. A search of the literature showed that the *cdc24* gene of yeast fulfilled both criteria [14]. The *cdc24* mutant shows a cell cycle arrest phenotype in that it is defective in bud formation at 37°C (but not at 30°C); and the post-
Start phase cell cycle arrest that it exhibits is accompanied by a large influx of calcium [14]. With this information in hand we decided to find out whether both defects could be overcome by transforming cdc24-4 yeast cells with DNA from D. discoideum. We first describe how a D. discoideum gene capable of substituting for cdc24 and other cell cycle mutants in S. cerevisiae was identified and characterised. An account of the unexpected consequences of over- or under-expressing the gene in D. discoideum follows next. The outcome led to a hypothesis regarding the manner in which the relevant gene products might function. Experiments were carried out to test the hypothesis, initially in S. cerevisiae and then in D. discoideum.

Results

cDNA encoding the D. discoideum S4 gene suppresses specific cell cycle mutations in S. cerevisiae

We looked for complementation in an attempt to find functional equivalents of cell cycle genes in D. discoideum. Multiple S. cerevisiae mutants with defects in cell cycle progression were transformed with a vegetative phase cDNA library of D. discoideum (a kind gift from Dr. Catherine Pears). The D. discoideum cDNA library was constructed to function under the yeast alcohol dehydrogenase promoter. To begin with we used temperature-sensitive cell cycle mutants in cdc24, cdc42 and bem1. Since the mutants were temperature sensitive for growth at 37°C and uracil auxotrophs, the transformants were screened at 37°C on a uracil-deficient plate [15]. Of the several independent transformants that were obtained, one was followed up for further study since it rescued the cell cycle defect in the cdc24-4, cdc42-1 and bem1 mutants (Figure 1). The 870 bp cDNA that rescued cdc24-2, cdc42-1 and bem1 was found to encode for the D. discoideum ribosomal protein DdS4. The rescuing DNA constituted a full-length gene with no introns and was identical to the S4 sequence reported in the literature [16]. A messenger RNA encoding D. discoideum S4 was reported to be expressed selectively in prestalk cells [17]. However, that mRNA encodes a different ribosomal protein, S2; it does not encode S4 [18]. Another S4 mRNA, which is said to be specifically expressed during the growth-to-differentiation transition in D. discoideum, is transcribed from the mitochondrial genome, and its

Figure 1. Rescue of temperature sensitive cell cycle mutants with DdS4. Growth of S. cerevisiae strains cdc24-4, (B) cdc42-1, (C) bem1 transformed with control vector or DdS4 or ScS4 at 30°C and 37°C. (A) shows that ScS4 is unable to rescue the cell cycle defect in cdc24-4 (A). (D) Cell cycle phases monitored by FACS on propidium iodide-stained cells. Complemented strains show 2 peaks corresponding to 1 N and 2 N DNA content as compared to the mutants which show just the 2 N peak (indicative of cell cycle arrest).

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sequence does not overlap to any significant extent with that of DdS4 [19]. We examined whether the *S. cerevisiae* S4 (ScS4) was also able to rescue the yeast mutants mentioned above. Unlike DdS4, ScS4 failed to complement any of the three (Figure 1A). This suggested to us that the observed rescue was specific to DdS4.

**D. discoideum** S4 specifically suppresses cdc24-4, cdc42-1 and bem1 in *S. cerevisiae*

ScS4 is a component of the small subunit of the ribosome. It is present at a position where it can affect codon-anticodon interactions during translation and, thereby, translational fidelity. Mutations in S4 have been reported to result in non-specific increase in errors (missense, nonsense and frameshift) during translation in both *S. cerevisiae* and *Escherichia coli* [20,21]. Since the phenotypes of cdc24-4 and cdc42-1 and bem1 (disruptant) were all rescued by DdS4 expression, we wondered whether DdS4 was inducing nonspecific errors during translation in *S. cerevisiae*. We addressed this concern by testing the effect of DdS4 expression on the phenotypes of eight independent mutations comprising both missense and nonsense categories and located on different linkage groups in *S. cerevisiae*. None of the eight mutations were suppressed by DdS4 (Table 1), suggesting that the rescue of the three cell cycle mutants we observed was a specific effect. These results pointed to an as yet unknown function for DdS4.

DdS4 can form a complex with proteins that regulate bud formation in *S. cerevisiae*

DdS4 had been picked up on the basis of its ability to rescue the *S. cerevisiae* cell cycle mutants cdc24-4, cdc42-1 and bem1, which influence cytoskeletal polarisation and depolarisation at specific phases of the cell cycle (Figure 1). Polarity establishment requires that the small GTPase Cdc42p, its exchange factor Cdc24p, and a scaffold protein, Bem1p function co-ordinately as part of a complex. Subsequently, Cdc42p recruits a number of effectors, among them a kinase, Cla4p, which phosphorylates Cdc42p and renders it inactive. It is believed that ScCdc24p functions via a complex formed by ScBem1p, ScCla4p, ScCdc24p, and ScCdc42p [22]. Given the ability of DdS4 to rescue the cdc24-4, cdc42-1 and bem1 cell cycle mutants, we wondered whether DdS4 could substitute for one or the other member of the complex formed by ScCdc42p, ScCdc24p and ScBem1p.

![Image](image)

**Table 1.** DdS4 does not affect translational fidelity.

| Marker | Nature | Error rate/cell |
|--------|--------|-----------------|
| his3-11 | nonsense | <10^{-4} |
| trp1-1 | ? | 1.5x10^{-3} |
| lys2 | missense | <10^{-4} |
| leu2-3 | nonsense | <10^{-4} |
| cdc7-1 | missense | <10^{-4} |
| cdc31-1 | missense | <10^{-4} |
| cdc36-16 | nonsense | <10^{-4} |
| esp1 | missense | <10^{-4} |
| cdc24-4 | 1 |

Rescue of *S. cerevisiae* cdc24-4 by *D. discoideum* S4 is not due to non-specific translational error. Eight different *S. cerevisiae* strains (kindly made available by Dr. U. Surana) harbouring the mutant alleles shown were transformed with the *E. coli* ADH: DdS4 plasmid. The transformants were analysed for reversion (10^6 transformants for each allele).

We tested this hypothesis by using polyclonal anti-DdS4 antibody on whole cell lysates from *S. cerevisiae* cells that expressed DdS4 along with GST-tagged ScCdc24p, ScCdc42p, ScBem1p and ScCla4p proteins. Anti-DdS4 antibody was able to immunoprecipitate the yeast proteins ScCdc24p, ScCdc42p, ScBem1p and ScCla4p (Figure 2A & Figure S2B, I, II, III). In the case of ScCdc42p and ScCdc24p, this was based on DdS4 interacting separately with each, as shown by GST-pull down assays using either GST-tagged Cdc42p or Cdc24p (Figure 2B). Endogenous ScCdc42p and ScCdc24p in yeast lysates could also be immunoprecipitated by antibodies against DdS4 (Figure 2C & 2D). Anti-DdS4 antibodies were used to pull down proteins from *D. discoideum* cell lysates; the immunoprecipitate could be recognised by anti-human Cdc42 and anti-human Cdc24 antibodies (Figure 2C & 2D). Finally after immunoprecipitation of *D. discoideum* cell lysates with anti-DdS4 antibody we could detect the presence of the RacGEF GscDD (Figure 2C); DdRacGEF proteins are the putative equivalents of ScCdc42p [23]. We went on to delineate the features of DdS4 that enabled it to specifically interact with the three cell cycle proteins in *S. cerevisiae* and *D. discoideum*.

A shared SH3 domain between ScBem1p and DdS4 has functional implications

In principle, DdS4 could participate in the yeast bud site selection complex by acting like a scaffold protein – for example, like ScBem1p, whose over-expression rescues the *Sccdc24* and *Sccdc42* mutant phenotypes [22,24]. Since the *bem1* knockout in *S. cerevisiae* is lethal (Dr. D. Johnson, personal communication) [25], the following strategy was devised to test the possibility. A haploid *S. cerevisiae* strain harbouring a plasmid encoding either ScBem1p or DdS4 was used to generate a knockout of *bem1* at the chromosomal locus. The reasoning was that if DdS4 is able to substitute for *bem1* it would rescue the lethality due to absence of *bem1*. The expression of DdS4 was indeed able to restore growth ability to the otherwise lethal *bem1Δ−/−* strain (ΔScBem1) as, expectedly, was *ScBem1* (data not shown) (Figure 3A). Anti-DdS4 antibody was able to pull down endogenous Cdc42p and Cdc24p in *S. cerevisiae* cells lacking Bem1p (Figure 2C).

The genetic complementation and pull-down studies with yeast led us to ask if there could be a structural basis for the functional similarity between DdS4 and ScBem1p. Using hidden Markov models of protein domain families we carried out an analysis of the amino acid sequence of ScBem1p and DdS4 multi-domain proteins (Figure S1A) [26]. The C-terminal region of DdS4 comprising of the KOW motif is predicted to lie within the β3.4.5 superfamily which corresponds to the all β-SH3-like translation protein domain folds (Figure S1A). We further analysed the C-terminal region of DdS4 to explore the extent to which it can mimic the role of SH3 domains in ScBem1p. Secondary structure prediction for the C-terminal region of DdS4 (150–267) showed predominantly extended β-sheets (Figure S1B). Residues 246–260 were predicted to be helical. The structural similarity of the predicted C-terminal portion of DdS4 to an SH3 domain was not obvious at the amino acid sequence level. The overall topology of the secondary structure was conserved between the domains of these two proteins based on fold recognition and modelling of the C-terminal region of DdS4 (Figure S1C). When compared with the SH3 domains from ScBem1p, a model of the DdS4 C-terminus resembled the second SH3 domain of ScBem1p more than it does the first (28.7% and 5.2% similarity respectively as obtained using Superpose [27]); the two SH3 domains in ScBem1p are considerably divergent in sequence. Multiple sequence alignment of close homologues of ScBem1p showed that the
second SH3 domain and the following C-terminal region were well conserved. No other D. discoideum protein was identified that resembled ScBem1p in sequence, though the search did pick up other SH3 domain-containing proteins that resembled ScBem1p weakly (data not shown).

Figure 4 shows the predicted model structure of the second SH3 domain of ScBem1p and C-terminus of DdS4. All the residues conserved in Bem1p are labelled on the DdS4 model. Region 178–235 of DdS4 aligns with both the SH3 domains and defines the structural core of the SH3-like region in DdS4. To test if the predicted structural core SH3-like region of DdS4 was functionally important we carried out a deletion analysis. If the DdS4 structural core SH3 domain with similarities to Bem1p was functionally relevant, its deletion should abolish the observed ability of DdS4 to rescue the cell cycle mutants. Deletion of amino acids 178–235 at the C terminal end of DdS4 failed to rescue the temperature sensitive phenotype of cdc24-4, cdc42-1 and bem1 mutants (Figure 3B & 3C), indicating a functional role for this amino acid residue which is conserved in all higher eukaryotes starting from D. discoideum but is absent in S. cerevisiae. We next examined the possible roles of DdS4 in D. discoideum development.

DdS4 expression and localization in D. discoideum

The DdS4 gene, which was isolated from a vegetative phase D. discoideum (AX2) cDNA library, showed a high level of expression during the growth phase (Figure 5A). DdS4 genomic DNA 1 Kb upstream of the start codon was cloned in-frame to a GFP reporter and used to transform cells (DdS4prm:GFP). Growth phase transformants were strongly fluorescent (Figure 5B I). The PSORT software program which predicts subcellular protein localization [29] assigned a cytoplasmic localisation (p = 76%) to the DdS4 protein. Subcellular fractionation revealed that DdS4 was also associated with the TritonX-100 soluble and insoluble cytoskeletal fraction (Figure S2C).

Next we looked at the expression profile of DdS4 during D. discoideum development. RT-PCR analysis showed that the expression of DdS4 declined during post-starvation development and increased again later in the slug (Figure 5A). DdS4prm:GFP cells showed weak GFP expression during aggregation and the spatial pattern became restricted to the posterior prespore region at the tipped mound stage and beyond; fluorescent spores (but not stalk cells) were observed subsequently (Figure 5B II, III, IV).
*in situ* hybridization confirmed this prespore and spore specific expression of *DdS4* (Figure S2A). The developmental profile of the DdS4 protein mirrored that of the transcript (Figure 5A). Western blot analysis using DdS4 specific antibody on disaggregated cells from slugs confirmed the predominantly prespore localization of DdS4 (Figure 5C I & Figure S2B - I & II; prespore-specific down-regulation of DdS4 resulted in fruiting bodies with fewer and smaller than normal spores; Figure 5C II and Table 2).

Similar developmental aberrations are seen irrespective of whether DdS4 is up- or down-regulated under a constitutive promoter

In order to understand the role of DdS4 in *D. discoideum* development, we attempted to knock out the S4 gene by homologous recombination. Numerous attempts to do so failed. We reasoned that a total lack of DdS4 function could compromise ribosomal function sufficiently to affect cellular viability. To circumvent this problem, we tried to modify the level of DdS4 expression without eliminating it entirely.

Towards this end DdS4 cDNA was cloned in antisense orientation under the *D. discoideum* actin15 promoter. Since DdS4 was expressed during the vegetative as well as the developmental phase, we choose the actin 15 promoter which is known to be expressed during both these stages [30]. Relative to controls (i.e. empty vector-containing transformants), the antisense construct (AX2:A15S4) caused a lowering in both transcript and protein levels (Figure 6A, for control see Figure 5A). The AX2:A15S4 cells divided faster than the controls and were smaller (Figure 6B and Table 3 & Table 4). They aggregated over a similar time-course (8–10 hours) as
controls; however, the aggregation streams fragmented into small aggregates that proceeded to form slugs and, eventually, erect undifferentiated finger-like structures that occasionally had a tiny spore mass at the top (Figure 6C & 6D and Table 5). The spores were spherical, not elliptical as normally, and of lowered viability (Figure 6D and Table 2). Next we sought to see the effects of \textit{DdS4} overexpression. We cloned the \textit{DdS4} cDNA in the sense orientation, again under the Actin 15 promoter, and confirmed that 'sene' transformants (AX2:A15S4) had increased levels of the \textit{DdS4} transcript and \textit{DdS4} protein at all developmental stages (Figure 6A, for control see Figure 5A). To our surprise, the \textit{DdS4} over-expressors had phenotypes strikingly similar to the antisense driven under-expressors (Figure 6B–D and Tables 2, 3, 4, 5).

Ribosomal function remains largely unperturbed when \textit{DdS4} is up- or down-regulated

Our analysis in \textit{S. cerevisiae} indicated that the \textit{DdS4}-induced rescue of mutant phenotypes was unlikely to be due to aberrations in ribosomal function. We wanted to verify that this was true also in the case of \textit{D. discoideum}. We used polyribosomal profiling as a means of measuring overall translational efficiency [31]. Sucrose density gradient centrifugation of stalled ribosome complexes (harbouring peptidyl tRNAs) resolves them into fractions corresponding to

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**Figure 4. Structural model of the second SH3 domain of \textit{S. cerevisiae} Bem1 and the C-terminal region of \textit{D. discoideum} S4.** (A) The structures have been color-coded based on the extent of conservation of residues amongst their homologs. Blue depicts the most conserved region while red represents the least conserved regions (annotation obtained from ConSurf) [76]. Residues conserved amongst Bem1 homologs (residues lying in blue region) are labeled on structurally equivalent regions on the S4 C-terminal modeled structure. Note that not all the labeled residues are conserved amongst the S4 homologs (green, blue color). Many of the labeled residues lie in variable regions (orange, yellow, red colors). (B) Sequence alignment between the C-terminal region of Dds4 and the second SH3 domain of ScBem1 for which above modelled structures were generated. The secondary structural elements predicted for both the sequences are highlighted in red (helix), green (beta sheet/strand) and grey (coil) colours respectively. Residue positions in the alignment which are identical or conservatively substituted are highlighted in yellow and white for DdS4 and ScBem1 respectively. The black outlined box highlights the conserved Asn in the C-terminus which has been implicated in playing an important role in the functioning of the proteins.

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monosomes (70S ribosomes) and polysomes (disomes, trisomes, tetrasomes, etc). The monosome peak corresponds to both the free and mRNA-bound ribosomes, and the polysome peak corresponds to the mRNA bound ribosomes [31]. Polyribosome isolation [32] was carried out from growing as well as starved cells. Upon density gradient centrifugation, the expected profile in control consists of a peak corresponding to monosomes followed by several peaks corresponding to polysomes. Polysome profiles of all three types (Control, AX2:A15S4+ and AX2:A15S4−) resembled each other sufficiently for us to conclude that the effects of over- or under-expressing DdS4 were unlikely to be caused by changes in ribosomal function at least with regard to gross translational efficiency (Figure 7A). We also carried out western blot analysis of the various fractions obtained after density gradient centrifugation to check where DdS4 primarily resides. We found equal representation of the DdS4 protein in the 40S, 60S and monosomes and the polysomes fraction when compared across control, AX2:A15S4+ and AX2:A15S4− cells (Figure 7B). Interestingly, we also found DdS4 in the free protein fraction (not associated with the ribosomes). The free fraction isolated from AX2:A15S4+ contained more DdS4 as compared to controls while the fraction from AX2:A15S4− contained a negligible amount of the protein (Figure 7B & 7C). This suggests that when over- or under expressed, only the free pool of the DdS4 protein is perturbed and the pool dedicated for ribosomes remains unaltered. We also carried out a S35 pulse chase experiment as an index of overall protein synthesis rates. Under conditions of DdS4 over- and under-expression, AX2, AX2:A15S4+ and AX2:A15S4− cells showed no discernible differences as indicated by three most prominent bands seen on the autoradiograph (Figure S3), indicating that overall protein turnover rates were unchanged.

**Table 2.** The effect of DdS4 perturbation on spore formation and viability.

| Strain     | Spore forming efficiency (%) | Spore viability (%) |
|------------|------------------------------|---------------------|
| AX2        | 76.2±4.6                     | 93.5±3.69           |
| AX2:A15S4+ | 7.8±1.5                      | 5.3±0.75            |
| AX2:A15S4− | 9.2±0.5                      | 3.6±0.2             |
| AX2:pspS4  | 24±2.5                       | 23±2                |

Spore forming efficiency and viability of AX2, AX2:A15S4+ and AX2:A15S4−. (Estimated on the basis of counting about 5000 spores per experiment for each strain. Mean ± S.D., N = 4). doi:10.1371/journal.pone.0030644.t002

DdS4 over- and under-expressers display a dosage effect

As described earlier under- or over-expression of DdS4 had similar phenotypic consequences during vegetative growth and...
development. Besides, in both cases the effects on the phenotype were graded similarly. Independent clones of AX2:A15S4+ and AX2:A15S4- cells that were subjected to varying selection intensities showed differences in phenotype that were positively correlated with the concentration of antibiotic used (Figure 7D and Table 6). Three independent clones falling under each class (over- or under-expressor), labelled ‘low’, ‘medium’ and ‘high’, were monitored. ‘Low’ cells were designated with the suffix ‘l’ as AX2:A15S4+l and AX2:A15S4-l depending on whether they carried the DdS4 in the sense (+) or antisense (−) orientation; ‘medium’ and ‘high’ cells were designated similarly but with the suffixes m and h. ‘Low’ cells (AX2:A15S4+l and AX2:A15S4-l)
could grow on no more than 5 μg/ml of neomycin sulphate, ‘medium’ cells (AX2:A15S4+ and AX2:A15S4−) tolerated 10 μg/ml and ‘high’ cells (AX2:A15S4+ and AX2:A15S4−), up to 15 μg/ml. Western blots using antibodies to DdS4 confirmed that the levels of S4 protein in the low, medium and high classes differed as expected in both sense and antisense transformants (Figure 7E). During vegetative growth, the clones showed concentration-dependent differences in doubling times (Table 6). During development, all clones reproduced the two traits reported in the earlier section: aggregation streams broke up and aggregation territory sizes were significantly smaller than usual. The terminal phenotype of ‘low’ cells consisted of a misshapen spore mass atop a short stalk with a broad base; ‘medium’ cells formed short club-shaped terminal structures with thickened stalks and a misshapen spore mass; and the development of ‘high’ cells ended development as finger-like erect structures with no discernible sori (Figure 7D). Additionally, the classes differed with regard to spore formation efficiency and spore viability (Table 6).

The phenotypic effects seen by graded over and under expression of DdS4 may be because of the participation of DdS4 in a multi-protein complex. A simple application of the law of mass action shows that the imbalance caused by raising or lowering the concentration of any component of the complex can lead to similar consequences [33,34]. DdS4 is known to be a component of the ribosome, a multi-subunit RNA-protein complex; however, polyribosome profiles ruled out the possibility that what we were seeing was a consequence of ribosomal malfunction. These observations confirmed our hypothesis that effects produced by DdS4 over- or under-expression are caused by its participation in the actin cytoskeleton remodelling complex.

Budding in yeast involves a remodelling of the actin cytoskeleton [35]. Do the consequences of over- or under-expressing DdS4 in DdS4 being compromised? Eukaryotic ribosomal protein-encoding genes are known to have pleiotropic roles. They are associated with transcription, splicing, translation and DNA repair; their deficiency has been linked to developmental disorders in humans, fruit flies and plants [37,38,39]. In fish, ribosomal protein genes act as haplo-insufficient tumour suppressors and are likely to be involved in regulating normal development [40]. Interestingly, a knock-down mutation of the zebrafish S4 gene leads to developmental defects, predominantly of neurological origin [41]. S19 deficiency in zebrafish leads to hematopoietic and developmental abnormalities due to dysregulation of delta Np63 and p53 [39,42]. L13a, a ribosomal protein binds to the 3′-UTR of ceruloplasmin mRNA and inhibits its translation - but only after it has been phosphorylated and released from the ribosome [39]. Of particular interest to our study, RPL41, a ribosomal subunit protein, associates with several cytoskeleton components including tubulin β, Y and myosin IIA [43]. In the case of D. discoideum, it has been reported that S4 binds inositol 6-phosphate [16]. The implications remain unknown, but the finding suggests that DdS4 also has a role in the plasma membrane or in signal transduction.

DdS4 is rather different from ScS4 in sequence; the latter groups with other fungal S4 proteins (Figure S4A & S4B). Sequence analysis points to similar domain architectures; a largely conserved N-terminus region and significant variations in the C-terminal end (Figure S4A & S4B). Some residues are conserved between ScBem1p homologues and DdS4 but vary between DdS4 and other S4’s. These features explain why DdS4, but not ScS4, can substitute for the absence of one of the proteins in the bud site selection complex in yeast. Since DdS4 can compensate for the lack of ScCdc24p, ScCdc42p or ScBem1p in yeast, one possibility is that DdS4 could substitute for any of these proteins in the

**Table 3.** The effect of DdS4 perturbation on doubling time.

| Strain         | Doubling time (hrs.) |
|----------------|----------------------|
| AX2            | 5.5                  |
| AX2:A15S4+     | 3.9                  |
| AX2:A15S4−     | 4.3                  |

The mean doubling time for each of the strains was estimated from the exponential portion of the growth curves in Fig 2B (N = 3).

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**Table 4.** The effect of DdS4 perturbation on cell size.

| Strain         | Cell size (arbitrary units) |
|----------------|-----------------------------|
| AX2            | 0.194±0.01                  |
| AX2:A15S4+     | 0.102±0.02                  |
| AX2:A15S4−     | 0.105±0.03                  |

Comparison of diameters of growth phase amoebae of AX2, AX2:A15S4+ and AX2:A15S4− made by taking images at 10× magnification. An average of the cell lengths was measured transversely and longitudinally. 0.1 arbitrary unit equals 10 microns. Mean ± S.D. N = 3.

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**Table 5.** The effect of DdS4 perturbation on aggregate size.

| Initial cell density | No. of aggregates | AX2 | AX2:A15S4+ | AX2:A15S4− |
|----------------------|-------------------|-----|------------|------------|
| 2 × 10^5/cm^2        | 2340±43           | 9739±383 | 8740±1820  |
| 5 × 10^5/cm^2        | 2250±116          | 9739±374 | 9737±252   |
| 8 × 10^5/cm^2        | 2272±38           | 10500±2126 | 12082±26  |

Number of aggregates formed/20 cm^2 at the indicated cell densities by AX2, AX2:A15S4+ and AX2:A15S4−. Mean ± S.D. (Based on ~300 fruiting bodies (for AX2) and terminal structures (AX2:A15S4+ and AX2:A15S4−) counted in replicate plates; N = 4.)

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**Figure 8A** [36]. However, the staining was significantly more intense in the case of AX2 cells than with those of AX2:A15S4+ or AX2:A15S4− (Figure 8B).
Figure 7. AX2:A15 transformants show normal ribosomal function and display phenotypic variability. (A) Polysome profiles of AX2, AX2:A15S4\textsuperscript{+}, and AX2:A15S4\textsuperscript{−} (intermediate under-expressor of DdS4) cells. i) Free protein ii) 40S subunit iii) 60S subunit iv) Monosomes v) Polysomes. The analysis was repeated twice with independently isolated polysomes for each strain. (B) Fractions corresponding to different peaks in the polysome profile were collected and subjected to TCA precipitation. Equal amounts of protein from each fraction were run on a 12% SDS PAGE. Western blot analysis was carried out using anti-DdS4 antibody. In all panels the protein band in the lane below is the normalising control. (C) Quantitation of DdS4 in terms of net intensity of the free fraction has been represented in histograms. The band intensities of independent exposures of Western blots were measured using Quantity One analysis software from BioRad. p values were calculated using one-way ANOVA and were <0.05. (D) Terminal structures at low and high magnification ('low', 'medium' and 'high' over-expressors on the right).
Western blots showing expression levels of DdS4 in AX2, AX2:A15S4+ and AX2:A15S4- cells. The total protein taken for over-expressors is less than that taken for under-expressors so as to prevent saturation of the band intensities.

Table 6. The copy-number effect of DdS4 perturbation on spore formation, spore viability and doubling time.

|                  | AX2:A15S4+ (Over-expressors) | AX2:A15S4- (Under-expressors) |
|------------------|------------------------------|-------------------------------|
|                  | Low | Medium | High | Low | Medium | High |
| Spore forming efficiency | 11±0.2% | 8±1.5% | 5±2.5% | 15±0.8% | 9±0.5% | 4±0.2% |
| Spore viability    | 9±0.5% | 5±0.75% | 3±0.3% | 7±0.8% | 4±0.2% | 3±0.4% |
| Doubling time (hrs.) | 4.2±0.5 | 3.9±1.75 | 3.5±1.2 | 5.3±1.8 | 4.3±2 | 3.9±1.3 |

Quantitative differences amongst the DdS4 over- and under-expressors in terms of spore forming efficiency, spore viability (estimated on the basis of counting about 5000 spores per experiment for each strain. Mean ± S.D., N = 4) and doubling time (Mean ± S.D., N = 4).

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We note that the varied consequences of DdS4 under- or over-expression (on cell size, spore shape and multicellular morphology) could all be due to a defective cytoskeleton. How might DdS4 interact with ScCdc24 and ScCdc42? The presence of a domain similar to the SH3 domain in the C-terminus of Bem1p (Figure 4) may enable DdS4 to interact with Cdc42 in a manner analogous to ScBem1p. Sequence analysis also indicates that the probable mode of interaction between DdS4 and Cdc24 is via the SH3-like domain in the C-terminus (Figure S1D). In support of this, it turns out that D. discoideum sequences that resemble ScCdc24 (and contain CH, PH and RhoGEL domains) do give proline-rich stretches which could be potential sites for binding to an SH3 domain (Figure S1D).

Relative to normal development in AX2 cells, we observe a number of differences when DdS4 is under- or over-expressed. They include a reduction in cell size, faster cell division, smaller aggregates, aberrant-looking fruiting bodies, fewer, rounded and less viable spores. Each of the differences has been reported in some mutant of D. discoideum (see http://www.dictybase.org/db/cgi-bin/dictyBase/phenotype), but no mutant phenotype includes all of them. Similar to our findings (Figure 8A & 8B), the actin cytoskeleton is implicated in at least two cases. Pleiotropy or ‘moonlighting’ is a pervasive feature of D. discoideum, extensive cytoskeletal rearrangements are involved in morphogenesis [46]. On the basis of sequence similarity, D. discoideum has at least 16 Rac GTPases that resemble ScCdc42p and at least 10 proteins that appear to possess guanine nucleotide exchange activity that resemble ScCdc24p [47] (www.dictybase.org). Rac GTPases have been studied extensively in D. discoideum and at least 10 proteins that appear to possess guanine nucleotide exchange activity that resemble ScCdc24p and ScCdc42p are a consequence of DdS4 functioning as a scaffold protein in yeast. Being temperature-sensitive, Scdc24-4 and Scdc42-1 are likely to be missense mutations. Though present in the cell at the restrictive temperature, the corresponding proteins will be unable to function normally because most molecules have an inappropriate conformation. When over-expressed, a surrogate scaffold protein can bind the misfolded protein and permit a functional complex to form. Alternatively, the surrogate could help to efficiently mobilise any residual properly folded protein and so restore normal function.

The developmental defects caused by expressing DdS4 in antisense (AX2:A15S4−) or sense (AX2:A15S4+) orientations under a constitutive promoter were comparable and graded in parallel with the level of expression (Table 6). This finding strengthens the conjecture that DdS4 affects morphogenesis in D. discoideum by functioning as part of a multi-protein complex. Specifically, we were led to the balance hypothesis, which relates to a stoichiometry-related effect on the equilibrium concentration of the complex. The hypothesis is that when similar phenotypic defects result from under- or over-expression of a protein, the protein may be functioning as a component of a multimeric complex. In such a situation the imbalance caused by increasing or decreasing the concentration of a component of the complex can be similar and therefore the consequences can be similar too [33,34,45]. In our context, a change in the cellular level of total DdS4 in either direction has similar consequences because DdS4 functions as part of a multi-subunit protein complex that is analogous to the bud site selection complex in yeast.

Even though there is nothing like ‘bud site selection’ in D. discoideum, extensive cytoskeletal rearrangements are involved in morphogenesis [46]. On the basis of sequence similarity, D. discoideum has at least 16 Rac GTPases that resemble ScCdc42p and at least 10 proteins that appear to possess guanine nucleotide exchange activity that resemble ScCdc24p [47] (www.dictybase.org). Rac GTPases have been studied extensively in D. discoideum and play unique roles in regulating actin polymerisation [47,48,49]. RacB, in particular, is of interest since its loss recapitulates some of the phenotypes that we report in this study, namely aggregate break up and abnormal terminal structures [50].
accommodate substantial variations in DdS4 levels (morphogenesis is affected and sporulation efficiency is lowered in the transformants, but not all the way to zero; Table 2). The presence of a second role for the same protein means that its basal levels are higher than they would have been in its absence. The fact that variations in the level are more or less tolerated with regard to the second role means that the performance of the first role is buffered against mutationally- or environmentally-induced changes that might have been lethal otherwise. In our case, the second role acts as a built-in safeguard against the potentially lethal consequences of sub-optimal ribosomal activity that might be caused by spontaneous variations in DdS4 levels. This observation adds to the list of selective advantages for the evolution of multifunctionality in proteins [57].

Materials and Methods

Unless stated otherwise, all chemicals were purchased from HiMedia, India or Difco, USA and are of analytical grade.

Cell culture and development

Dictyostelium strains were grown on Phosphate buffer (15 mM KH2PO4, 2 mM K2PO4, pH 6.4) agar plates with Klebsiella aerogenes at 22°C. Neomycin resistant transformants were maintained with bacteria on PBA agar plates containing 12 μg/ml of neomycin. The number of viable spores was determined by harvesting all cells after 4 days of development, heating the cell suspension to 45°C for 5 minutes followed by treatment with 0.1% NP-40 for 10 minutes and counting the number of resistant cells that form plaques on bacterial lawns. For growth rate measurements, the DdS4 over- and under-expressors were grown with bacteria on agar plates containing appropriate antibiotic concentrations.

Molecular biology

The DdS4 gene was amplified from AX2 genomic DNA using primers, 5’ CAGGATCCAAAGATGGCTCGTCCA3’ and 5’TCTAGATTTATTTATTTAAGCAACG3’. The gene was then cloned in pACT15XPT1 vector (a kind gift from Dr. David Knecht) to get DdS4 both in sense and antisense orientations with respect to the Actin 15 promoter. The constructs were then electroporated into AX2 to obtain AX2:A15S4+ and AX2:A15S4−. For generating the promoter-GFP construct, the putative promoter was amplified from AX2 genomic DNA with primers, 5’ TTCTCGAGTACCCACACCCTAATGGTT3’ and 5’ AATCTAGATTTTTCTTTGGACCACGA3’ and sub cloned into pDRIVE vector (Qiagen TA cloning kit) and subsequently into pREMIGFP vector.

Figure 8. Cytoskeletal remodelling seen by actin-phalloidin staining. (A) Aggregating D. discoideum cells (AX2, AX2:A15S4+ and AX2:A15S4−). (I) Cells at 60 x magnification with images at the top taken with green filter; corresponding bright field images are in the panels below. (II) Image of a typical cell after 3-D stacking. (B) The histograms indicate the mean phalloidin fluorescence intensity (calculated per μm² of cells using Image J software). The p values were calculated using one-way ANOVA and were <0.05.

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Saccharomyces cerevisiae transformation was done by the Lithium acetate- Poly ethylene glycol method [58]. For complementation studies, DdS4 and ScS4 (using primers 5’ CGGATCCATGGCTCGTCCA3’ and 5’ CTTAGTTCTAGTTTTTACCAAGG3’) were cloned in pYES2. Temperature shift was used to synchronise cdc mutants of Saccharomyces cerevisiae. Briefly cells were grown to a density of 106/ml in YPD at 30°C under shaking conditions using an overnight grown culture as inoculum. The culture was then shifted to a shaker maintained at 37°C and incubated for a period of two and half hours to arrest the entire population in a cell cycle specific manner. The arrest was confirmed by FACS analysis. Cell cycle by FACS was carried out following the method described by Hutter and Eipel [59]. The 2ADH library obtained was amplified following the method described by Miller to obtain a titre of 1011 pfu/ml [60]. For translational fidelity assay, strains with different auxotrophic or cell cycle mutation either due to missense
and nonsense mutations were used. In brief, these were transformed with the plasmid encoding the ribosomal protein and the untransformed and the transformed cells were plated on auxotrophic media and maintained under selective conditions. The number of colonies appearing under each condition were analysed after 72 hours and this was used to calculate the translational error rate, a function inverse to translational fidelity, with the wild type error rate as a background control. 

Bem1 knockout in haploid S. cerevisiae was created by amplifying the Leu cassette from pRS405 using the primers 5’ GTTCTTCAC-TATATACCTAAACCTATGGAAGGCGTGAAGGATGAAAGGCTC-TGTGTAAGAACGGGATAATATATCTATCTTAATGAGAATTTGGT-AATGGTTCAGCCTATTTATTTATGAAATCTCA 3’. The wild type yeast was transformed with either DdS4 or yeast bem1 [amplified using primers 5’ ATGCGGATCCATGGTG-TGA Arenacgttcctgcagttcagctctgaa and 5’ GATCCCTGCAGGAAATATCGTG-GAC 3’ and cloned in pYES2 vector]. The untransformed yeast strain was the control. Colonies that grew on the minimal plate containing URA3 were chosen for transformation with the linear DNA (the PCR product). The colonies that grew on LEU2-URA3-plates at 30°C were selected. The clones are confirmed using the primers 5’ TGGCGAAGGGCCCCATTCAT 3’ and 5’ GATCCCTGCAGGAAATATCGTG-GAC 3’ and cloned in pYES2 vector). The untransformed yeast strain was the control. Colonies that grew on the minimal plate containing URA3 were chosen for transformation with the linear DNA (the PCR product). The colonies that grew on LEU2-URA3-plates at 30°C were selected. The clones are confirmed using the primers 5’ TGGCGAAGGGCCCCATTCAT 3’ and 5’ GATCCCTGCAGGAAATATCGTG-GAC 3’ and cloned in pYES2 vector). The untransformed yeast strain was the control. Colonies that grew on the minimal plate containing URA3 were chosen for transformation with the linear DNA (the PCR product). The colonies that grew on LEU2-URA3-plates at 30°C were selected. The clones are confirmed using the primers 5’ TGGCGAAGGGCCCCATTCAT 3’ and 5’ GATCCCTGCAGGAAATATCGTG-GAC 3’ and cloned in pYES2 vector). The untransformed yeast strain was the control. Colonies that grew on the minimal plate containing URA3 were chosen for transformation with the linear DNA (the PCR product). The colonies that grew on LEU2-URA3-plates at 30°C were selected. The clones are confirmed using the primers 5’ TGGCGAAGGGCCCCATTCAT 3’ and 5’ GATCCCTGCAGGAAATATCGTG-GAC 3’ and cloned in pYES2 vector).

Antibody generation and Western blot analysis

Full length coding region of DdS4 was cloned in pET16b and transformed into E. coli BL21. The His- protein was then used for generation of polyclonal antibodies in rabbit.

For Western blot analysis, cells were harvested at various developmental stages, washed, solubilized in lysis buffer [100 mM NaCl, 5 mM EDTA, 50 mM Tris pH 7.5, 1 mM protease inhibitor cocktail (Roche)], and freeze thawed 3–4 times. Equal amount of cellular proteins (as determined by Bradford assay) were loaded on a 12% polyacrylamide SDS gel and subsequently transferred to PVDF membrane. After blocking, the blots were incubated for 3 hrs with the primary anti-DdS4 antibody at 1:2000 dilution, washing 3 times with PBS-T, and incubating with secondary anti rabbit goat antibody (Millipore) at 1:2000 dilution. After washes, the bound antibody was detected with an ECL Western blotting kit (Perkin Elmer). For commercial antibodies, anti-CDC24 (MCF2/Dbl antibody, Cell Signaling Technology) and anti-CDC42 (Cell Signaling Technology) dilution used was 1:1000 and the buffer used was PBS-T.

E. coli BL21 over-expressing Cdc42p-GST, Cdc24p-GST, Bem1p-GST and Gla4p-GST proteins (the constructs were kind gift from Dr D. Lew) were harvested and washed with lysis buffer. After pelleting the cells the supernatant was used for interaction with GSH beads for 1 hr in a rocker at 4°C. For specific interaction, the beads were incubated with the supernatant containing the over-expressed protein. This was incubated on a rocker at 4°C for 2 hrs. The beads are then washed with interaction buffer [50 mM HEPES, 100 mM NaCl, 1 mM Triton X-1, 1 mM DTT, 5 mM EDTA, IX protease inhibitor Cocktail (Roche)] followed by a PBS wash. The beads are then boiled in the SDS loading dye or in loading dye without β-mercaptoethanol for blots where anti-CDC42 was used and loaded on a 12% SDS-PAGE. The commercial antibodies used for identifying DdCdc24 and DdCdc42 were raised against the human MCF2/Dbl which has ~69% similarity with full length ScCdc24p and the residues surrounding Lys135 of human Cdc42 respectively which is known to be crucial for the GTPase activity of Cdc42 in several species including S. cerevisiae.

Co-immunoprecipitation studies

For co-immunoprecipitation, the supernatants of the over-expressing clones upon lysis with glass beads [425–600 microns (Sigma Chemicals, USA)] were pooled. This cocktail was subjected to preclearing with 10 μg pre-immune serum IgG and Sepharose-protein G (Sigma Chemicals, USA) slurry on a rocker at 4°C for 3 hrs. DdS4 protein antibody was added and incubated overnight at 4°C on the rocker. Subsequently sepharose-protein G beads were added and allowed to interact for 2 hrs. The pellet was then loaded after boiling on SDS-PAGE.

Immunohistochemical staining

For actin phalloidin staining, AX2 cells were fixed with 4% paraformaldehyde for 30 mins at room temperature [61]. Washes with TBS-T (0.1% Triton-X in TBS) were followed by blocking with 5% milk for half an hour. Rhodamine-phalloidin staining (1:375 dilution) (Molecular Probes) at 37°C for 65 mins was carried out. Three washes with TBS-T were given and cells were mounted in vectamount (Vector Laboratories) on slides and viewed in confocal microscope.

DNA in situ Hybridisation

The DNA probe (1–3 μg) was labelled for 20 hrs by random priming by incorporation of dUTP-digoxigenin using the ‘DIG High prime’ labelling kit from Roche Molecular Biochemicals, Germany (cat. no. 1 585 606) according to the manufacturer’s instructions. Probes were purified by ethanol precipitation. The protocol used for hybridization, washing and color development was essentially as described by Escalante and Loomis (1995) with minor modifications. For separation of prestalk and prespore cells, slugs were harvested and cells were disaggregated in KK2 buffer containing 20 mM EDTA. A gradient of 70% Percoll (Sigma, USA) in KK2 buffer containing 20 mM EDTA was made in 15 ml Corex tube at 27,200 g for 40 mins. 1010 cells in 250 μl was layered on this gradient and spun at 17,400 g for 3 mins. The cells were recovered by washing them with KK2 buffer. For subcellular fractionation the procedure has been described previously [23].

Polyosomal assay

Amoebae were harvested from nutrient medium at ~5×106–107 cells/ml by centrifuging at 300 g for 3 mins and immediately chilled on ice. Cells were resuspended in buffer followed by addition of cycloheximide at a concentration of 150 μg/ml and allowed to incubate on ice for additional 5 mins. The cells are spun at 4000 rpm for 5 mins in cold. The cells are washed once with lysis buffer (50 mM HEPES pH 7.5, 5 mM MgCl2, 40 mM Magnesium acetate, 20 mM potassium phosphate, 0.5 mM DTT, 0.1 mg/ml cycloheximide, 5% sucrose and 0.4% NP-40) and subsequently the pellet is frozen in liquid nitrogen. 200 μl of lysis buffer and an equal volume of glass beads were added to the pellet. This is vortexed at full speed for 2 mins followed by incubation in ice for 5 mins. Repeat this thrice. Spin down the cells at 4000 rpm for 2 mins in cold. The concentration of RNA was determined spectrophotometrically and 20 units of RNA was layered onto 11 ml of 10–50% linear sucrose gradient. Centrifugation was carried out at 28000 rpm for 4 hrs at 4°C. Gradients were fractionated by upward displacement in a gradient fractionator equipped with a UV monitor.

For S35 labelling, 1×105 cells were spotted on filter paper and allowed to stand for 15 mins. The filter paper was then placed on...
50 microlitre droplets of 750 µCi label (Perkin Elmer) in an empty petridish and incubated for an hour. The filter paper was placed in an eppendorf and spun down to collect the cells. The pellet was resuspended in buffer without the label and frozen. This was time t = 0. For later time points, the cells in buffer were plated on SM/5 plates with bacteria and collected at times indicated.

Computational sequence analysis and structure prediction

Sequences of ScBem1p, DdS4, ScS4 and their related proteins were obtained from Uniprot [62]. Domain assignments based on functional similarity was obtained from Pfam [26] family database by doing a HMM based search in the protein family database. Structural domain assignments were obtained by using 3D-Jigzaw [63], an automated server for comparative modeling and Superfamily database system [64] which queries the sequence against SCOP [65] families and superfamilies. Subsequent to identification of the domain boundaries, sequence fragments encompassing the domain and variable flanking regions were analysed using 3 d Jura, a metaserver [66] which provides consensus based structure prediction. Such an approach has higher accuracy than individual structure prediction algorithms. From the server a consensus base secondary structure prediction was obtained. Using the server structural fold was predicted for the submitted sequence by different approaches such as ngenthreader [67], FUGUE [68]. Independently the sequences were also submitted to phyre [69] for fold prediction. An appropriate structural template was chosen based on consensus from highly significant and reliable hits obtained from different fold prediction methods. Structural modeling and modeled structure validation was done using modeler version 9 [70] and PROSA [71] respectively. In order to obtain functionally critical residues conserved across other homologues sequences CONSURF [72] was used. CONSURF takes up a structure as an input extracts its sequence and searches for its homologs (e-value below 0.0001; database uniprot). These homologous sequences are then aligned (MUSCLE [73]) and residue-wise conservation is obtained using Bayesian method. To obtain locally similar regions between ScBem1p and DdS4, MEME [74] was used. MEME detects conserved, statistically significant local patterns (motifs) amongst a group of sequences. In order to identify SH3 binding regions in DdCdc24 SH3 hunter [75] was used.

Supporting Information

Figure S1 Structural insights into DdS4 interactions. (A) Domain organization of ScBem1p and DdS4p. SCOP superfamilies were obtained from Superfamily.org database [77]. Definitions for SCOP ids are as follows: b.34.2 : All Beta protein, SH3 like barrel, SH3 domain; b.34.5 : All Beta, SH3-like barrel, Translation proteins SH3-like domain; d.189.1 : Alpha and beta proteins, PX domain; d.15.2 : Alpha and beta proteins, beta-Grasp(ubiquitin like), CAD & PB1 domain. (B) Consensus based secondary structure prediction of DdS4. C-terminal region, c = coil, e = extended sheet, h = helix. Gray shaded box depicts the model for the DdS4 C-terminal region. The color coding of the structure is based on the extent of conservation of residues amongst the S4 homologs (as obtained from ConSurf). Blue colored regions of the structure are most conserved, while red colored regions are least conserved. (C) JOY alignment [78] between the template structure 1JJ2 [79] and the c-terminal region of S4 (150 residue onwards). Protein bank id 1JJ2 corresponds to the structure of the protein encoding the Large Ribosomal Subunit from Haloarcula marismortui. The S chain from this structure was used for modelling the C-termini of S4. This chain is annotated as 50S ribosomal protein L24P and bears structural resemblance to SH3. Different secondary structural elements of 1JJ2 are labeled as helix (a), Sheets (b) and 310 helix (3). Solvent accessible residues are represented in lower case, solvent inaccessible residues in upper case, residues hydrogen bonded to main-chain amide are in bold, residues hydrogen bonded to main-chain carbonyl are underlined and positive phi torsion angle are represented in italics. (D) PXXPX motifs in Cdc24 proteins from Dictyostelium. Regions in bold depict proline rich regions in Cdc24 proteins form Dictyostelium as obtained from uniprot. PXXPX is involved in recognition and binding to SH3 domain. Regions with high significance score are highlighted in red (significance score obtained from SH3 hunter [75]).

Figure S2 Spatial localization DdS4 mRNA, anti-DdS4 antibody specificity and sub-cellular localization of DdS4. (A) In situ hybridisation of developmental stages using a DdS4 DNA probe. (B) Specificity of anti-DdS4 antibody was determined by western blot using (I) E. coli Bl21 lysate and probing with anti-His antibody (DdS4 is expressed as a His-tag protein) and (II) D. discoideum lysates where increasing amounts of lysates were loaded from either control AX2 or AX2::A15S4+. (III) Specific interactions between the GST-tagged proteins and DdS4 are observed in the IP lane. Refer Figure 2A legend for details. This is an over-exposed blot to visualize pull downs of Bem1p and Cia4p more clearly. (C) Subcellular fractionation of cells. Aggregating AX2 cells were subjected to high speed centrifugation to yield cytosolic and membrane fractions. The soluble and insoluble cytoskeletal fractions were also checked for DdS4. Equal loading was checked by Coomassie staining.

Figure S3 AX2::A15 mutants display normal protein synthesis. DdS4 cells were pulse-labelled for 1 h with [35S]methionine and chased for 4 h. The protein bands indicate equal rate of incorporation and subsequent chase of [35S]label.

Figure S4 DdS4 is different from ScS4 at the C-terminal end. (A) Motifs conserved across S4 homologs. (B, II) The C-terminal region from few DdS4 homologs (S.cerevisiae, P.falciparum, S.pombe, Leolongsorpus, C.cinerea okayama) depicting motif 11 (purple), 15 (yellow) and 16 (blue). S4 homologs were submitted in MEME [80] to identify locally conserved regions (depicted by different colored rectangular boxes numbered according to the e-value, 1 showing the lowest e-value). N-terminus is largely conserved while variation largely lies in the C-terminus. DdS4 C-terminus lacks conserved motifs 15 and 16; purple region depicts motif 11 while yellow and blue regions show motifs 15 and 16 respectively. Motif 15 and 16 are not conserved in DdS4 showing a very high e-value. (B) Tree depicting all the S4 homologous sequences. All sequences considered are of euakaryotic origin. Tree was generated using Neighborhood joining method and validated by bootstrapping for 500 iterations (values indicated on the branches). Nodes have been clustered and color coded based on the kingdom, all the Metaozoa sequences are represented in Purple, Plants in green and fungi in Blue. ScS4 (Dark Blue edge) clusters with other fungal S4 sequences (sky blue edge), DdS4 (dark blue edge) shows variation from other S4 (more similar to Plants). Tree generation and statistical testing done using MEGA [81].
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References

1. Bonner JT (1967) The cellular slime molds. Princeton, N.J.: Princeton University Press. pp x, 205.
2. Raper KB, Rahn AW (1984) The dictyostelids. Princeton, N.J.: Princeton University Press. pp x, 453.
3. Leach CK, Ashworth JM, Garrod DR (1973) Cell sorting out during the differentiation of mixtures of metabolically distinct populations of Dictyostelium discoideum. J Embryol Exp Morphol 29: 637-661.
4. McDonald SA, Durston AJ (1984) The cell cycle and sorting behaviour in Dictyostelium discoideum. J Cell Sci 66: 195-204.
5. Saran S, Arhar M, Manogaran PS, Pand G, Nanjundiah V (1994) The level of sequestered calcium in vegetative amoebae of Dictyostelium discoideum can predict post-aggregative cell fate. Differentiation 57: 163-169.
6. Azhar M, Kennedy PK, Pande G, Espiritu M, Holloman W, et al. (2001) Cell cycle phase, cellular Ca2+ and development in Dictyostelium discoideum. J Dev Biol 45: 405-414.
7. Beach D, Durkacz B, Nurse P (1982) Functionally homologous cell cycle control genes in budding and fission yeast. Nature 300: 706-709.
8. Michaelis C, Wecke G (1993) The isolation from a unicellular organism, Dictyostelium discoideum, of a highly-related cd2 gene with characteristics of the PCTAIRE subfamily. Biochem Biophys Acta 1179: 117-124.
9. Souza GM, Lu S, Kuspa A (1998) YakA, a protein kinase required for the transition from growth to development in Dictyostelium. Development 125: 2291-2302.
10. Fields SD, Conrad MD, Clarke M (1998) The S. cerevisiae CUL1 and D. discoideum ducA genes are functional homologues that influence mitochondrial morphology and distribution. J Cell Sci 111(Pt 12): 1717-1277.
11. Butty AC, Perrinquant N, Petit A, Jaquenoud M, Segall JE, et al. (2002) A conserved protein kinase regulates the guanine-nucleotide exchange factor Cdc24 at sites of polarization. EMBO J 21: 1563-1576.
12. Casamayo A, Snyder M (2002) Budsite selection and cell polarity in budding yeast. Curr Opin Microbiol 5: 179-186.
13. Irazoqui JE, Lev DJ (2000) Polarity establishment in yeast. Curr Opin Cell Biol 12: 2169-2171.
14. Anand S, Prasad RS (1987) Status of calcium influx in cell cycle of S. cerevisiae. Biochem Int 14: 963-970.
15. Shoat BF, Adams A, Pringle JR (1981) Roles of the CDC24 gene product in cellular morphogenesis during the Saccharomyces cerevisiae cell cycle. J Cell Biol 89: 395-405.
16. Tapparo A, Sann M, Klein G (1998) Cloning, sequencing and developmental expression of the genes encoding S4 and S10 ribosomal proteins in the cellular slime mould Dictyostelium discoideum. Curr Genet 34: 410-418.
17. Corney AJ, Richards AJ, Phillips T, Hames BD (1990) Developmental regulation of cell-type-enriched mRNAs in Dictyostelium discoideum. Mol Microbiol 4: 613-623.
18. Proffin JA, Jagger G, Wilson GA, Donovan JT, Widdowson DC, et al. (1991) A developmentally regulated gene encodes the dihydroxyacetone homolog of yeast ribosomal protein S4 and mammalian LRRP3 proteins. Nucleic Acids Res 19: 3067-3073.
19. Inoue Y, Chae GC, Maeda Y (1999) Transient expression of a mitochondrial gene cluster including rpl6 is essential for the phase-shift of Dictyostelium cells from growth to differentiation. Dev Genet 25: 339-352.
20. Atkins JF, Eileviers D, Gorsin L (1972) Low activity of -galactosidase in a temperature-sensitive -galactosidase strain. Nature 236: 210-231.
21. Bender A, Pringle JR (1989) Multicopy suppression of the cdc24 budding defect. Mol Gen Genet 229: 2291-2302.
22. Pollitt AJ, Insall RH (2008) Abi mutants in Dictyostelium reveal specific roles for the SCAR/WAVE complex in cytokinesis. Curr Biol 18: 203-210.
23. Mondal S, Neelamegan D, Rivero F, Noegel AA (2007) GxcDD, a putative actin bundling protein. Curr Biol 17: 210-213.
54. Wright S (1964) Pleiotropy in the evolution of structural reduction and dominance. Am Nat 98: 65–69.

55. Hodgkin J (1998) Seven types of pleiotropy. Int J Dev Biol 42: 501–505.

56. Huberts DH, van der Klei I (2010) Moonlighting proteins: an intriguing mode of multitasking. Biochim Biophys Acta 1805: 529–525.

57. Erijman A, Aizner Y, Shifman JM (2011) Multispecific recognition: mechanism, evolution, and design. Biochemistry 50: 602–611.

58. Klebe RJ, Harris JV, Sharp ZD, Douglas MG (1983) A general method for polyethylene-glycol-induced genetic transformation of bacteria and yeast. Gene 25: 333–341.

59. Hutter KJ, Eipel HE (1979) Microbial determinations by flow cytometry. J Gen Microbiol 113: 569–575.

60. Miller JH (1992) A Short Course in Bacterial Genetics: A Laboratory Manual and Handbook for Escherichia coli and Related Bacteria. New York: Cold spring harbor laboratory press.

61. Watts DJ, Ashworth JM (1970) Growth of myxameobae of the cellular slime mould Dicystostelium discoidum in axenic culture. Biochem J 119: 171–174.

62. Boutet E, Lieberherr D, Tognolli M, Schneider M, Bairoch A (2007) UniProtKB/Swiss-Prot. Methods Mol Biol 406: 89–112.

63. Bates PA, Kelley LA, MacCallum RM, Sternberg MJ (2001) Enhancement of protein modeling by human intervention in applying the automatic programs 3D-JIGSAW and 3D-PSSM. Proteins Suppl 5: 39–46.

64. Wilson D, Madera M, Vogel C, Chothia C, Gough J (2007) The SUPERFAMILY database in 2007: families and functions. Nucleic Acids Res 35: D308–313.

65. Andreeva A, Howorth D, Chandonia JM, Brenner SE, Hubbard TJ, et al. (2008) Data growth and its impact on the SCOP database: new developments. Nucleic Acids Res 36: D419–425.

66. Kajan L, Rychlewski L (2007) Evaluation of 3D-Jury on CASP7 models. BMC Bioinformatics 8: 304.

67. Jones DT (1999) GenTHREADER: an efficient and reliable protein fold recognition method for genomic sequences. J Mol Biol 287: 797–815.

68. Shi J, Blundell TL, Mizuguchi K (2001) FUGUE: sequence-structure homology recognition using environment-specific substitution tables and structure-dependent gap penalties. J Mol Biol 310: 243–257.

69. Bennett-Lovey RM, Herbert AD, Sternberg MJ, Kelley LA (2008) Exploring the extremes of sequence/structure space with ensemble fold recognition in the program Phyre. Proteins 70: 611–625.

70. Eswar N, Eramian D, Webb B, Shen MY, Sali A (2000) Protein structure modeling with MODELLER. Methods Mol Biol 426: 145–159.

71. Wiederstein M, Sippl MJ (2007) ProSA-web: interactive web service for the recognition of errors in three-dimensional structures of proteins. Nucleic Acids Res 35: W407–410.

72. Guttenberg O, Erez E, Nimrod G, Ben-Tal N (2009) The ConSurf-DB: pre-calculated evolutionary conservation profiles of protein structures. Nucleic Acids Res 37: D323–327.

73. Edgar RC (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. Nucleic Acids Res 32: 1797–1799.

74. Bailey TL, Williams N, Muleh C, Li WW (2006) MEME: discovering and analyzing DNA and protein sequence motifs. Nucleic Acids Res 34: W369–373.

75. Ferraro E, Pezuo D, Via A, Anielle G, Helmer-Citterich M (2007) SH3-Hunter: discovery of SH3 domain interaction sites in proteins. Nucleic Acids Res 35: W451–454.

76. Glaser F, Pupko T, Paz I, Bell RE, Bechor-Shental D, et al. (2005) ConSurf: identification of functional regions in proteins by surface-mapping of phylogenetic information. Bioinformatics 19: 163–164.

77. Gough J, Karplus K, Hughey R, Chothia C (2001) Assignment of homology to genome sequences using a library of hidden Markov models that represent all proteins of known structure. J Mol Biol 313: 903–919.

78. Mizuguchi K, Deane CM, Blundell TL, Johnson MS, Overington JP (1998) JOY: protein sequence-structure representation and analysis. Bioinformatics 14: 617–623.

79. Klein DJ, Schneising TM, Moore PB, Steltz TA (2001) The kink-turn: a new RNA secondary structure motif. EMBO J 20: 4214–4221.

80. Bailey TL, Boden M, Buske FA, Frith M, Grant CE, et al. (2009) MEME SUITE: tools for motif discovery and searching. Nucleic Acids Res 37: W202–208.

81. Kumar S, Nei M, Dudley J, Tamura K (2008) MEGA: a biologist-centric software for evolutionary analysis of DNA and protein sequences. Brief Bioinform 9: 299–306.