ZizB, a novel RacGEF regulates development, cell motility and cytokinesis in *Dictyostelium*.

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Abbreviations: DIC differential interference contrast; GEF, guanine nucleotide exchange factor; DHR, Dock-homology region; CZH, CDM (Ced-5, Dock180, myoblast city) zizimin homology.
Summary

Dock (Dedicator of Cytokinesis) proteins represent a family of Guanine nucleotide Exchange Factors (GEFs) that include the well studied Dock180 family and the poorly characterised zizimin family. Our current understanding of Dock180 function is to regulate Rho small GTPases, playing a role in a number of cell processes including cell migration, development and division. Here, we have employed a tractable model for cell motility research, Dictyostelium discoideum, to help elucidate the role of the related zizimin proteins. We show that gene ablation of zizA causes no change in development whereas ablation of zizB gives rise to an aberrant developmental morphology and a reduction in cell directionality and velocity, and altered cell shape. Fluorescently labeled ZizA protein associates with the microtubule organizing centre (MTOC), whereas the ZizB protein exhibits cortical enrichment. Overexpression of ZizB also causes an increase in the number of filopodia and a partial inhibition of cytokinesis. Analysis of ZizB protein binding partners indicates it interacts with Rac1a and a range of actin-interacting proteins. In conclusion our work provides the first insight into the molecular and cellular functions of zizimin GEF proteins playing a role in cell movement, filopodia formation and cytokinesis.
Introduction

The Rho family of small GTPases (Rho, Rac and Cdc42) act as small molecular switches during cellular signalling, cycling between their active and inactive state. These small G-proteins are involved in the regulation of many cellular processes within the cell such as cytoskeletal organisation, cytokinesis, cell morphogenesis, cell migration and development (Dumontier et al., 2000; Jaffe and Hall, 2005; Kolsch et al., 2008; Mondal et al., 2007; Para et al., 2009). The activity of these small GTPases is regulated via the exchange of GDP/GTP (Guanosine-5'-diphosphate/Guanosine-5'-triphosphate) catalysed by guanine nucleotide exchange factors (GEFs), GTPase-activating proteins (GAPs) and guanine nucleotide-dissociation inhibitors (GDIs) (Jaffe and Hall, 2005). GEFs regulate GTPases by facilitating the dissociation of GDP, allowing GTP to bind and activate the protein. There are two main families of GEFs for Rho GTPases, the conventional Dbl homology-pleckstrin homology (DH-PH) domain GEFs and the dedicator of cytokinesis (Dock) GEFs (Cote et al., 2005; Cote and Vuori, 2002; Meller et al., 2005). Although considerable research has investigated the function of DH-PH GEFs, the roles of Dock proteins remain poorly understood. A better understanding of these proteins is important, since the Dock signaling pathway has been implicated in a number of diseases such as lung cancer and immunodeficiency diseases (Engelhardt et al., 2009; Ruusala and Aspenstrom, 2004; Takahashi et al., 2006; Zhang et al., 2009).

Mammalian Dock proteins can be subdivided into four main classes, Dock180-related, Dock4-related, zizimin and zizimin-related (Cote and Vuori, 2002). The Dock proteins are composed of two main domains, the Dock-Homology Region 1 (DHR1) and the Dock-Homology Region 2 (DHR2) domains. The exact function of the DHR1 domain has been shown to bind phospholipids (Cote et al., 2005; Kobayashi et al., 2001; Para et al., 2009). The DHR2 domain interacts with the target GTPase and is responsible for the GEF activity (Brugnera et al., 2002; Cote and Vuori, 2002; Cote and Vuori, 2006). In regards to target specificity, the zizimin and zizimin-related Dock family sub-groups have been shown to have a preference for Rac and Cdc42 (Meller et al., 2002).

Eight Dock proteins have been identified in D. discoideum, four Dock-180/Dock4 related (Doc A-D) and four zizimin/zizimin-related (ZizA-D) proteins. The D. discoideum Dock180 related proteins, DocA and DocD regulate the actin cytoskeleton and cell motility (Para et al., 2009). In our study, we explore the role of the highest expressing D. discoideum zizmins, zizA and zizB and show both novel and conserved functions of these members of the ancient zizimin protein family.
Results

Evolutionary conservation, domain characterisation and expression analysis of zizimin proteins

Eight Dock family proteins were identified within the *D. discoideum* genome using homology screening. Phylogenetic analysis of these proteins showed that they comprise of four zizimin-related proteins (ZizA-D), and four Doc proteins (DocA–D) that are homologous to the Dock180-related clade (Fig. 1A). This division into either Dock or Zizimin-type homologues is supported by boot strap analysis, confirming the early separation of these proteins. The four *D. discoideum* zizimin-related proteins have DHR1 and DHR2 domains and show greatest sequence similarity to the human zizimin-related proteins (Dock6-8) (Fig. 1B), with the human Dock7 providing the closest homologue to *D. discoideum* ZizA (with a 36% identity and 57% similarity), and the human Dock8 providing the closest homologue to *D. discoideum* ZizB (28% identity and 49% similarity). Comparison of the full length *D. discoideum* ZizA and B proteins shows a 26% identity and 44% similarity (Fig. 1C), where the DHR2 domains (the putative GTPase interacting domain) shows highest homology, with slightly reduced homology in the DHR1 (the putative phospholipid binding domain) and the interdomain region, and low homology in the N-terminal region. The domain structure of these zizimin proteins support a similarity of function with zizimin related proteins, rather than human Dock180 proteins that have an additional SH3 domain.

Since the Rho family of small GTPases are known to play a role in development and cell motility (Zigmond *et al.*, 1997), and these processes are often controlled by regulated gene expression (Loomis and Shaulsky, 2011), we examined the expression of *D. discoideum* zizA-D over the 24 hr developmental cycle. In these experiments wild type cells were developed on a nitrocellulose filter, from which RNA samples were prepared at 4 hr time intervals (Fig. 2). Analysis of the transcription level of zizA-D by reverse-transcription PCR showed that all four genes were expressed throughout growth and development. ZizB has the highest expression level, in agreement with the RNA sequence profiling resource (http://dictyexpress.biolab.si/), where mRNA levels are shown to peak at an average of 30 copies per cell during development, which puts it within the top 5% of the most highly expressed genes (Parikh *et al.*, 2010; Rot *et al.*, 2009). Since the *D. discoideum* ZizA and B proteins grouped within the zizimin/zizimin related clade of the phylogenetic tree, have greatest homology to human Dock7 and Dock8 (which share a similar domain structure), and have the highest expression levels of the four *D. discoideum* zizimins, these
two were chosen for further investigation. The zizA and B genes were disrupted by homologous integration of a knockout cassette in multiple independent cell lines (Supplementary Fig. 1; zizA− and zizB−). Gene disruptions were confirmed by PCR analysis and subsequent loss of gene expression by reverse transcriptase PCR (Supplementary Fig. 1).

**ZizB plays a role in development and cell migration**

During the process of *D. discoideum* development, cells release a chemoattractant cAMP, to which surrounding cells migrate towards, initiating the formation of a multi-cellular fruiting body (Schaap and Wang, 1986). We analyzed development of cells following both ablation and overexpression of zizA and B genes at various stages throughout the developmental cycle (8, 13 and 24 hr) (Fig. 3). In overexpressing both genes, a highly active constitutive promoter (Actin 6) was used to express each full length cDNA-derived open reading frame linked to a C-terminal green fluorescent tag (to create ZizA-GFP and ZizB-GFP expression constructs). Cells lacking ZizA protein, or overexpressing either tagged protein in wild type cells (to form zizA+ and zizB+ cells) did not show altered development, thus producing mounds at 8 hr, and developing to a first-finger stage at 13 hr, then mature fruiting bodies with morphology similar to that of wild type cells at 24 hr. In contrast, cells lacking the ZizB protein were slightly delayed in early development compared to wild type cells (still showing late streaming) at 8 hr, and were again delay at first finger stage (13 hr) compared to the wild type development. Finally the zizB null cells showed sparse collapsed fruiting bodies with thickened stalks and in comparison to wild type cells at 24 hr (Fig. 3). We confirmed this aberrant morphology was caused by gene ablation of zizB, since overexpression of ZizB-GFP in zizB− cells (producing zizB+/− cells) rescued aberrant fruiting body morphology.

During the formation of fruiting bodies, the movement of individual cells both towards the chemoattractant cAMP and within an immature fruiting body are necessary to enable these structures to be formed (Schaap and Wang, 1986). To identify whether the developmental defect exhibited by the zizB− cells was due to a cell motility defect, we investigated the ability of the zizB− and zizB+ cells to chemotax along a cAMP gradient (Fig. 4). In these experiments, we forced cells to a common developmental point by pulsing with cAMP for 5 hr prior to chemotaxis analysis. These cells were analysed using time lapse imaging to record the tracks of single cells during chemotaxis in a shallow cAMP gradient (Dunn chamber). Analysis of migration towards cAMP was used as a measure of cell directionality, illustrated by the X-Y coordinate plots (Fig. 4A). Each line represents the track of a single cell over a 5 minute period. In this assay, the zizB− mutant showed a strong reduction in the ability to move along the chemotactic gradient (compare
Supplementary movie 1 and 2), in addition to a significant reduction in cell velocity (where P < 0.05, Fig. 4A, B, E). In contrast, ZizB-GFP overexpression in a wild type background did not grossly alter cellular movement during chemotaxis compared to wild type cells (Fig. 4C, E) but reversed the chemotactic deficits shown following loss of ZizB (Fig. 4A, D, E). In addition, analysis of cell shape (aspect) from wild type, zizB−, zizB+ and zizB+/− cells showed that the zizB− mutant had a more rounded cell shape during chemotaxis (represented by a reduced ratio of cell length-to-breadth) (Fig. 4B, C, D, E). This phenotype was reversed upon overexpression of zizB. These results confirm a role of ZizB in development and chemotaxis in D. discoideum, and show that ZizB-GFP can catalyse the cellular events carried out by the endogenous ZizB in D. discoideum.

Overexpression of ZizB produces increased filopodia and a cytokinesis defect
Cellular localization of both zizimin proteins was then examined using GFP-tagged proteins by live cell imaging. ZizA-GFP was found to be distributed throughout the cytosol (Fig. 5A), with enrichment in a structure resembling the microtubule organising centre (MTOC, white arrow) adjacent to the nucleus during cell movement (also see supplementary movie 5). This ZizA MTOC localization was then confirmed by expression of ZizA-RFP in cells containing α-tubulin-GFP, where live cell fluorescence showed colocalisation (Fig. 5B). In contrast, ZizB-GFP shows enrichment at the cortex (Fig. 5A). The zizB+ cells also showed a significant 2-fold increase in filopodia formation compared to wild type and zizA+ cells (P< 0.0001) (Fig. 5A, C, D). The D. discoideum Dock180-related Dock A and Dock D proteins have been previously shown to rapidly translocate to the cell cortex upon global stimulation with cAMP (Para et al., 2009). We investigated if the D. discoideum zizimin proteins are also involved in the chemotactic signaling response. Time lapse images of cells following a single 1 µM cAMP pulse show no change in ZizA-GFP localisation (Fig. 5E, F). Unexpectedly ZizB-GFP had the exact opposite behaviour as the D. discoideum Dock proteins. Stimulation with cAMP leads to a transient removal from the cortex to the cytosol and then returned back to the cortex 8 seconds after stimulation (Fig. 5E, F).

We next used live cell imaging to investigate the localisation of both proteins during cell motility, within a cAMP gradient and during random cell movement. In these experiments, ZizA-GFP showed no change in localisation during either chemotaxis or random movement (Supplementary movie 3 and 5). However, ZizB-GFP was excluded from the trailing edge in both conditions and appeared enriched on the cortex at the front and sides of the cell (Fig. 6A, B and C;
Supplementary movie 4 and 6). These data support a role for ZizB in general cell motility (rather than chemotaxis) with a potential cortical function.

Since Dock proteins have been widely associated with a role in cytokinesis, we also examined this process in zizA−, zizB−, zizA+ and zizB+ cells by extended (5 day) growth in shaking suspension. These growth conditions reduced traction-mediated cytokinesis, giving rise to an increase in average cell nuclei number associated with cytokinesis effects. Under these conditions, zizB+ cells were commonly unable to complete cell division, where the cytoplasmic bridge between dividing cells was not cleaved during cytokinesis to form daughter cells (Fig. 7A, B) compared to wild type, zizA−, zizB− or zizA+. Visualisation of nuclei number per cell using DAPI staining using zizA+ and zizB+ cells (Fig. 7C-H) showed a significant (P>0.05) increase in the number of nuclei in zizB+ cells compared to wild type and zizA+ cells (Fig. 7I). This increase was associated with an increase in cell size, and both these effects are consistent with a partial inhibition of cytokinesis in zizB+ cells. A decrease in growth rate was only seen in zizB− cells (not zizB+ cells) however the overexpression of ZizB protein showed a highly significant increase in cell density in the stationary phase (P< 0.0001).

ZizB interacts with Rac1a

To further examine the cellular mechanisms of zizB, we then sought to identify potential binding partners. In this approach, lysates from zizB+ cells were immunoprecipitated with anti-GFP antibody coated beads, and coimmunoprecipitated proteins were visualised by Coomassie stain to identify interacting proteins. Mass spectrometry analysis of unique bands was then used to identify these interacting proteins (Fig. 8, Supplementary Table 1). This approach showed zizB bound a range of actin and/or myosin associated proteins including tubulins, TubA and B (Trivinos-Lagos et al., 1993) (Supplementary Table 1), consistent with a role in controlling cortical function and cell movement (Rivero and Somesh, 2002). In addition, ZizB interacted with a Rac1a, a small GTPase, formin A, a protein that functions as an actin filament nucleator (Kitayama and Uyeda, 2003), Cap32 and Cap34, which are capping protein subunits (Schleicher, Gerisch, and Isenberg, 1984; Eddy et al., 1997; Hug et al., 1995) and the ArpC1 and ArpC2 proteins (coded for by arcA and arcB genes) which form part of the Arp2/3 complex (Langridge and Kay, 2007). These potential binding partners position ZizB in a central role in actin cytoskeletal organisation. To confirm the ZizB interaction with Rac1a, ZizB-GFP protein was washed through a column containing bound (bacterially produced) Rac-GST proteins. The protein complex was separated on a SDS-page gel.
and visualised by western blot analysis (Supplementary Fig. 2). This approach confirmed a direct binding of ZizB to Rac1a, and also showed an interaction with RacA, RacC and RacG.

Discussion

ZizB is essential for normal development and cell migration

_**D. discoideum**_ contains four zizimin GEF proteins, with all four containing the characteristic DHR1 and DHR2 catalytic domains associated with the Dock family of proteins (Fig. 1). Expression of all four genes was found to be constitutive throughout development (Fig. 2), rather than showing developmental regulation as seen in other _D. discoideum_ GEF proteins such as the putative RacGEF, GxCDD (Mondal et al., 2007; Shaulsky et al., 1996) or Trix (Strehle _et al._, 2006). The expression patterns of the zizimin genes therefore suggest that their cellular roles are more likely related to a potential role in cytoskeletal regulation during movement (rather than just development).

The analysis of the role of zizA and B proteins in development showed that cells lacking ZizA had no gross change in developmental phenotype (i.e. in fruiting body formation); however loss of ZizB gave rise to aberrant fruiting body formation (Fig. 3). The lack of developmental changes following ZizA loss does not preclude an important role for the protein in development, since the cellular roles for the protein may be, at least partially, compensated for by the other zizimin proteins. This functional redundancy has been shown in other small GTPase related signaling families of _D. discoideum_ Doc proteins (Para _et al._, 2009), RacB and RacGEF1 (Park _et al._, 2004) and Trix RacGEF (Strehle _et al._, 2006). However, ablation of zizB did perturb development, suggesting a critical role that cannot be replaced by related proteins, or that the relatively low expression levels of ZizA, C and D cannot effectively complement loss of ZizB.

ZizB is involved in filopodia formation and cytokinesis

Overexpression of ZizB-GFP identified a second role for the protein in controlling filopodia formation (Fig. 5). Increased filopodia formation (and microspike production) compared to wild type has also been shown following zizimin1 (p220) overexpression in fibroblasts, and this protein was shown to bind to the activated Rac enzyme, Cdc42 (Lin _et al._, 2006). Furthermore an increase in the number of filopodia has been shown following the overexpression of DocD in _D. discoideum_, that also binds Rac1a (Para _et al._, 2009). We also showed a third role for ZizB, where
overexpression caused a large increase in cells unable to complete daughter cell separation as demonstrated by measuring the frequency of multinucleate cell formation following growth in shaking suspension in the absence of traction-mediated cell division (Fig. 7). Interestingly, a similar cytokinesis defect was found in the dynamin A null strain (Gopaldass et al., 2012; Wienke et al., 1999). This phenotype also suggests a role for ZizB in regulation of the actin/myosin cytoskeleton involved in cell division (Rivero et al., 2002; Rivero and Somesh, 2002). It is noteworthy that similar phenotypes (increased number of filopodia and cytokinesis defect) can be elicited by the overexpression of Racla (Dumontier et al., 2000). Combined with the fact that Rac1a was identified as a potential binding partner for ZizB, our data strongly suggests that ZizB plays a role in activating Rac1a in filopodia formation, cytokinesis and chemotaxis (Rivero et al., 2002).

**Zizimin localisation**

Our current model for the function of RacGEF proteins during chemotaxis is that, upon cAMP stimulation, these proteins are rapidly translocated to the cortex (or leading edge in chemotaxing cells) to initiate F-actin polymerisation through the regulation of Rac GTPases, enabling forward movement (Para et al., 2009). In contrast to this model, our data shows ZizB moves off the cortex into the cytosol following global cAMP stimulation thus showing the opposite behaviour of previously described RacGEF proteins such as DRG (Knetsch et al., 2001), DocD (Para et al., 2009) and RacGEF1 (Park et al., 2004). One explanation for this movement would be a cortex-stabilising mechanism for ZizB, between cyclic periods of reorganisation caused by cAMP waves during chemotaxis, which is consistent with ZizB localisation at the front and side of the cell with exclusion from the trailing edge or retracting pseudopods, during both random cell movement and in chemotaxis (Fig. 6). This localisation is distinct to proteins involved in initiating cell movement that are enriched at the leading edge (regulating and driving F-actin polymerisation) (Han et al., 2006). Cortical localization may occur through DHR1 (phospholipid binding) activity, but domain-specific localization studies would be needed to confirm this.

**Zizimin B forms complexes with Racla and actin-binding proteins**

The binding partners for the *D. discoideum* zizimin proteins remain unknown. In other model systems, the zizimin family of proteins is known to regulate the Rho family of small GTPases, which have a number of roles within the cell including regulating the actin cytoskeleton during cell
movement and other processes (Dumontier et al., 2000; Para et al., 2009; Rivero and Somesh, 2002). In relation to cell movement, dynamic regulation of F-actin polymerisation at the leading edge, the cell enables cytoskeleton reorganization, pseudopod formation and cell movement. Small GTPases play a key role in this process (Sasaki and Firtel, 2006). Here we demonstrate that ZizB binds Rac1a within the natural environment of the cell – using coimmunoprecipitation – in addition to a number of actin and myosin associated proteins. We further showed a direct binding of ZizB to Rac1a by binding of the bacterially expressed Rac1a protein, confirming this interaction in vitro, and in agreement with a role for ZizB in regulating the cytoskeleton (Fig. 8 and Supplementary Table 1 and Fig. 2). A direct binding has also been shown for the mammalian ZizB homologues (Dock7 and 8), with an interaction with Rac1, which is in agreement with zizimin proteins providing a crucial role of regulating Rac1 as a central small GTPase, shown to promote filopodia and membrane ruffles in multi model systems (Ruusala and Aspenstrom, 2004; Watabe-Uchida et al., 2006a; Watabe-Uchida et al., 2006b). The binding, in vitro, of other Rac proteins in this approach will need to be confirmed in cellular function through further studies, since (http://dictyexpress.biolab.si), and this may explain why only Rac1a was found in the direct, cellular co-immunoprecipitation reaction.

ZizB showed an interaction with ARPC1 and 2, two D. discoideum Arp2/3 subunits (Langridge and Kay, 2007). These subunits form the core of the Arp2/3 complex, which drives pseudopod formation and cell movement by catalyzing nucleation of new actin filaments and thus overcoming the kinetic barrier to actin polymerization (Insall & Machesky, 2009) and this complex represents one of many actin modifying enzymes that accumulate at the leading edge driving pseudopod formation and cell movement (Sasaki and Firtel, 2006). Other ZizB binding partners identified here include forA, cap32, cap34 and severin. ForA is a formin protein that functions as an actin filament nucleator. The knockout of forA did not show any distinct phenotype for growth and development (Kitayama and Uyeda, 2003), however another D. discoideum formin ForH (dDia2), has been implicated in filopodia formation, the knockout mutant showed numerous defects in development, pseudopodia formation, filopodia formation and a decrease in cell motility and chemotaxis (Schirenbeck et al., 2005; Van Haastert and Bosgraaf, 2009). ForA could therefore be involved in filopodia formation by forming a complex with Rac1a and ZizB. Cap32 and cap34 are subunits of the heterodimeric actin capping protein (Eddy et al., 1997). Capping proteins cap but do not sever (nor nucleate) actin filaments and thereby prevent the addition or loss of actin subunits at the barbed filament end (Hug et al., 1995). Severin is a protein that severs the actin filament and remains bound to the barbed end (Eichinger et al., 1991). Both the cap32/34 and severin play key
roles in the regulation of the actin cytoskeleton, further supporting the role for ZizB in the dynamic regulation of the actin cytoskeleton.

Conclusion

Zizimun related proteins are relatively poorly characterised members of the important Dock family of GEF proteins. In this paper, we examined the cellular function of ZizA and ZizB proteins in *D. discoideum*. We show that the *D. discoideum* ZizB has a significant role in development, and is necessary for normal cell movement and shape. We show that ZizB has an elevated association with the cortex (compared to the cytosol), and shows an unusual cortex de-localisation following global chemotactic stimulation. Furthermore, an elevated level of ZizB gives rise to an increase in the number of filopodia and partially interrupts cytokinesis. Finally, in agreement with these cellular functions (Dumontier *et al.*, 2000), ZizB forms a complex with a number of cell cytoskeletal proteins, including Rac1a, and this is consistent with the role of mammalian zizimin proteins in preferentially binding Cdc42 and Rac small GTPases (Allen *et al.*, 1998; Ruusala and Aspenstrom, 2004; Watabe-Uchida *et al.*, 2006c). Our data therefore illustrates an important role for zizimin proteins in controlling development, cell shape and motility, filopodia formation and cytokinesis.
Materials and Methods

Materials
Axenic medium was purchased from ForMedium Ltd (Norfolk, UK). All restriction enzymes, First Strand cDNA synthesis kit were purchased from Fermentas Ltd (St. Leon-Rot, Germany). Nonidet P-40 (NP40), Trizma hydrochloride (Tris-HCl), sodium chloride (NaCl), ethylene glycol tetraacetic acid (EGTA), ethylenediaminetetraacetic acid (EDTA), dithiothreitol (DTT), phenylmethanesulfonyl fluoride (PMSF), 4’, 6-diamidino-2-phenylindole (DAPI), cyclic adenosine monophosphate (cAMP), potassium phosphate monobasic (KH2PO4), potassium phosphate dibasic (K2HPO4), methanol and caffeine were purchased from Sigma-Aldrich (Dorset, UK). The High Pure RNA isolation kit and the protease cocktail inhibitor was purchased from Roche (West Sussex, UK). Antibiotics, penicillin/streptomycin and blasticidin were purchased from PAA Laboratories Ltd (Somerset, UK) and hygromycin from Invitrogen (Paisley, UK). The DNasefree kit was purchased from Ambion Inc (Austin, USA). The anti-GFP antibody and the GFP trap agarose bead were purchased from ChromoTek GmbH (Planegg-Martinsried, Germany).

Cell Culture, Strains and Plasmids
All D. discoideum strains were grown at 22°C in Axenic medium containing 100µg/ml penicillin and 100µg/ml streptomycin. A wild type (Ax2) strain was used to generate all the mutants.

Knockout constructs were created using methods as described previously (Terbach et al., 2011). Briefly, 5’ and 3’ fragments flanking the gene of interest were amplified by PCR (peqSTAR 96 Universal Gradient, Erlangen, Germany) from Ax2 genomic DNA. The 5’ and 3’ PCR fragments were cloned into the plPBLP expression vector (Faix et al., 2004) using the BamHI / PstI and the NcoI /KpnI restriction sites, respectively, incorporating the blasticidin resistance cassette. The knockout cassette was linearised and transformed into Ax2 wild type cells via electroporation (Gene Pulser Xcell, Bio-Rad, Hertfordshire, UK). Positive transformants were selected in nutrient media containing blasticidin (10µg/ml). Independent clones were screened for homologous integration by PCR, using a genomic and vector control as well as a diagnostic knockout band. Loss of gene transcription was confirmed using reverse transcription PCR, where RNA was extracted from the independent positive transformants using the High Pure RNA isolation kit according to the manufacturer’s instructions. Contaminating DNA was removed using the DNasefree kit prior to cDNA synthesis, using the First Strand cDNA synthesis kit and 1 µg of RNA per sample. The cDNA was analysed by PCR to confirm loss of gene transcription. Primers were designed, where
possible, to flank an intron, thus confirming cDNA amplification owing to the decrease in size of the cDNA-derived product (in comparison to genomically derived product).

Overexpression constructs were prepared using full length open reading frames of zizA and zizB that were amplified from cDNA with BamHI and NheI as flanking restriction sites. Endogenous BamHI sites were removed (silent mutations). The PCR products were cloned into a PCR ligation vector and sequences of the inserts were compared with the reference sequence on DictyBase. Correct clones were digested with BamHI and NheI and ligated into the *D. discoideum* GFP / RFP expression vector pDM450 / pDM451, respectively under the control of Actin6 promoter (Veltman *et al.*, 2009). Constructs were transformed into appropriate cell lines via electroporation and selected for using hygromycin (50µg/ml). GFP / RFP overexpressor cell lines were confirmed by fluorescence microscopy and western blot analysis using an anti-GFP or anti-RFP antibodies.

**Development Assays**

Filter assays were performed as described previously (Boeckeler *et al.*, 2006; Williams *et al.*, 2002). Briefly, cells were harvested at a density of 1x10^7 cells/ml before being washed in potassium phosphate buffer and evenly distributed on a 47mm nitrocellulose filter (Millipore catalog number FDR-296-040N, Watford, UK). The filter was incubated, for 24hrs, on an absorbent pad soaked in potassium phosphate buffer. Images were captured using a dissection microscope (MZ16 Leica Microsystems (UK) Ltd, Milton Keynes, UK) and a QImaging RetigaExi Fast1394 digital camera (QImaging, BC, Canada).

**Cell Movement and Image Acquisition**

Chemotaxis assays were preformed as described previously (Robery *et al.*, 2011). In brief, cells were pulsed at a density of 1.7x10^6 cells/ml with 30nM cAMP at 6-min intervals for 5 hr before being allowed to adhere to a coverslip. After cells had adhered, the coverslip was inverted onto a Dunn chamber (Hawksley, Sussex, UK) containing 5µM cAMP in the outer well. The response of the cells was recorded using time lapse imaging and ImagePro 6.3 software (Media Cybernetics, Bethesda, MD, USA) (one image every 6s for 5 mins). Experiments were repeated in at least triplicate with an average of 20-30 cells. Computer-assisted analysis of cell movement and cell shape was performed using ImagePro 6.3, measuring the velocity (µm/s) and the aspect. The aspect parameter is a measure of roundness, perfectly round cells have a value of 1, whereas elongated cells have a value of >1. For fluorescence chemotaxis, aggregation competent cells expressing either ZizA-GFP or ZizB-GFP were loaded into an Insall chemotaxis chamber (Muinonen-Martin *et al.*, 2010).
al., 2010). The phosphate buffer in the outer well was replaced by 1 µM cAMP and chemotaxing cells were visualised on a Nikon confocal microscope as above.

For localisation, fluorescently tagged GFP fusion proteins were cultivated in nutrient media, washed with potassium phosphate buffer and allowed to adhere to a glass coverslip before being visualised with an Olympus IX71 microscope (U-RFL-T laser, 543nm emission, Olympus UPlanFL 60x oil immersion objective with NA 1.25) with an QImaging RetigaExi Fast1394 digital camera and ImagePro6.3 software. To investigate cytokinesis defects, cells were cultured in a shaking suspension for 3 days and fixed with 100% methanol at -20°C for 15mins before being fluorescently labeled with 4’, 6-diamidino-2-phenylindole (DAPI) to visualise and count the number of nuclei per cell. All specimens were analysed using an Olympus IX71 microscope (U-RFL-T laser, 350nm and 543nm emission, respectively, Olympus UPlanFL 60x oil immersion objective with NA 1.25) with a QImaging RetigaExi Fast1394 digital camera. Data were processed using Adobe Photoshop or ImageJ software packages. To analyse the number of filopodia, time lapse images were taken every 5s over a 10min period (Nikon Eclipse TE2000-E with a 1.4 NA Plan Apo 60x objective) using a Q Imaging RetigaEXi camera. To quantify the number of filopodia for wild type, zizA+ and zizB + cells an average of 27 individual cells were counted for three time points (0mins, 5mins and 10mins) over the 10 min period. A filopodia is identified as a thin projection that extends from a pseudopod. The time lapse movies were analysed and an average number of filopodia over the 3 time points was calculated for statistical analysis (unpaired, two-tailed student t-test). To show the MTOC / ZizA co-localisation, zizA+ cells were co-expressed with α-tubulin-RFP (King et al., 2010) and visualized using live cell fluorescent imaging with a Nikon A1R confocal microscope, 543nm and 647nm emission with a 1.4 NA Plan Apo 60X objective using a Q Imaging RetigaEXi camera.

For global stimulation, aggregation competent cells expressing either ZizA-GFP or ZizB-GFP were loaded into a µ-slide (Ibidi, Martinsried, Germany) and allowed to adhere to the surface. Cells were perfused with 200 µl of 1 µM cAMP and images were recorded on a Nikon A1R confocal microscope with a 1.4 NA Plan Apo 60X objective.

In Vivo Pull-down and Purification of ZizA and ZizB-interacting Proteins and MS Analyses
Aggregation competent cells were washed with potassium phosphate buffer (KK2: 16.5 mM KH2PO4, 3.8 mM K2HPO4, pH 6.2), resuspended at a density of 3x10^8 cells/ml in potassium phosphate buffer, before being shaken for 20 mins at 250rpm with 2.5mM caffeine. Cells were lysed (0.5% NP40, 40mM Tris-HCl pH 7.5, 20mM NaCl, 5mM EGTA, 5mM EDTA, 10mM DTT, 1mM PMSF, 2x protease cocktail inhibitor). The cell lysate was incubated with GFP-Trap agarose beads as per manufacturer’s instructions. Briefly, the lysate was incubated with the GFP-Trap agarose beads at 4°C for 1 hr before being collected and washed with the wash buffer (10mM Tris-HCl pH 7.5, 150mM NaCl, 0.5mM EDTA, 1mM PMSF, 2 x protease cocktail inhibitor). Immunocomplexes were dissociated from the beads by incubating at 95°C for 10 mins before the beads were collected by centrifugation. The coimmunoprecipitated proteins were separated by SDS-page (Invitrogen, Paisley, UK) and analysed by Coomassie staining and mass spectrometry (MS) analysis.

GST pulldown was performed as described previously by Mondal et al (2007). Briefly, GST-Rac proteins were expressed in E.coli and bound to glutathione-sepharose beads (GE Healthcare). For the interaction of ZizA and ZizB with Rac proteins, 3x10^8 D. discoideum cells expressing ZizB-GFP were lysed in a lysis buffer (25mM Tris, pH7.5, 150mM NaCl, 5mM EDTA, 0.5% Triton X-100, 1mM NaF, 0.5mM Na3VO4, 1mM DTT, 1mM PMSF, 2x protease cocktail inhibitor (Roche)) and incubated with equal amounts of GST-Rac bound beads for 1hr at 4°C. Beads were washed with the wash buffer (25mM Tris, pH 7.5, 150mM NaCl, 5mM EDTA, 2x protease cocktail inhibitor (Roche)). The eluate of the pull down was immnoblotted, the zizimin protein was detected using the GFP specific monoclonal antibody (ChromoTek GmbH) and the rac proteins were identified with a GST specific monoclonal antibody (Millipore # Calbiochem OB03). Cells expressing only GST were used as a control. The immunoblot was visualised using the Odyssey Sa infrared imaging system.

**Acknowledgments**

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Reference List


Figure 1. Phylogenetic and domain structure analysis of the Dock family of proteins. (A) Phylogenetic tree comparing the evolutionary conservation of the *D. discoideum* Dock proteins against Dock proteins from other species. All analysis was performed using Mega5 software. The evolutionary distance was inferred using the neighbour joining method. The percentages of replicate trees in which the associated taxa clustered together in the bootstrap test (500 replicates) are shown next to the branches. The box illustrates the *D. discoideum* ZizA-D. (B) Schematic of the *D. discoideum* zizimin domain structures compared to the human zizimin related proteins (Dock7/8). (C) Table of the identities and similarities of the ZizA and ZizB DHR1 and DHR2 domains, the intervening sequences (N-terminal intervening sequence and the middle intervening sequence) and the complete protein.

Figure 2. *D. discoideum* zizimin gene expression throughout development. RNA samples were prepared from *D. discoideum* cells during growth (0 hr) or at 4 hr intervals during development, with derived cDNA used to amplify specific ziz genes. L – Illustrates the 1Kb ladder used as a molecular weight marker. (A) Agarose gel electrophoresis of zizA–D cDNA-derived PCR products throughout *D. discoideum* development. Ig7 was used as an expression control. (B) Quantification of the expression levels of zizA-D from three independent samples.
Figure 3. Development of zizimin A and B null and overexpressor cells. Wild type (Ax2), zizA−, zizA+, zizB− and zizB+ cells were developed on nitrocellulose filters over 24 hr. Fruiting body morphology was recorded at 8 hr, 13 hr and 24 hr from a side angle perspective. The zizA−, zizA+ and the zizB+ cells all developed into mature fruiting bodies, comprising a stalk elevating a spore head off the substratum and did not show any defects in early development compared to wild type cells. However, the zizB− mutant developed aberrant fruiting bodies with thickened, often horizontal stalks and showed a delay in develop. The size bar represents 1mm.

Figure 4. Chemotactic effects of zizimin B null and overexpressor cell lines where cells overexpress ZizB-GFP in wild type and zizB null background. Cell movement for wild type, zizA and zizB− mutants was recorded by time lapse photography over a 5 min period (at 6 second intervals) in a Dunn Chamber. Computer-generated cell outlines using Image Pro6.3 software enabled recording of individual cell movement over this period, illustrated here by X-Y coordinate plots. (A) X-Y coordinate plots (directionality) of the zizB−, zizB+ and zizB−/+ cells compared to wild type Ax2 cells. Each line represents the track of a single cell chemotaxing towards cAMP (5 μM). (B - D) Analysis of the velocity (μm/s) and aspect (roundness) of the zizB−, zizB+ and zizB−/+ cells (grey), respectively, compared to wild type Ax2 cells (black). (E) Quantitative analysis of cell migration. Aspect refers to the shape of the cell; a perfectly round cell will have an aspect of 1 whereas a more elongated polarized cell will have a number greater than 1. Statistical analysis was performed using a student t-test. All experiments were performed at least in triplicate with an average of 20-30 cell analysed per experiment. * indicates P-value < 0.05. The size bar represents 1mm.

Figure 5. Cellular localisation and filopodia induction of ZizA-GFP and ZizB-GFP proteins. (A) Live cell fluorescent imaging of zizA+ cells showing ZizA-GFP localisation to the cytosol and a small region associated with the nucleus (indicated by the white arrows), and zizB+ cells showing ZizB-GFP localisation to the cytosol with enrichment at the cortex. The zizB+ cells also show an increase in filopodia formation, indicated by the grey arrows. (B) Live cell fluorescent imaging of ZizA-RFP co-localised with α-tubulin-GFP at the MTOC in a mono and dinucleate cell. (C) DIC images illustrate filopodia in wild type, zizA+ and zizB+ cells. (D) Quantification of filopodia/cell for wild type, zizA+ and zizB+ cells, time lapse images were analysed (averaging 27 cells) and
compared using an unpaired, two tailed student t-test (*** indicates P<0.0001). (E) Time-lapse images of zizA+ and zizB+ cells following global stimulation with 1µM cAMP, with time points indicating seconds after cAMP addition, with (F) analysis by quantification of relative fluorescent levels within the cytosol in zizA+ and zizB+ cells. Size bars represent 10µm.

**Figure 6.** Localisation ZizB-GFP protein during chemotaxis and random cell motility. (A) zizB+ cells were used in live-cell time lapse imaging experiments during chemotaxis towards cAMP (time indicated in seconds and the large arrow represents the direction of the cAMP gradient) and (B) during random cell motility (arrows indicate the front of the moving cell) showing ZizB-GFP increased cortical association towards the front and sides of the cell with a reduction in cortical localisation at the trailing end. (C) The absence of zizB-GFP in the trailing end is confirmed in 3-D reconstruction of zizB+ cells during random cell motility where retracting pseudopods (Grey arrow) lack ZizB-GFP cortical localisation. Size bar represents 10µm.

**Figure 7.** ZizB overexpression mutants have a defect in cytokinesis. (A & B) Live cell images of zizB+ cells illustrating a cytokinesis defect where (A) cells have failed to break the cytoplasmic bridge during repeated cell divisions or (B) the cleavage furrow begins to develop filopodia. Cells were cultivated in nutrient media for 5 days in shaking suspension before being fixed and stained with 4′-6-diamidin-2-phenylindole (DAPI) to investigate whether they were multinucleate. (C, E & G) DAPI-stained wild type Ax2, zizA+ and zizB+ cells, respectively, where zizB+ shows an increase in the number of multinucleate cells, indicated by the arrows (G). (D, F & H) Shows the corresponding cells under the 543nm emission (GFP). (I) Histograms show the distribution of nuclei in wild type Ax2, zizA+ and zizB+ cells. There was a significant increase in the number of nuclei in the zizB+ mutant. The * indicates P-value <0.05. Size bar represents 10µm.

**Figure 8.** Immunoprecipitation of ZizB interacting proteins. (A) Extracts from wild type (cont) or ZizB+ cells (containing zizB-GFP) were used to identify specific binding partners. The purified complex was separated on a SDS-page gel and visualised with Coomassie stain. Binding partners for ZizB (not found in extracts purified from wild type cells) were ForA, TubA and B, ArpC1 and C2, AcpA and B and Racla. A complete list of binding partners is shown in supplementary table 1.
Supplementary data figure legends

Supplementary Table 1. List of the potential ZizB (and ZizA) binding partners.

Mass spectrometry was used to analyse the GFP Trap pull down data. The MS/MS data was analysed using scaffold 3 software, here the analysis parameters were set to a minimum protein match of 95% and the minimum peptide threshold was set to 4 with a 90% match. This analysis indicated ~300 interactions which were carefully analysed to eliminate non-specific candidates. The numbers indicate the number of unique peptides. Initially all proteins were identified using their Dictybase ID so as to identify their known functions. From here all candidates that were not unique to the ZizA or ZizB, compared to wild type control, were removed. From there the ribosomal and general housekeeping proteins were eliminated as potential candidates, leaving only specific ZizB interactors. The remaining potential interaction proteins have been grouped according to cellular function, blue show the GTPases, pink shows actin associated proteins, orange show candidates involved in actin and myosin association, green indicates myosin association and white are other interactors that had a high peptide hit but different functions.

Supplementary Figure 1. Generation of zizA− and zizB− cells. (A) Schematic illustration of the strategy used to generate and screen the knockout mutants. (B) N and C terminal PCR screening analysis of zizA− used to confirm positive transformants, showing genomic (G) and vector (V) controls as well as the diagnostic knockout band (KO). (C) N and C terminal PCR screening analysis of zizB− used to confirm positive transformants, showing genomic (G) and vector (V) controls as well as the diagnostic knockout band (KO). (D&E) PCR analysis of cDNA from multiple independent zizA− or zizB− cell lines for a control PCR product (+) and specific zizimin genes (ziz).

Supplementary Figure 2. Direct Rac-GST/ZizB-GFP binding. Rac1a-GST, RacA-GST, RacC-GST and RacG-GST were co-immunoprecipitated with extracts from wild type (cont) cells or cells overexpressing ZizB-GFP. The purified complex was separated on a SDS-page gel and visualised by Western blot analysis to indicate Rac-proteins present in each immunoprecipitation (using an anti-GST antibody antibody - lower panel), and the resulting bound ZizB-GFP protein (using an anti-GFP antibody - upper panel). GST–tagged Rac1a, RacA, RacC and RacG showed a ZizB interaction, whereas extracts from cells expressing GST only showed no binding (analysis under identical conditions and run on a separate gel due to lane constraints). L indicates the molecular maker and molecular masses are shown in KDa.
**Supplementary movie 1.** Chemotaxis movie of Ax2 wild type cells. Time lapse imaging was used to record Ax2 wild type cells chemotaxing in a cAMP gradient Dunn chamber. Images were taken at 6s time intervals for 10mins.

**Supplementary movie 2.** Chemotaxis movie of zizB− cells. Time lapse imaging was used to record cells chemotaxing in a cAMP gradient Dunn chamber. Images were taken at 6s time intervals for 10mins.

**Supplementary movie 3.** Chemotaxis movie of zizA+ cells. Fluorescent time lapse imaging was used to visualise the localisation of the ZizA-GFP protein during chemotaxis towards cAMP (1 µM) using an Insall chemotaxis chamber. Images were taken every 5s for 10 mins.

**Supplementary movie 4.** Chemotaxis movie of zizB+ cells. Fluorescent time lapse imaging was used to visualise the localisation of the ZizB-GFP protein during chemotaxis towards cAMP (1 µM) using an Insall chemotaxis chamber. Images were taken every 5s for 10 mins.

**Supplementary movie 5.** Random cell movement movie of zizA+ cells. Fluorescent time lapse imaging was used to visualise the localisation of the ZizB-GFP protein during random cell movement. Images were taken every 5s for 10 mins.

**Supplementary movie 6.** Random cell movement movie of zizB+ cells. Fluorescent time lapse imaging was used to visualise the localisation of the ZizB-GFP protein during random cell movement. Images were taken every 5s for 10 mins.
A

B

Hs Zizimin1  PH  DHR1  DHR2  2069 aa
Hs Dock7/8  DHR1  DHR2  2140/2031 aa
Dd ZizA     DHR1  DHR2  2284 aa
Dd ZizB     DHR1  DHR2  2147 aa
Dd ZizC     DHR1  DHR2  2621 aa
Dd ZizD     DHR1  DHR2  2162 aa

C

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A. Wild type, zizB−, zizB+, zizB−/+ growth under a CAMP gradient.

B. Time course of CAMP-activated cell migration velocity (μm/s) for wild type and zizB−.

C. Time course of CAMP-activated cell migration velocity (μm/s) for wild type and zizB+.

D. Time course of CAMP-activated cell migration velocity (μm/s) for wild type and zizB−/+.

E. Table of velocity and aspect ratios:

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A  
\[ \text{A} \quad \text{zizA}^+ \quad \text{zizB}^+ \]

B  
\[ \text{B} \quad \text{zizA}^+ \quad \alpha\text{-tubulin} \quad \text{merge} \]

C  
\[ \text{C} \quad \text{Wild type} \quad \text{zizA}^+ \quad \text{zizB}^+ \]

D  
\[ \text{D} \quad \text{Graph}\]  
\[ \text{Number of filopodia/cell} \]
\[ \text{Wild type} \quad \text{zizA}^+ \quad \text{zizB}^+ \]

E  
\[ \text{E} \quad \text{zizA}^+ \]
\[ \text{zizB}^+ \]

F  
\[ \text{F} \quad \text{Relative Fluorescence} \]
\[ \text{Time (s)} \]

\[ \quad \text{zizA}^+ \quad \text{zizB}^+ \]